Increased Hepatic Levels of the Insulin Receptor Inhibitor, PC-1/NPP1, Induce Insulin Resistance and Glucose Intolerance

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The ectoenzyme, plasma cell membrane glycoprotein-1 (PC-1), is an insulin receptor (IR) inhibitor that is elevated in cells and tissues of insulin-resistant humans. However, the effects of PC-1 overexpression on insulin action have not been studied in animal models. To produce mice with overexpression of PC-1 in liver, a key glucose regulatory organ in this species, we injected them with a PC-1 adenovirus vector that expresses human PC-1. Compared with controls, these mice had two- to threefold elevations of PC-1 content in liver but no changes in other tissues such as skeletal muscle. In liver of PC-1 animals, insulin-stimulated IR tyrosine kinase and Akt/protein kinase B activation were both decreased. In this tissue, the IR-dependent nuclear factor Foxo1 was increased along with two key gluconeogenic enzymes, glucose-6-phosphatase and phosphenolpyruvate carboxykinase. The PC-1 animals had 30–40 mg/dl higher glucose levels and twofold higher insulin levels. During glucose tolerance tests, these animals had peak glucose levels that were >100 mg/dl higher than those of controls. These in vivo data support the concept, therefore, that PC-1 plays a role in insulin resistance and suggest that animals with overexpression of human PC-1 in liver may be interesting models to investigate this pathological process. *Diabetes* 54:367–372, 2005

I nsulin resistance is a clinical abnormality that may affect >25% of the population of the U.S. and other countries (1,2). Insulin resistance is also a major contributor to both type 2 diabetes and the metabolic syndrome, which is a cluster of abnormalities that includes hypertension, dyslipidemia, and coronary artery disease (1,2). It has been proposed (3) that insulin resistance may result from multiple causes that include genetic and acquired influences. In most insulin-resistant individuals, however, the molecular cause(s) of insulin resistance are either unknown and/or unproven (3). Thus, specific therapy to treat this condition is not available.

One candidate molecule to cause insulin resistance is the ectoenzyme, plasma cell membrane glycoprotein-1 (PC-1/NPP1), a nucleotide pyrophosphatase-phosphodiesterase (NPP) that scavenges ATP under physiologic conditions (4). In insulin-resistant subjects, PC-1 is elevated in both tissues and fibroblasts (5–9). In addition, overexpression of PC-1 in cultured cells reduces both insulin-stimulated insulin receptor (IR) activation and downstream signaling (10). PC-1 binds to the IR but does not block insulin binding (5,11). Rather, PC-1 inhibits the insulin-induced conformational changes that lead to IR autophosphorylation and tyrosine kinase activation (11). Although these data suggest that PC-1 is involved in insulin resistance, a complete understanding of the role of PC-1 in this pathological process has been hampered by the lack of a convenient animal model of PC-1 overexpression.

In most mammals, the hormone insulin via the IR regulates fasting and postprandial glucose levels. In humans, during fasting, insulin and other hormones play a major role in regulating glucose levels by controlling hepatic gluconeogenesis (1,2). In humans, during the postprandial state, glucose levels are also regulated to a large extent by insulin-mediated glucose uptake into skeletal muscle (1,2). In rodents, however, hepatic regulation of glucose metabolism is important for both fasting and postprandial glucose regulation (12). In mice, for example, gene knockout of the IR in muscle is virtually without effect on insulin sensitivity (13,14), whereas IR gene knockout in liver leads to insulin resistance (15). Therefore, to investigate candidates for insulin resistance in this species, the liver must be targeted (13–15).

Herein, to investigate the effect of increased levels of PC-1 on glucose metabolism in mice, we employed an adenoviral-mediated gene delivery system to transfer human PC-1 cDNA into the liver of adult mice (16). This approach results in the infection and transduction of the majority of hepatocytes in the liver. There is little infection of cells in extrahepatic tissues, including muscle, adipose, spleen, pancreas, and kidney (17). Compared with mice transfected with a control (empty viral vector), animals
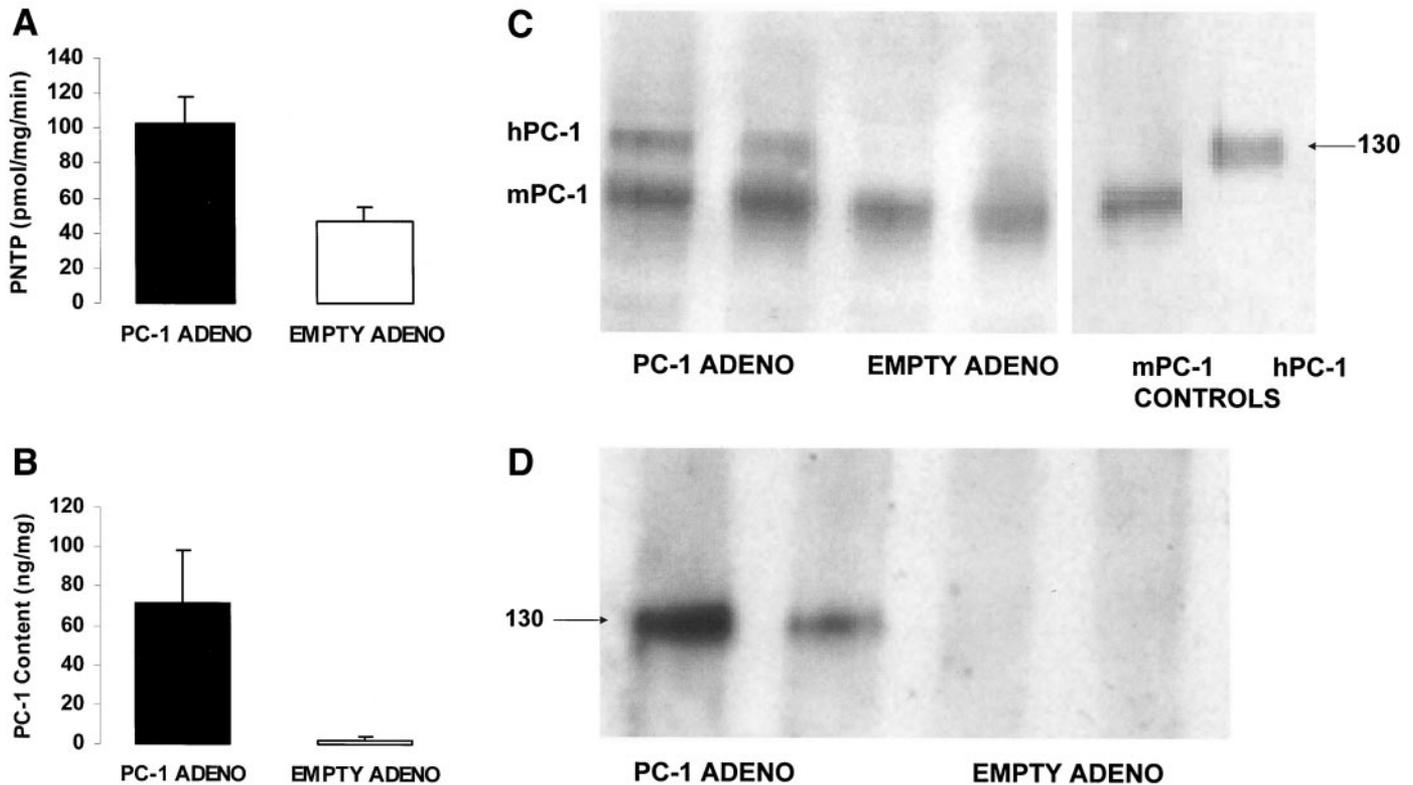
FIG. 1. Analysis of human PC-1 in liver. In these studies, seven PC-1 adenovirus–treated animals and seven empty virus controls were studied 1 week after infection. Livers were removed, and a solubilized high-speed membrane fraction was prepared (9). *A*: PC-1 activity was measured using the substrate PNTP. Results are means ± SE (*P* < 0.01). *B*: Human PC-1–specific ELISA. *C*: Western blot in liver fractions of human (h) and mouse (m) PC-1 using an antibody that recognizes both proteins. Standards were prepared from cultured cells transfected with either human or mouse PC-1. *D*: Western blot of human PC-1 expression in liver using an antibody specific to human PC-1.

with increased PC-1 had hyperglycemia, hyperinsulinemia, impaired glucose tolerance, and the increased expression of key hepatic gluconeogenic enzymes. These results indicate, therefore, that PC-1 plays a role in the insulin resistance of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Adenovirus PC-1. The wild-type, full-length human PC-1 K allele was subcloned into pACCMVpLpA (which contains the genomic sequence of the replication-defective E1 mutant of adenovirus 5 and the SV 40 poly A tail) as previously described (16). Adenoviral vectors were produced in HEK293 cells and purified as described (18). The titer of the PC-1 vector is 2.5×10^{12} viral particles/ml (9.8×10^{10} pfu/ml). The control vector devoid of the PC-1 cDNA (empty vector) has a titer of 2.4×10^{12} viral particles/ml (8.8×10^{10} pfu/ml).

Hepatic PC-1 gene transfer. Male C57 BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and fed with standard rodent diet and water ad libitum in a barrier animal facility with a 12-h light/dark cycle. Before treatment, animals were stratified by body weight and randomly assigned to two groups (*n* = 7 per group) to ensure a similar mean body weight (10 weeks old, 25.4 ± 1.7 g) among groups. The two groups of mice were treated with intravenous administration of 3×10^{12} viral particles/kg of the PC-1 vector or an equivalent dose of the control vector without PC-1 (empty vector), respectively. This vector dose has previously been defined to result in ~70% transduction of hepatocytes in the liver by adenovirus following intravenous injection (17,18). Animals were killed following 1 week after PC-1 adenovirus treatment, and the liver tissue was collected for PC-1 analysis (see below) and preparation of total hepatic RNA.

Glucose tolerance test. Mice were fasted for 5 h, followed by an intraperitoneal injection dose of glucose (3 g/kg body wt). Blood glucose levels were determined both before and at different times after the glucose.

Insulin tolerance test. Fed mice were given an intraperitoneal injection of insulin (0.5 units/kg body wt). Blood glucose levels were determined both before and at different times after the injection.

PCR. Real-time quantitative RT-PCR assay was employed to quantify the relative abundance of mRNA for the following enzymes: phosphenolpyruvate

carboxykinase (Pck1), glucose-6-phosphatase (G6pc), glucokinase, and the forkhead transcription factor Foxo1. β-Actin mRNA was used as an internal standard as described (18).

Tissue preparation. Mouse tissue was pulverized under liquid nitrogen, and the resultant powder was homogenized in a polytron homogenizer (Kinematica, Lucerne, Switzerland) for 10 s. Homogenates were then centrifuged at 100,000*g*, solubilized for 1 h at 4 °C, then recentrifuged at 100,000*g* for 1 h (9).

Thymididne-5′-monophosphate-*p*-nitrophenyl ester assay. Alkaline phosphodiesterase I activity was assayed by the hydrolysis of thymididne-5′-monophosphate-*p*-nitrophenyl ester (PNTP; Sigma) as previously described (9).

Western blot for PC-1. Direct analysis of human and mouse PC-1 was as follows: 100 μg protein from each sample was treated with PAGEprep (Pierce Chemicals) and boiled in the presence of 5% β-mercaptoethanol. Proteins were resolved on a 4–12% Tris-glycine gel (Cambrex) and transferred to nitrocellulose. Blots were blocked in SuperBlock (Pierce Chemicals) and then incubated with anti–PC-1 antibody (gift from Dr. Scott Hickman) that recognizes both human and rodent PC-1. After washing, blots were incubated with anti-rabbit horseradish peroxidase. Signal was developed by using SuperSignal reagent (Pierce Chemicals).

Immunoprecipitation and analysis of human PC-1. A total of 100 μg protein was precleared with protein A Sepharose, and affinity-purified anti–PC-1 (human specific) was added. The precipitates were analyzed by Western blot as above using human-specific PC-1 antibody.

PC-1 enzyme-linked immunosorbent assay. Human PC-1 was measured as described (16). This PC-1 enzyme-linked immunosorbent assay (ELISA) is specific to human PC-1 with no significant cross-reactivity with mouse PC-1.

IR autophosphorylation and Akt/protein kinase B phosphorylation. Western blots of IR autophosphorylation and Akt/protein kinase B (PKB) phosphorylation were measured in immunoprecipitates as described by Michael et al. (15). IR autophosphorylation in liver particles was measured by ELISA as described (11). Incubation conditions were as follows: 10 μmol/l ATP, 150 mmol/l NaCl, 2 mmol/l $MnCl_2$, 10 mmol/l $MgCl_2$, 50 mmol/l TRIS pH 7.4, and 50 μg/ml membrane protein. Insulin was measured using an insulin ELISA kit from Crystalchem.

Statistics. Statistical analyses of data were performed by ANOVA using StatView software (Abacus Concepts, Piscataway, NJ). An unpaired ANOVA *t*

test was conducted to study statistical significance between PC-1 and control vector treatment groups. Data were expressed as means ± SE. *P* values < 0.05 were considered significant.

RESULTS

Increased PC-1 expression in liver. After PC-1 adenovirus administration, we employed several distinct approaches to document the expression and function of PC-1 in mouse liver (Fig. 1). Since PC-1 has phosphodiesterase activity, the protein is able to hydrolyze the substrate PNTP (9) (the enzyme activity of PC-1, however, is not necessary for its inhibition of the IR [10]). In PC-1 adenovirus–treated mice, PNTP hydrolysis was over twofold greater than control animals treated with empty vector (Fig. 1*A*). To determine whether this activity was due to expression of human PC-1, we performed two types of experiments. First, we evaluated PC-1 content by human-specific PC-1 ELISA (Fig. 1*B*). Human PC-1 was detected in PC-1 adenovirus–treated animals but not in controls. Second, we carried out two types of Western blots. Initially, we probed a solubilized, particulate fraction of liver with an antibody that recognizes both mouse and human PC-1 (Fig. 1*C*). Mouse PC-1 monomer has a molecular size of 120 kDa and was detected in livers from both PC-1 adenovirus animals and controls. In contrast, human PC-1 monomer, which has a molecular size of 130 kDa, was detected in the livers of only the PC-1 adenovirus–treated animals. Next we employed a species-specific antibody that recognized only human PC-1. Human PC-1 was only detected in the PC-1 adenovirus–treated animals (Fig. 1*D*).

Reduced IR function and signaling in liver. In view of the fact that PC-1 inhibits IR autophosphorylation (5), we studied insulin activation of this function and the downstream mediator of IR signaling, Akt/PKB, in liver from adenovirus PC-1 animals in the fed state (see below). Western blots revealed that the PC-1 mice had decreased tyrosine autophosphorylation of the IR and decreased serine phosphorylation of Akt/PKB (Fig. 2*A*). Next, we studied the activity of the IR in vitro by employing liver particles. Both basal and insulin-stimulated IR autophosphorylation were significantly decreased by 25–35% in liver from the PC-1 mice (Fig. 2*B*).

Key gluconeogenic enzymes are elevated in liver. In view of the decreased IR signaling in liver, we measured the expression of two key enzymes: Pck1 and G6pc by quantitative PCR. Both are negatively regulated by insulin at the transcriptional level (18). In mice with elevated hepatic levels of PC-1, the levels of Pck1 and G6pc were twofold higher than in controls (Fig. 3*A* and *B*). In contrast, the hepatic enzyme glucokinase, which is not regulated by insulin, was unchanged in the animals with elevated PC-1 (Fig. 3*C*). These observations indicate that the livers of these animals were resistant to suppression of G6pc and Pck1 expression by insulin and are in concert with the observation that insulin-induced IR autophosphorylation was decreased.

It is now recognized that forkhead transcription factor, Foxo1, mediates the negative transcriptional effects on G6pc and Pck1 by the IR (18,19). The activation of the IR by insulin leads to activation of the serine kinase Akt/PKB. Akt/PKB reduces both Foxo1 content and its presence in the nucleus. Accordingly, Foxo1 expression was measured (Fig. 3*D*). In mice with elevated hepatic expression of
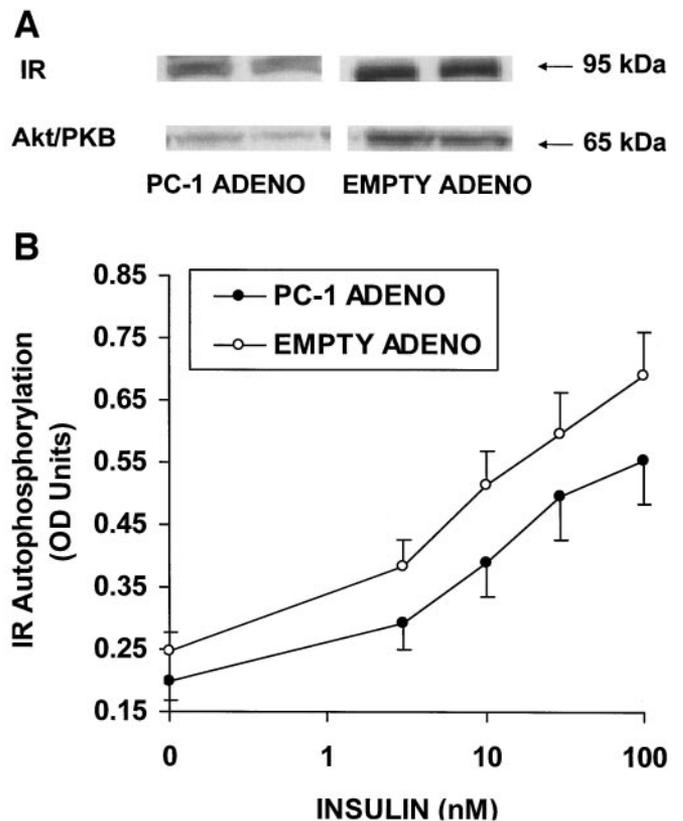

FIG. 2. Insulin receptor signaling in liver. *A*: Effect of PC-1 expression on IR autophosphorylation in vivo. Liver fractions were obtained from four fed animals and immunoprecipitated with antibodies to either the IR or Akt/PKB, normalized to equal amounts of protein, and Western blotted against an antibody to phosphotyrosine. *B*: Effect of PC-1 expression on IR autophosphorylation in vitro. Liver fractions from the animals in Fig. 1 were incubated with insulin, ATP, and metals and IR autophosphorylation measured.

PC-1, we observed that the expression of this nuclear regulatory factor was significantly increased, indicating that this tissue was resistant to the effect of insulin on function.

PC-1 mice have elevated glucose and insulin levels and abnormal glucose tolerance. Next, we characterized the metabolic status of mice with elevated levels of PC-1 in liver. In the fed state, mice with increased hepatic PC-1 showed glucose levels that were 30–40 mg/dl higher than those of controls (Fig. 4*A*). Moreover, they had an over twofold increase in insulin levels, indicating that they were resistant to the action of insulin (Fig. 4*B*). To study the mechanisms through which elevated PC-1 levels in liver induced insulin resistance, we performed glucose tolerance tests. Because the liver is the primary site of glucose regulation in rodents (12,15) and in view of elevated content of hepatic PC-1 with decreased IR autophosphorylation, we predicted that these mice would be glucose intolerant. Indeed, during glucose tolerance tests, glucose levels were significantly higher in mice with increased hepatic PC-1 at all time points (Fig. 4*C*). At 30 min after glucose administration, the glucose levels were >100 mg/dl higher in the animals with elevated PC-1; at 2 h, there was still an elevation in this function.

Studies in skeletal muscle and heart. As mentioned above, in contrast to humans, most rodent models of muscle insulin resistance are not glucose intolerant, re-
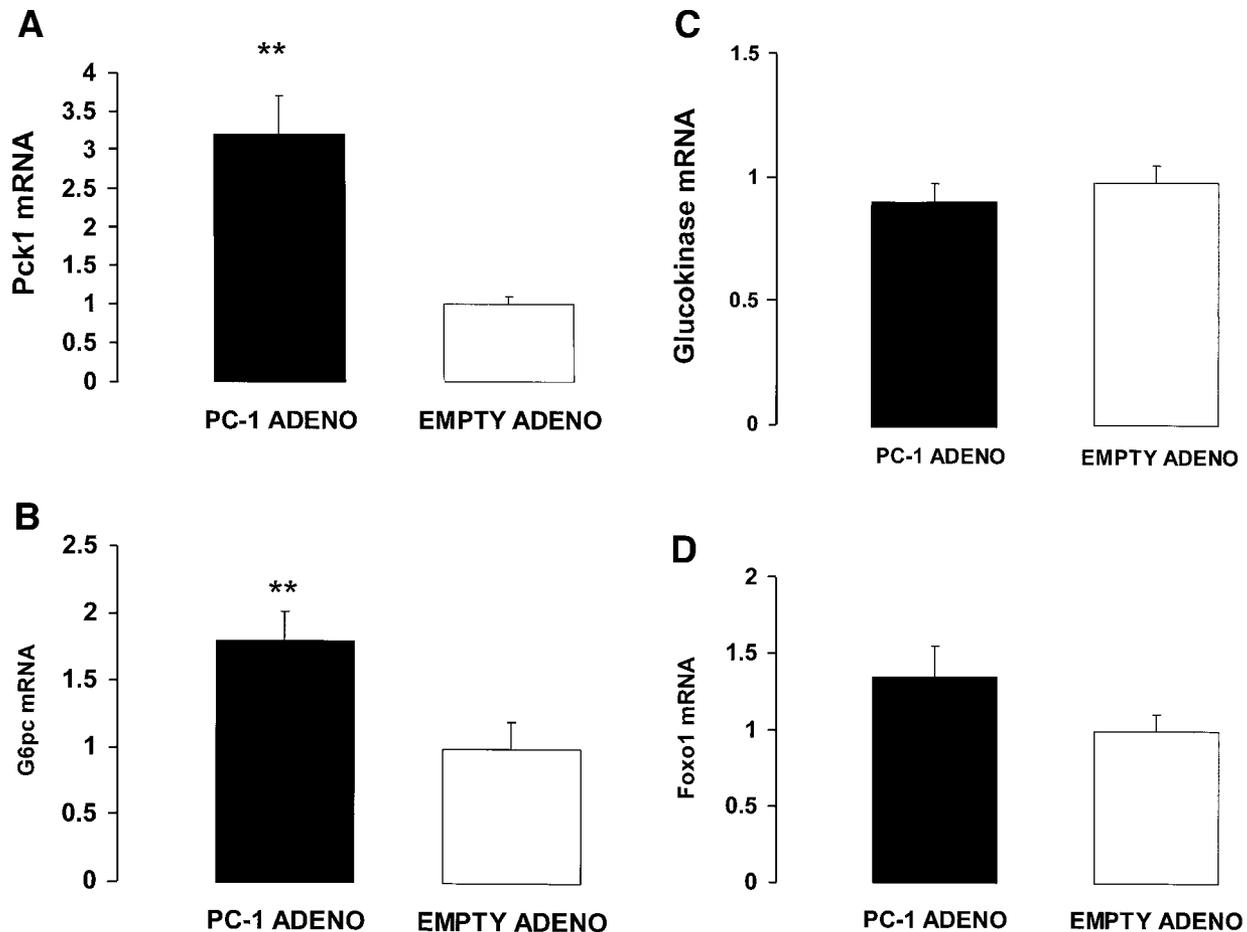
FIG. 3. Analysis of liver enzymes by RT-PCR. mRNAs for four hepatic enzymes were measured in fed animals 7 days after infection. *A*: Pck1. *B*: G6pc. *C*: Glucokinase. *D*: Foxo1. Except for glucokinase, the differences between PC-1 adenovirus and empty adenovirus were significant (*P* < 0.01 for Pck1 and G6Pase and *P* < 0.05 for Foxo1). Data are expressed relative to β-actin.

flecting major species-specific differences (12). In contrast to liver, in skeletal muscle and heart, there was no evidence for increased PC-1 activity as evidenced by PNTP hydrolysis (Fig. 5), PC-1 ELISA (<1 ng/mg protein), and Western blot (data not shown). Moreover, IR autophosphorylation was not altered in these tissues (data not shown). Because muscle is the major tissue for glucose disposal in response to insulin, we predicted that insulin tolerance tests would be normal in these animals. Indeed, when these tests were performed, there was no difference between PC-1 and control mice (Fig. 6).

DISCUSSION

In humans, there is considerable evidence linking insulin resistance with PC-1. PC-1 is elevated two- to threefold in muscle and adipose tissue of insulin-resistant humans (7,9,20). These two key tissues for regulating insulin-mediated glucose metabolism are resistant to insulin action in humans with type 2 diabetes (2). Moreover, PC-1 is elevated in cultured fibroblasts from many insulin-resistant subjects (5,6,8). This observation indicates that there may be a genetic or intrinsic overexpression of this protein in certain insulin-resistant subjects. There are two alleles of PC-1, the common arginine-encoding (K) allele at amino acid 121 and the less-common glutamine-encoding (Q) allele at this site (21). The latter binds to the IR with higher

affinity than the K allele and is more potent in inhibiting IR autophosphorylation. Moreover, in some ethnic populations, the Q allele is strongly associated with insulin resistance (21). The PC-1 gene is at locus 6q22–23. Duggirala et al. (22), studying Hispanic families in San Antonio, Texas, have found linkage with the q22–23 locus and insulin resistance. Also, in a genome-wide scan, Meyre et al. (23) have found linkage between 6q22.31-q23.2 and childhood obesity traits and insulin resistance.

The present study indicates that modest (two- to three-fold) overexpression of human PC-1 in mouse liver by adenovirus infection leads to insulin resistance in this tissue. IR autophosphorylation and downstream signaling were impaired, as was glucose tolerance. There was no detectable human PC-1 expression in muscle, and IR signaling was not affected in this tissue. A comparison of these data and the IR knockout data in liver and muscle (24) are of interest. In the liver knockout mice, like the mice studied herein, the glucose tolerance test was impaired. Since the impairment of the IR in the adenovirus PC-1 mice was much less severe than in the knockout mice, these data suggest that this function is very sensitive to the status of the liver IR. In the liver knockout, but not in the animals studied herein, there was also impairment of the insulin tolerance test. There are several possible explanations for these seemingly discordant observations.
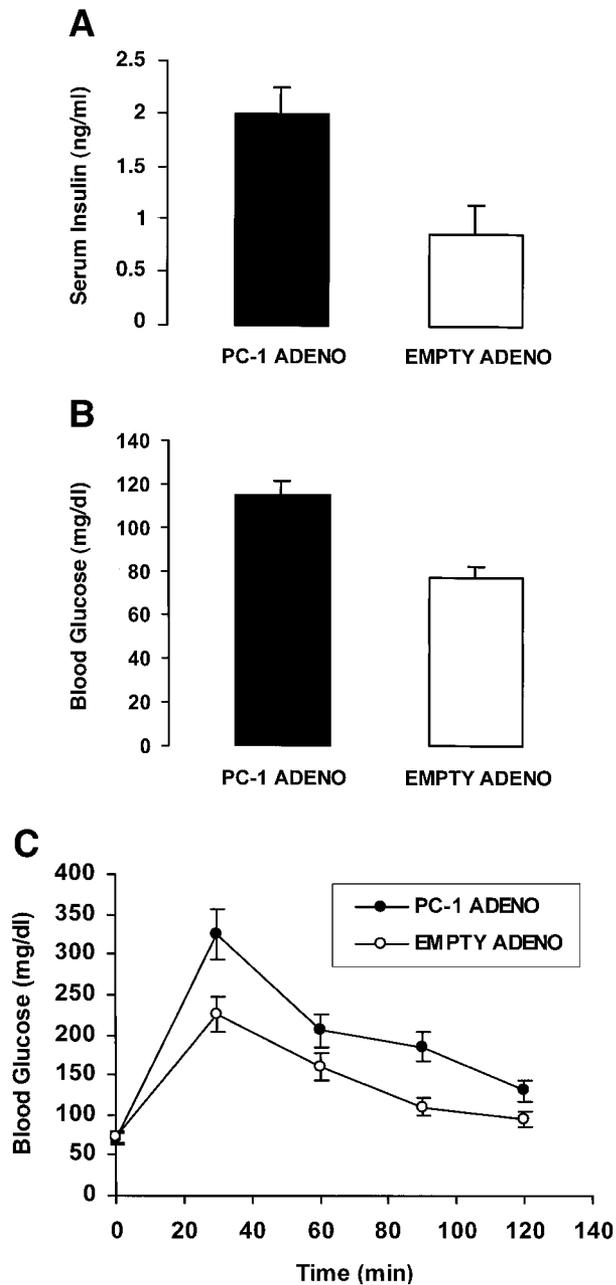
A

B

C

FIG. 4. Metabolic effects of increased PC-1 levels. *A* and *B*: Serum was obtained from fed animals 7 days after infection, and glucose and insulin were measured. The differences in these parameters between PC-1 adenovirus and control animals were significant (insulin and glucose levels $P < 0.01$). *C*: In fasting mice, glucose tolerance tests were performed in groups of animals at 3 and 5 days after infection, and the same results were obtained on both days. Data from day 5 are shown. Differences in glucose levels were significant at 30, 60, 90, and 120 min ($P < 0.05$ or 0.01).

FIG. 5. Analysis of human PC-1 activity in heart and skeletal muscle. In these studies, seven PC-1 adenovirus–treated animals and seven empty virus controls were studied 1 week after infection. Skeletal muscle and heart were removed, and a solubilized high-speed membrane fraction was prepared (9). PNTP activity was measured. In both tissues, no significant changes were observed between PC-1 and control animals.

In contrast to the PC-1 adenovirus animals, the knockout animals have the lesion from very early in development. Also, the impairment of the liver IR signaling pathway in the knockout animals is much greater than with the PC-1 adenovirus animals. Thus, in the liver knockout animals, it is likely that other unrecognized factors (i.e., developmental or compensatory) may be present leading to the loss of muscle function.

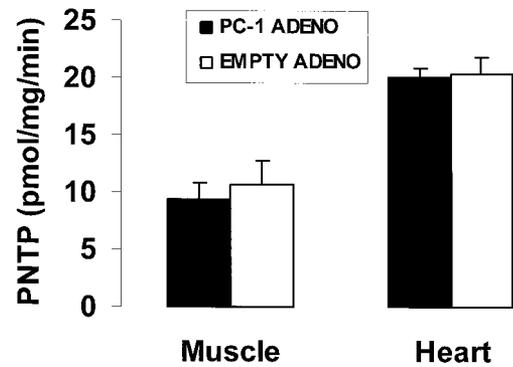Although there is considerable evidence that PC-1 may contribute to insulin resistance, a major drawback in investigating the role of PC-1 has been the lack of convenient animal models for studying the effects of elevated PC-1 content on insulin and glucose economy. The obese, insulin-resistant rhesus monkey has elevated PC-1 levels in muscle that correlate with insulin resistance (25), but it is not an animal that can be easily investigated. For two reasons, therefore, the present studies with mice having elevated liver content of PC-1 are important. First, they support the concept that PC-1 plays a role in clinical insulin resistance. Second, they present an animal model in which agents that antagonize PC-1 can be tested. Thus, with these and similar types of engineered animals having PC-1 overexpression, it should be feasible to test the effects of anti–PC-1 agents, such as small molecules (26) and antibodies (11), on impaired insulin action in vivo. Thus, an in vivo approach may have the potential to lead to the development of new agents for the treatment of clinical insulin resistance.

PC-1 is a class II transmembrane protein that is expressed in most cell types and is a member of the NPP family (4,27). These members include PD-1α (autotaxin) and PD-1β (gp103RB). PC-1 is the same protein as liver nucleotide pyrophosphatase-alkaline phosphodiesterase. PC-1 exists as a disulfide-linked homodimer of 230–260 kDa; the reduced form of the protein has a molecular size of 114–135 kDa, depending on the cell type and species. The physiological role of PC-1 is not completely defined, but there is evidence in bone and cartilage metabolism that PC-1 scavenges ATP and in doing so produces inorganic pyrophosphate (28). Pyrophosphate modulates pathologic calcification (28). Humans without PC-1 are severely ill, with abnormal bone deposition in the aorta and some periarticular tissues (29,30). Conversely, cartilage PC-1 expression and NPP activity become markedly upregulated in association with the aging-associated cartilage degenerative disorders osteoarthritis and chondrocalcinosis, where PC-1 overactivity appears to be part of an adaptive response to chondrocyte ATP depletion (31).

In conclusion, we have developed a mouse model of modest liver overexpression of the IR inhibitor PC-1. These animals are insulin resistant and glucose intolerant, mimicking early type 2 diabetes. Since PC-1 overexpression is common in humans with insulin resistance, our results suggest that PC-1 dysregulation is one cause of
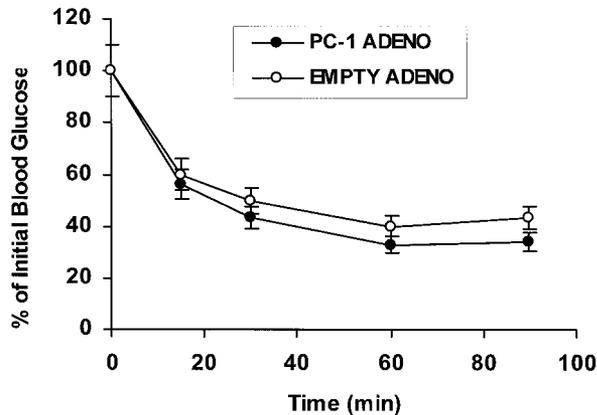
FIG. 6. Insulin tolerance tests. Tests were performed 7 days after infection in fed animals. Data are expressed as the percent of the control glucose values shown in Fig. 4. Differences in glucose levels were not significant at any time point.

human insulin resistance. It should be pointed out, however, that given the major differences in the metabolism of glucose and insulin between mice and humans as stated above, further studies of PC-1 overexpression in muscle will be needed to support this hypothesis.

ACKNOWLEDGMENTS

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