Overexpression of the insulin receptor inhibitor PC-1/ENPP1 induces insulin resistance and hyperglycemia

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TYPE 2 DIABETES MELLITUS (T2D) is a common disease in developing countries (2, 25). A major contributor to T2D is insulin resistance. It has been proposed that insulin resistance in T2D may result from multiple causes that include genetic and acquired influences (23, 26). In most insulin-resistant individuals, however, the molecular cause(s) of insulin resistance is either unknown and/or unproven (15, 23, 26). Thus specific therapy to treat this condition is unavailable.

One candidate molecule to cause insulin resistance is the ectoenzyme, plasma cell membrane glycoprotein-1 PC-1 (PC-1, ENPP1), a nucleotide pyrophosphatase-phosphodiesterase that, under physiological conditions scavenges ATP (10). In insulin-resistant subjects, PC-1 is elevated in both tissues and fibroblasts (5, 7, 18, 27, 28). In addition, overexpression of PC-1 in cultured cells reduces both insulin-stimulated IR activation and downstream signaling (11). PC-1 binds to the IR but does not block insulin binding (17, 18). Rather, PC-1 inhibits the insulin-induced conformational changes that lead to IR autophosphorylation and tyrosine kinase activation (17). Although these data suggested that PC-1 is involved in insulin resistance, a complete understanding of the role of PC-1 in this pathological process was hampered by the lack of a convenient animal model of PC-1 overexpression.

Previously, we (3) demonstrated that overexpression of human PC-1 in liver of mice produced via adenoviral transduction induced insulin resistance and glucose intolerance; however, the animals were not overtly diabetic. There are several possible reasons for this result. One reason is that diabetes is a chronic condition and adenoviral transduction is a transient approach. Another is that we targeted only liver, and it is possible that another major insulin-sensitive tissue, such as muscle, needs to overexpress PC-1 to induce hyperglycemia.

Herein, to investigate the effect of increased levels of PC-1 on glucose metabolism in mice, we produced transgenic mice with human PC-1 cDNA expressed in both liver and muscle. In addition, we also employed the potent q allele of PC-1 (24). Compared with control littermates, PC-1 mice had fed and fasting hyperglycemia with hyperinsulinemia. These results suggest, therefore, that PC-1 may play a role in the insulin resistance and hyperglycemia of T2D.

METHODS

PC-1 transgenic animals. The wild-type full-length human PC-1 q allele was subcloned into plasmid pRK7 under the control of the CMV promoter, as previously described (11). Transgenic animals were made as follows. Gene constructs were freed of plasmid sequences by gel electrophoresis after appropriate restriction enzyme digestion. The DNA was injected into the pronucleus of fertilized mouse eggs harvested from recently mated and superovulated, 4- to 6-wk-old female, white FVN mice and black B6D2 mice. After the animals were euthanized, the pancreas of each animal was removed and homogenized. PC-1 transgenic animals were then centrifuged at 100,000 × g for 1 h. The resultant supernatants were then centrifuged at 100,000 × g for 1 h.

Thymidine-5'-monophosphate-p-nitrophenyl ester assay. Alkaline phosphodiesterase 1 activity was assayed by the hydrolysis of thymi-
dine-5'-monophosphate-p-nitrophenyl ester (PNTP; Sigma) as previously described (28).

**Western blot for PC-1.** For direct analysis of human PC-1, 500 μg of protein from each sample were immunoprecipitated with an antibody that recognizes undenatured mouse and human PC-1 (3). The immunoprecipitates were 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 an anti-PC-1 antibody that recognizes denatured mouse and human PC-1 (3). After a washing, blots were incubated with anti-rabbit horseradish peroxidase. Signal was developed using SuperSignal reagent (Pierce Chemicals).

**Insulin ELISA.** Insulin was measured using a kit from Crystalchem.

**Glucose metabolic index.** To obtain an index of insulin-stimulated tissue-specific glucose metabolism (Rg), 5-h-fasted mice (5 transgenic and 7 controls) were studied at 5 days after chronic catheter implantation, as described previously (9). Surgical procedures and postoperative catheter maintenance of catheters were the same as those used previously by the Vanderbilt Mouse Metabolic Phenotyping Center (8, 9, 12). An infusion of 4 μU·kg⁻¹·min⁻¹ insulin was started at −120 min. Euglycemia was maintained during insulin infusion by measuring arterial blood glucose (~5 μl of whole blood) every 10 min and infusing 50% dextrose as necessary. The glucose infusion rates required to maintain euglycemia were 44 ± 5 and 42 ± 6 mg·kg⁻¹·min⁻¹ in control and PC-1 animals, respectively. Mice received saline-washed red blood cells from a donor mouse to minimize reductions in hematocrit (>5%). At 5 min, a 12-μCi bolus of 2-deoxy-[¹⁴C]glucose ([¹⁴C]2-DG) was administered to determine an index of muscle glucose uptake. At 7, 10, 15, 20, and 30 min, arterial blood was sampled to determine arterial blood glucose and plasma [¹⁴C]2-DG. At 30 min, mice were anesthetized with an infusion of pentobarbital sodium. Tissues were excised, immediately frozen in liquid nitrogen, and stored with plasma samples at −70°C until future tissue analysis. All procedures performed were approved by the Vanderbilt University Animal Care and Use Committee.

Plasma samples were deproteinized with Ba(OH)₂ and ZnSO₄, and then [¹⁴C]2-DG radioactivity was determined by liquid scintillation counting (TRI-CAR 2900TR; Packard, Meriden, CT) with Ultima Gold (Packard) as scintillant. Frozen muscle samples were weighed, homogenized in 0.5% perchloric acid, centrifuged, and neutralized with KOH. One supernatant aliquot was counted directly to determine radioactivity from free [¹⁴C]2-DG (5). [¹⁴C]2-DGP is calculated as the difference between the two aliquots. The accumulation of [¹⁴C]2-DG was normalized to tissue weight and tracer bolus in all experiments. Rg was calculated as previously described (16).

**Statistics.** Statistical analyses of data were performed by analysis of variance (ANOVA) using StatView software (Abacus Concepts, Berkeley, CA). An unpaired ANOVA t-test was conducted to study statistical significance between PC-1 and control vector treatment groups. Data are expressed as means ± SE. P values of <0.05 were considered significant.

**RESULTS**

**PC-1 is expressed in liver, muscle, and brain but not fat.** To generate transgenic mice, we employed a vector encoding the potent human PC-1 q allele under the control of the CMV promoter (11). We injected both FVB/N mice and B6D2 mice (a C57B-BDA hybrid). We obtained five lines of PCR-positive FVB/N mice and two lines of B6D2 mice. Two lines of FVB/N mice (but not B6D2 mice) expressed human PC-1 in tissues. Expression of PC-1 was similar in both, and only one line was studied further (Fig. 1). As assessed by the PNTP assay, which measures the enzymatic activity of PC-1 (3), an increase in PC-1 was observed in soleus muscle, liver, and brain, but not adipose tissue. Small increases in PC-1 activity were observed in lung and spleen (data not shown). Western blots for PC-1 in liver and muscle were then carried out (Fig. 2). As expected, mouse PC-1 was detected both in PC-1 and in control animals, whereas human PC-1 was detected only in the transgenic animals.

**Insulin and glucose levels.** Compared to controls, in the fed state PC-1 mice had sixfold higher fed insulin levels than controls. PC-1 mice had elevated glucose levels (>250 mg/dl), indicating that they were insulin resistant and diabetic (Fig. 3). In the fasting state, insulin levels were 0.7 ± 0.1 ng/ml in controls and 1.6 ± 0.1 ng/ml in PC-1 mice.

**Glucose tolerance tests.** PC-1 animals had fasting hyperglycemia and glucose intolerance (Fig. 4). Basal glucose levels were nearly 70 mg/dl higher than controls, and after glucose challenge they rose to over 150 mg/dl greater than controls.

**Glucose metabolic index (Rg).** Arterial glucose levels were maintained at ~160 mg/dl during constant insulin infusion. In PC-1 animals, Rg was decreased in oxidative muscles (soleus and diaphragm; Fig. 5). In contrast, the Rg in nonoxidative muscles (superficial vastus lateralis and gastrocnemius), as well as in fat, were not decreased in PC-1 animals. Interestingly, there was a small decrease of this function in brain.

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**Fig. 1.** PC-1 expression in liver and muscle of transgenic (TG) animals. In these studies, 6 male PC-1 TG animals and 6 male controls of the same age and weight were studied at 3 mo of age. Organs were removed, a solubilized high-speed membrane fraction was prepared (28), and PC-1 enzyme activity was measured with the substrate, thymidine-5'-monophosphate-p-nitrophenyl ester (PNTP). Soleus muscle, P < 0.001; liver, P < 0.01; brain, P < 0.05.

**Fig. 2.** Human (h)PC-1 overexpression in liver and soleus muscle by Western blot. Tissue extracts were Western blotted with an antibody that recognizes both human and mouse (m)PC-1. Purified PC-1 proteins alone and in combination are shown to demonstrate that hPC-1 has a relative molecular mass of 130 kDa and mPC-1 has a smaller relative molecular mass of 110 kDa.
**DISCUSSION**

In humans there is considerable evidence linking insulin resistance with PC-1. PC-1 is elevated two- to threefold in tissues of insulin-resistant humans (6, 7, 28). Moreover, PC-1 is elevated in cultured fibroblasts from many insulin-resistant subjects (5, 18, 27). 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 lysine-encoding k allele at amino acid 121 and the less common glutamine-encoding q allele at this site (24). The q allele binds to the IR with higher affinity than the k allele does and is more potent in inhibiting IR autophosphorylation (17). Moreover, in some ethnic populations, the q allele is strongly associated with insulin resistance (24).

The PC-1 gene is at locus 6q22–23. Stern and colleagues, studying Hispanic families in San Antonio, have found linkage with the q22–23 locus and insulin resistance (4). Also, in a genome-wide scan, Froguel and colleagues have found linkage between 6q22.31 and q23.2, and childhood obesity traits and insulin resistance (20). Recently, in collaboration with this group and studying a very large population, we (19) have identified an “at-risk” haplotype of the q allele that very strongly correlates with both T2D and childhood and adult obesity.

Although there is considerable evidence that PC-1 may contribute to insulin resistance, a major drawback in investigating the role of PC-1 had 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 (22), but it is not an animal that can be easily investigated. Our previous study (3) indicated that modest (2- to 3-fold) overexpression of human PC-1 in mouse liver by adenovirus infection leads to insulin resistance in this tissue with concomitant glucose intolerance. In the present study with PC-1 transgenic animals, we now find that the overexpression of PC-1 in muscle and liver leads to insulin resistance and both fasting and fed hyperglycemia. Most likely, both the chronic overexpression of PC-1 in two insulin-sensitive tissues combined with the use of the more potent q allele contributed to the diabetic state.

Herein, we also observed that PC-1 overexpression decreased glucose uptake in a tissue-specific manner. Rg was decreased in soleus and diaphragm, which are very oxidative and very sensitive to insulin (8, 12). In contrast the nonoxidative muscle groups (superficial vastus lateralis and gastrocnemius), which are less sensitive to insulin, were not affected. As expected, Rg fat, where PC-1 was not overexpressed, was not affected. Surprisingly, overexpression of PC-1 in brain caused a small impairment of Rg. Although responsive to insulin in selected regions (21), generally the brain glucose uptake is unresponsive to insulin. Whether this is an insulin-dependent effect on the brain is unclear. The lack of a parallel fall in the glucose infusion rate during the clamp was not surprising, given the selected group of tissues that were negatively impacted by PC-1 overexpression. Thus an effect of PC-1 that was marked when tissue specific glucose uptake was assessed was essentially undetectable when the glucose infusion rate was used to assess the effect. This finding emphasizes the added sensitivity obtained using tissue-specific isotopic methods specifically for evaluating tissue glucose metabolism.
PC-1 is a class II transmembrane protein that is expressed in most cell types and is a member of the NPP family (1, 10). 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 mass 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 (13, 14).

In conclusion, we have developed a transgenic mouse model of overexpression of the IR inhibitor PC-1. These animals are insulin resistant and hyperglycemic, mimicking T2D. Because PC-1 overexpression is common in humans with insulin resistance, our results suggest that PC-1 dysregulation could possibly be one cause of human insulin resistance.

**NOTE ADDED IN PROOF**

Abate et al. have recently reported that overexpression of the PC-1/ENPP1 q allele in adipose tissue in mice leads to insulin resistance and diabetes. (Abate N, Cook W, Chandalia M, and Livingston E. Metabolic and genetic determinants of insulin resistance. Proceedings of the First International Workshop on PC-1/ENPP1, Rome, Italy, December 9, 2005). These data are in concert with our current data and suggest that overexpression of PC-1 in adipose tissue, in addition to muscle and liver, can cause insulin resistance and diabetes.

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**REFERENCES**


