Communication

Inhibition of Insulin Receptor Phosphorylation by PC-1 Is Not Mediated by the Hydrolysis of Adenosine Triphosphate or the Generation of Adenosine*

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Individuals with insulin resistance show increased levels of PC-1 expression in skeletal muscle and fibroblasts, and in transfected cell lines that overexpress PC-1 there is a reduction in the insulin-stimulated insulin receptor tyrosine phosphorylation. As PC-1 is a type II transmembrane protein with extracellular phosphodiesterase and pyrophosphatase activity, increased expression of PC-1 at the cell surface will decrease extracellular adenosine triphosphate levels and increase extracellular adenosine levels. Consequently it is possible that PC-1-mediated insulin resistance could be caused either by a decrease in adenosine triphosphate or an indirect increase in adenosine levels. We have tested this hypothesis and find that the PC-1-mediated inhibition of insulin-stimulated insulin receptor autophosphorylation is not altered by agents that alter the level or action of adenosine. Further, a mutated PC-1 with a single amino acid change that abolishes the phosphodiesterase and pyrophosphatase activities is still able to inhibit insulin-stimulated insulin receptor phosphorylation. The results of these experiments indicate that the phosphodiesterase activity of PC-1 is not involved in the inhibition of insulin receptor autophosphorylation.

Insulin controls glucose homeostasis by regulating the production of glucose by the liver and the uptake of glucose into muscle and fat. If these actions of insulin are inadequate, glucose levels rise and diabetes mellitus develops. The most common form of diabetes mellitus is non-insulin-dependent diabetes mellitus (NIDDM). While the cause(s) of non-insulin-dependent diabetes mellitus is unknown, this form of diabetes is accompanied by insulin resistance, an inability of insulin to appropriately trigger cellular responses. Except in rare individuals, this insulin resistance is not the result of alterations of the number or structure of the insulin receptors; rather the defect lies distal to the insulin receptor in the insulin signaling pathway (for reviews see Refs. 1-4).

We have demonstrated that in muscle and fibroblasts of many patients with insulin resistance there is increased expression of PC-1 (5). When the mammary epithelial cell line, MCF-7, is transfected with a PC-1 cDNA expression vector, expression of PC-1 is increased and the cells become insulin-resistant (5). PC-1 is a type II (extracellular C-terminus) transmembrane glycoprotein (6). Although initially identified in plasma cells, it is now known to be expressed in a wide variety of cell types (7). PC-1 is also known as nucleotide pyrophosphatase/alkaline phosphodiesterase I, which has pyrophosphatase as well as phosphodiesterase activity (8). These activities of PC-1 suggested two hypotheses as to how PC-1 could decrease insulin receptor phosphorylation. One possibility is that the pyrophosphatase activity leads to a loss of ATP either at the cell membrane or in an intracellular trafficking pool, and this reduction in ATP could impact negatively on insulin signaling. Another possibility is that extracellular ATP hydrolysis could lead indirectly to an increase in extracellular adenosine, which could act through one of the adenosine receptors to reduce the response of PC-1 expressing cells to insulin. Both of these hypotheses rely on the pyrophosphatase activity of PC-1.

We have tested these hypotheses in two ways. First we have used pharmacological agents to either alter the level of extracellular adenosine or the signaling through the adenosine receptor. None of these manipulations altered the insulin resistance in either control MCF7 cells or in MCF7 cell lines that express PC-1. In addition we have introduced an amino acid change into PC-1 that abolishes the pyrophosphatase and phosphodiesterase activity. When an expression plasmid encoding this variant is introduced into MCF7 cells, the protein is expressed without these two enzymatic activities. This variant is, however, as active as the wild type PC-1 protein in its ability to reduce the insulin-stimulated autophosphorylation of the insulin receptor kinase. Taken together these data suggest that the phosphodiesterase activity of PC-1 is not involved in the inhibition of insulin receptor phosphorylation.

MATERIALS AND METHODS

Cell Lines—The human mammary epithelial cell line MCF7 was cultured in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal calf serum, nonessential amino acids, and antibiotics. To increase the level of PC-1, cultures of MCF7 cells were transfected by electroporation using plasmids with either the antibiotic resistance gene neo under the control of the cytomegalovirus promoter or this plasmid and one that contained the human PC-1 cDNA under the control of the cytomegalovirus promoter. The PC-1 cDNA used was one in which the 5'-most ATG was included (9). A plasmid that contained the more 3'-ATG was provided by Dr. Jim Goding (Monash University, Melbourne). The missing sequence for the 5'-ATG was added using a synthetic oligonucleotide. This part of the plasmid was sequenced. The numbering used this N-terminal-most methionine as 1 and indicates a protein of 925 amino acids in length. Clones of cells resistant to the antibiotic G418 were identified and grown under selective conditions and assayed for the expression of the PC-1 protein by Western analysis and by enzymatic assay.

ATP Pyrophosphatase Assay—The enzymatic assay for the PC-1 protein used a modification of the phosphoadenosine phosphosulfate hydrolysis assay (5). In this assay the radioactive substrate phosphoadenosine phosphosulfate was replaced by [γ-33P]ATP. The final concentration of ATP in the assay was 0.25 mM. The unlabeled ATP was supplemented with 0.01 mCi of [33P]ATP that had a specific activity of 2000 Ci/mmol. The reaction and determination of activity were carried out as described (5).

Insulin-stimulated Insulin Receptor Phosphorylation—MCF7 cells were grown to approximately 80% confluence in six-well plates. Eight...
The insulin receptor agonists and antagonists. Insulin at 100 nM induced a significant alteration in the sensitivity of either wild type or the PC-1-transfected cells. This lack of effect of adenosine was observed when the adenosine was present for 1 h at either 100 or 10 μM (Fig. 2 and data not shown) or for 1 h at 100 or 10 μM (Fig. 2 and data not shown). Extracellular adenosine is converted to inosine by exogenous adenosine deaminase. If PC-1 were leading to an increased level of extracellular adenosine and this was causing the insulin resistance, then PC-1-transfected cells would have an increase in their response to insulin in the presence of adenosine deaminase. As can be seen in Fig. 2 the presence of adenosine deaminase in the culture medium for the 19 h prior to the insulin response assay did not significantly alter the sensitivity of either wild type or the PC-1-transfected cells. We also tested whether the adenosine receptor antagonist 8-PT was able to alter the response of the cells to insulin. As shown in Fig. 2 the PC-1-induced insulin resistance was not altered by the presence of 8-PT when present for 19 h at 3 μM (Fig. 2) or 1 μM (data not shown).

These pharmacological interventions suggested that the insulin receptor (5). To facilitate an analysis of the mechanism by which the PC-1-mediated inhibition occurs we have developed a 96-well plate-based assay that specifically measures insulin-stimulated insulin receptor phosphorylation (Fig. 1). As described under “Materials and Methods” the assay is dependent on an insulin-mediated signal and is determined by the use of two well characterized monoclonal antibodies. The insulin-dependent response seen in this assay parallels the response determined by Western blotting analysis (data not shown). It has been reported that adenosine can alter insulin sensitivity (see below), and it is known that ATP is required for phosphorylation of the insulin receptor. Thus we hypothesized that the pyrophosphatase activity associated with PC-1 could cause insulin resistance by increasing adenosine or by decreasing ATP. This hypothesis was tested initially using adenosine receptor agonists and antagonists. Insulin at 100 nM induced a comparable phosphorylation of the insulin receptor whether or not adenosine was present. This lack of effect of adenosine was observed when the adenosine was present for 1 h at either 100 or 10 μM (Fig. 2 and data not shown) or for 1 h at 100 or 10 μM (Fig. 2 and data not shown). Extracellular adenosine is converted to inosine by exogenous adenosine deaminase. If PC-1 were leading to an increased level of extracellular adenosine and this was causing the insulin resistance, then PC-1-transfected cells would have an increase in their response to insulin in the presence of adenosine deaminase. As can be seen in Fig. 2 the presence of adenosine deaminase in the culture medium for the 19 h prior to the insulin response assay did not significantly alter the sensitivity of either wild type or the PC-1-transfected cells. We also tested whether the adenosine receptor antagonist 8-PT was able to alter the response of the cells to insulin. As shown in Fig. 2 the PC-1-induced insulin resistance was not altered by the presence of 8-PT when present for 19 h at 3 μM (Fig. 2) or 1 μM (data not shown).

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from both our pharmacological intervention studies and the autophosphorylation of the insulin receptor. In addition, data of adenosine on the response to insulin, as measured by the increase in intracellular pyrophosphate levels possibly within vesicles associated with the movement of PC-1 to the plasma membrane (16). A related hypothesis was that a PC-1-mediated increase in extracellular adenosine could mediate the effects on insulin-stimulated phosphorylation. Adenosine interacts with and signals through the P1-purinergic receptors, which are further categorized as either A1, A2, A3, or A4 adenosine receptors on the basis of selective binding of pharmacological agents. A further distinction between the various adenosine receptors is made on the basis of the intracellular signaling pathways used. The A2 receptor appears to couple exclusively to and activates adenylyl cyclase. In contrast A1 and A3 activation decreases adenylyl cyclase activity, and in addition the A1 receptor affects potassium and calcium channels and can couple to a variety of intracellular messenger systems including G proteins (17-19). That adenosine can influence insulin-mediated signals is well established although whether adenosine acts to increase or decrease insulin sensitivity appears to be very much dependent on the cells of the organ system used and which insulin-mediated event is measured. Adenosine increases insulin sensitivity in isolated fat cells as measured by insulin-mediated glucose phosphorylation and insulin-mediated anti-lipolysis (20–22). In contrast, the effects of adenosine on insulin-stimulated glucose uptake in muscle are less clear. Challiss and colleagues (23) have shown that adenosine receptor antagonists (adenosine deaminase, 8-PT) will increase the sensitivity of isolated muscle strips to insulin. In contrast, Vergauwen et al. (24) reported that adenosine receptor antagonists (caffeine, 8-cyclopentyl-1,3-dipropylxanthine) will decrease the effectiveness of insulin in vivo. In this latter study the adenosine had no effect on insulin action when the muscle was at rest but did decrease insulin-stimulated glucose uptake when the muscles were electrically stimulated.

At least for MCF7 cells, there does not appear to be any effect of adenosine on the response to insulin, as measured by the autophosphorylation of the insulin receptor. In addition, data from both our pharmacological intervention studies and the analysis of the cell lines expressing the pyrophosphatase-negative PC-1 are concordant in demonstrating that the PC-1-mediated inhibition of insulin-stimulated insulin receptor autophosphorylation is not mediated either by the hydrolysis of ATP or the generation of adenosine.

The mechanism by which PC-1 inhibits insulin receptor autophosphorylation is not known. It has been suggested that PC-1 has both intrinsic kinase and phosphatase activities (25, 26) and that PC-1 interacts directly with the fibroblast growth factor receptor (25). As it is likely that any kinase or phosphatase activity associated with PC-1 would be extracellular, it is unclear how these kinase/phosphatase activities would impact on insulin signaling. Furthermore, it has been demonstrated that PC-1 may not be a true kinase; the radiolabeling by $[\gamma-\text{32P}]$ATP may be due to the presence of a covalent ATP-PC-1 intermediate formed during the cleavage of the pyrophosphate bond in the ATP (27). The PC-1 protein also includes amino acid sequence motifs that are characteristic of tyrosine kinase phosphorylation sites, somatomedin B domain signature motifs, and divergent cation binding domains (28). We are currently determining whether these regions may be involved in the inhibition of insulin-stimulated phosphorylation of the insulin receptor.

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