Insulin and Insulinomimetic Agents Induce Activation of Phosphatidylinositol 3'-Kinase upon Its Association with pp185 (IRS-1) in Intact Rat Livers*

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The major cytosolic substrate of the insulin receptor is a 185-kDa phosphoprotein (IRS-1) that contains multiple putative attachment sites for the p85 regulatory subunit of phosphatidylinositol 3'-kinase (PI3K). To examine the possible interaction of pp185 with p85a in vivo, we injected insulin or insulinomimetic agents (a combination of H2O2 and vanadate (H/V)) into the portal vein of anesthetized rats. In this model system, H/V treatment and, to a lesser extent, injection of insulin resulted in rapid and sustained tyrosine phosphorylation of multiple cellular proteins, including pp185/IRS-1. The latter was found to undergo specific association with the p85a regulatory subunit of PI3K but not with two other proteins that contain src homology domains. As p85a was not detectably phosphorylated on tyrosine residues and did not appear to interact directly with the insulin receptor, we conclude that tyrosine phosphorylation of pp185 promotes its association with p85a and the catalytic subunit of PI3K. The recruitment of the holoenzyme may also involve its enzymatic activation and thus constitute an important step in the transduction of insulin signals.

The tyrosine kinase activity of several cell-surface receptors such as those for insulin and insulin-like growth factor-1, epidermal growth factor, PDGF, and colony-stimulating factor-1 receptors plays a central role in cell growth and differentiation (1, 2). Following ligand-induced tyrosine autophosphorylation of these receptors, phosphorylation of endogenous substrates occurs on tyrosine residues which is important in mediating the biological signals of these ligands. A detailed understanding of postreceptor signaling mechanisms requires the characterization of the endogenous substrates involved, a number of which have recently been identified, such as the phosphoinositide-specific phospholipase Cγ, ras-GTPase-activating protein (GAP), the Ser/Thr kinase Raf-1, the cytosolic protein pp185 (IRS-1), the mitogen-activated protein kinase (MAP-2 kinase), and phosphatidylinositol 3'-kinase (PI3K) (reviewed by Cantley (3)). The latter is a heterodimer composed of an 85-kDa regulatory subunit that contains src homology domains (4-6) and a 110-kDa catalytic subunit (7).

Most biological effects of insulin are mediated by the intrinsic tyrosine kinase activity of the insulin receptor (8, 9). One of its potential substrates is pp185, whose phosphorylation on tyrosine residues occurs immediately following insulin stimulation (10, 11). Recently, Sun et al. (12) cloned the pp185 (IRS-1) cDNA, and its predicted amino acid sequence revealed the presence of at least 10 potential tyrosine phosphorylation sites, 6 of which occur in YMXM motifs (12). This motif belongs to a common consensus sequence (D/E)(D/E)Y(M/V)(P/D/E)M present in the PDGF, colony-stimulating factor-1, and Kit receptors (3). Phosphorylation of this consensus sequence is believed to create a binding site for the src homology 2 (SH2) domain of the p85 regulatory subunit of PI3K. Indeed, it has been demonstrated that upon insulin stimulation, increased PI3K activity is associated with the insulin receptor and pp185 (13-15), raising the possibility that pp185 functionally links PI3K to the insulin receptor.

H2O2 and vanadate are both inhibitors of protein tyrosine phosphatases (16, 17) that mimic several of the metabolic effects of insulin and related growth factors. When added together, H2O2 and vanadate (H/V) act synergistically in inducing insulin’s metabolic effects with a potency that exceeds that of insulin itself (18, 19). These effects could be mediated, at least in part, by the marked increase in tyrosine phosphorylation of intracellular proteins, which results from enhanced tyrosine kinase activity and inhibition of phosphotyrosine phosphatase activities (16, 17). We have taken advantage of the use of H/V to amplify the tyrosine phosphorylation of endogenous proteins which may represent potential substrates of the insulin receptor.

To this end, we injected intact rat livers with either H/V or insulin and analyzed several potential substrates. Our results suggest that following in vivo phosphorylation of pp185 by the insulin receptor kinase, the former becomes physically associated with the regulatory and catalytic subunits of PI3K.

**EXPERIMENTAL PROCEDURES**

Insulin and H/V-treated Rat Livers—Male Sprague-Dawley rats (200-300 g) were injected intraperitoneally with Nembutal (60 mg/g of body weight) and were operated on 10–15 min later as soon as anesthesia was complete. The abdominal cavity was opened, the portal vein exposed, and 5 ml of phosphate-buffered saline with or without a combination of H/V (1.5 mM H2O2 and 0.5 mM vanadate) or 200 ml of insulin (2 × 10−6 M) was injected through a 21-gauge needle connected to a mechanical syringe pump. After infusion, the liver was excised rapidly and frozen in liquid nitrogen. Cytosolic extracts were prepared by adding buffer A (5 ml/g) containing 50 mM...
Hepes, pH 7.6, 150 mM sucrose, 80 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 2 mM Na3VO4, 10 mM sodium pyrophosphate, 50 mM NaF, 10 mg/ml aprotinin, 5 mg/ml leupeptin, 10 mg/ml trypsin inhibitor, 2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 1 h at 4°C at 100,000 × g, and aliquots of the supernatants were normalized for protein, mixed with concentrated (5×) Laemmli sample buffer (20), boiled for 5 min, and resolved on 7.5% SDS-PAGE under reducing conditions (20). Total liver extracts were prepared as above using buffer A supplemented with 1% Triton X-100.

Western Immunoblotting—Electrophoretic transfer of proteins from the gels to nitrocellulose papers was performed essentially as described in Ref. 21. Immunoblotting using different antibodies was performed as previously described (16). The ECL kit used for immunoblotting was purchased from Amersham (Amersham, Buckinghamshire, UK), and horseradish peroxidase-conjugated Protein A was used according to the manufacturer’s instructions (Amersham, Buckinghamshire, UK).

Antibodies—Affinity-purified anti-phosphotyrosine (anti-Tyr(P)) antibodies were generated as previously described (16). Antibodies to the 85-kDa subunit of type I PI3K (α isoform (4)) were raised against a synthetic peptide corresponding to positions 1305–1324 of the human insulin receptor. Polyclonal antibodies directed against pp185 were raised against a synthetic peptide segment 1305-1324 of the human insulin receptor. Polyclonal antibodies directed against pp185 were raised against a synthetic peptide segment 1305-1324 of the human insulin receptor.

Immunoprecipitation—Antibodies were added to 60 ml of 50% Protein A-Sepharose (Uppsala, Sweden) in 0.1 M Tris, pH 8.5, and incubated for 1 h at 4°C. The complex was pelleted at 12,000 × g (5 min) and washed with three times with 0.1 M Tris, pH 8.5, and once with buffer A containing 1% Triton X-100. 500 ml of liver extracts (0.8 mg) (prepared in buffer A, as described above) from control, insulin, or H/V-treated rats were incubated for 2 h with the antibody-Protein A-Sepharose complex. The immunocomplexes were pelleted by centrifugation at 12,000 × g (5 min) and washed twice with buffer A containing 1% Triton X-100 and twice with HTN buffer (150 mM NaCl, 50 mM Hepes, 0.1% Triton X-100, pH 7.6). The final pellets were then suspended in Laemmli sample buffer (20), resolved by 7.5% SDS-PAGE, and transferred to nitrocellulose papers followed by Western immunoblotting.

In Vitro PI3K Assay—Liver extracts were subjected to immunoprecipitation as described above. PI kinase activity in the washed immunoprecipitates was assayed according to Whitman et al. (23).

RESULTS AND DISCUSSION

H/V Enhance in Vivo Protein Tyrosine Phosphorylation—We have previously shown (24) that injection of insulin into the portal vein of rats enhanced tyrosine phosphorylation of two hepatic proteins, the 95-kDa β-subunit of the insulin receptor and pp185. Phosphorylation of these proteins was maximal 30 s following injection and then rapidly declined. By contrast, injection of H/V resulted in even higher (20-fold that of insulin) and significantly more sustained tyrosine phosphorylation of several proteins with apparent molecular masses ranging from 55 to 200 kDa (Fig. 1). Their maximal phosphorylation was detected 30 min after injection, and it decreased thereafter. Thus, acute inhibition of tyrosine-specific phosphatase activity following injection of H/V enabled detection of proteins that escaped notice in insulin-injected rats. To determine whether at least some of these proteins represent potential targets of the insulin receptor kinase, we immunoprecipitated cytosolic fractions from H/V-injected livers with antibodies directed against pp185 (IRS-1), a known effector of insulin receptor kinase (11, 12). These antibodies quantitatively precipitated IRS-1 from the cytosolic fraction (Fig. 1B, compare lane 2 versus 3) revealing that pp185 represents only a minor fraction of the total tyrosine-phosphorylated proteins (Fig. 1B, lane 1 versus 3). As shown in Fig. 2A, tyrosine phosphorylation of pp185 was stimulated at least 50-fold following H/V injection with kinetics similar to that of the other phosphoproteins, reaching a maximum by 30 min and then declining. Consistent with our studies in cultured Fao cells (16), pp185 migrated more slowly as its extent of Tyr phosphorylation increased. Since pp185 contains multiple tyrosine phosphorylation sites (12) their successive phosphorylation could result in the apparent increase in its molecular mass. Additional tyrosine-phosphorylated proteins, with molecular masses of 98, 114, and 150 kDa, were detected in the pp185 immunoprecipitates (Figs. 1B and 2A).

It is presently unknown whether these represent degradation products of pp185 or whether they are unidentified phosphoproteins which tightly associate with IRS-1.
In Vivo Association between pp185 and p85α—Since pp185 associates with PI3K in cultured cells and cell-free systems (13–15), we studied the possible association between the two proteins in intact rat liver. By Western immunoblotting with antibodies directed against the α isoform of the 85-kDa subunit of PI3K (anti-p85α) (4) we could demonstrate (Fig. 2B) the presence of p85α in the pp185 immunoprecipitates, although no tyrosine-phosphorylated protein having a molecular mass of 85 kDa could be detected in these immunocomplexes (Fig. 2A). Maximal association between the two proteins occurred 30 min following H/V injection at the time when tyrosine phosphorylation of pp185 was at its peak. Importantly, a fraction (15%) of the cellular p85α became associated with pp185 as revealed by Western blotting (with anti-p85α) of anti-pp185 immunoprecipitates (Fig. 3A). Thus, the association of p85α with pp185 in vivo appears to be proportional to the extent of tyrosine phosphorylation of pp185 and is most likely due to the interaction between the SH2 domains of p85α and the Tyr(P) residues within the YMXM consensus motifs of pp185 (12). To examine the possibility that other SH2-containing proteins also form complexes with pp185, we analyzed the presence of phospholipase Cγ and GAP in pp185 immunoprecipitates (Fig. 3B). We failed to detect either protein by Western blotting with specific antibodies, implying that the association between pp185 and p85α is specific and does not merely depend upon the presence of a src homology domain.

Next, we attempted to evaluate whether tyrosine phosphorylation of p85α occurs upon its association with pp185 in H/V-treated rat liver. As seen in Fig. 3A, p85α was precipitated to the same extent by Tyr(P) and pp185 antibodies. In contrast, when the original liver extracts were precipitated with anti-p85α antibodies and then blotted with anti-Tyr(P) antibodies, we failed to detect p85α. These findings suggest that p85α is not detectably phosphorylated on tyrosine residues, but it associates with one or more tyrosine-phosphorylated proteins such as pp185. This conclusion is compatible with the results of Hu et al. (25) and McGlade et al. (26), who demonstrated that association of p85α with the PDGF receptor occurs in the absence of significant tyrosine phosphorylation of p85α. Nevertheless, the amount of p85α that coprecipitated with antibodies to pp185 or to Tyr(P) was significantly lower than that in direct immunoprecipitates of p85α (Fig. 3A), implying that only part of the pool of p85α interacts with pp185.

Insulin Treatment of Intact Livers Induces Rapid Interaction between pp185 and p85α—To confirm our hypothesis that the interaction between pp185 and p85α in H/V-injected rats is related to the insulinomimetic potential of these agents, we compared their effects with those of insulin. To this end, we removed livers 30 s after insulin injection or 30 min after H/V injection and immunoprecipitated the liver extracts with either anti-pp185 or anti-insulin receptor antibodies. Western blotting of these immunoprecipitates with anti-p85α antibodies revealed that both insulin and H/V treatment resulted in association of p85α with pp185 (Fig. 4). Consistent with the notion that H/V acts with a potency greater than insulin...
alone, a higher amount of p85α (3-fold) was found associated with pp185 in livers from H/V-injected rats. In contrast, we were unable to detect p85α in immunoprecipitates of anti-insulin receptor antibodies, although these antibodies readily precipitate the hepatic insulin receptor. One possible interpretation of these results is that a simultaneous complex of p85α with insulin receptor kinase, probably via pp185, either does not exist or that it undergoes very rapid dissociation. Our findings are consistent with those of Endemann et al. (13) who demonstrated that in cultured cells, p85α could not be precipitated by insulin receptor antibodies.

**Insulin and H/V Treatment of Intact Liver Induces Association of PI3K Activity with pp185**—To determine whether the p85α that becomes associated with pp185 is complexed with the catalytic subunit of PI3K (p110) (7), liver extracts were precipitated with a panel of antibodies, and the PI3K activity in the immunoprecipitates was assayed (Fig. 5, top) and quantitated (Fig. 5, bottom). In extracts from control, non-treated livers, trivial amounts of PI3K activity were detectable following immunoprecipitation with anti-Tyr(P) or with anti-insulin receptor antibodies. Only basal activity was found associated with anti-p85α immunocomplexes, while a ~5-fold higher activity was detected following immunoprecipitation with anti-pp185 antibodies. Consistent with the data presented in Fig. 4, where injection of insulin increased the amount of pp185 associated with pp185 immunoprecipitates, a similar 2-fold increase was found when PI3K activity was assessed in these precipitates. Injection of H/V further elevated the PI3K activity that was associated with pp185. Similarly, injection of H/V, but not insulin, increased ~16-fold the PI3K activity associated with anti-Tyr(P) antibodies. PI3K activity was hardly detectable in immunoprecipitates made with anti-insulin receptor antibodies regardless of whether the extracts were derived from treated or untreated rats. Interestingly, in extracts derived either from non-injected or from injected rats, the PI3K activity associated with pp185 antibodies exceeded by far that associated with the p85α antibodies (2.5- and 10-fold in insulin and H/V-injected rats, respectively). This occurred in spite of the fact that p85α antibodies precipitated a ~6-fold higher amount of this protein (Fig. 3). Hence, the specific activity of PI3K associated with anti-pp185 antibodies exceeds that associated with p85α antibodies at least by 15- and 60-fold in insulin or H/V-injected rats, respectively. Several possibilities could account for these results. For example, non-quantitative precipitation of PI3K by our p85α antibodies could reduce its specific activity in the immune complexes, if only partial activation of the total pool takes place. Alternatively, p85α antibodies themselves could inhibit the activity of the enzyme in the complex, or the p85α antibodies may not recognize certain active isoforms which may associate with pp185. We cannot, however, rule out the intriguing possibility that the interaction of PI3K with pp185 leads to catalytic activation of the former. Even if pp185 acts only to promote the association between p85α and the 110-kDa catalytic subunit, it could result in enhanced PI3K activity in the pp185 immunocomplexes.

Taken together, our findings are consistent with a model that assumes functional association between pp185 and p85α following insulin treatment. Presumably these physical associations involve tyrosine residues of pp185 that are found in the context of YMXM motifs (27) and the SH2 domains of p85α (28). The increased PI3K activity in these complexes could either reflect the fact that tyrosine-phosphorylated pp185 recruits more catalytic units into the complex, or, alternatively, could result from the activation of the PI3K holoenzyme by the tyrosine-phosphorylated pp185. According to our model, PI3K is itself minimally phosphorylated (if at all) on tyrosine residues, and its precipitation by anti-Tyr(P) antibodies reflects its co-precipitation with other tyrosine-phosphorylated proteins such as pp185. The data presented in Fig. 3A and the correlation between the extent of phosphorylation of pp185 and the amount of PI3K associated with it (Fig. 2B) strongly support this hypothesis. The limited association of PI3K with the insulin receptor suggests that a ternary complex between the insulin receptor, pp185, and PI3K is unstable in vivo and fails to precipitate as a complex. Nevertheless, the binary pp185-p85α complex preexists in untreated rat livers.

In summary, we have shown that a specific association occurs in vivo between pp185 and PI3K following stimulation of liver cells with insulin or potent insulinomimetic agents. This non-covalent association of pp185 with PI3K, which activates this enzyme, is specific since an interaction of pp185 with PI3K does not take place with other signal transduction elements like phospholipase Cγ or GAP. The powerful capability of H/V to enhance in vivo protein tyrosine phosphorylation, as was demonstrated in the present work, may be most instrumental in future attempts to identify additional proteins that may be associated with pp185 and could play a role in the insulin receptor signaling system.

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