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Combination of Insulinomimetic Agents H_2O_2 and Vanadate Enhances Insulin Receptor Mediated Tyrosine Phosphorylation of IRS-1 Leading to IRS-1 Association With the Phosphatidylinositol 3-Kinase

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Abstract To analyze the mechanism of action of the insulinomimetic agents H_2O_2, vanadate, and pervanadate (H_2O_2 and vanadate), CHO cells or CHO cells that overexpress wild-type or mutant insulin receptor and/or the insulin receptor substrate (IRS-1) were used. H_2O_2 or vanadate treatment alone had little or no effect on tyrosine phosphorylation of cellular proteins; however, pervanadate treatment dramatically enhanced tyrosine phosphorylation of a number of proteins including the insulin receptor and IRS-1. However, the insulin receptor and IRS-1 coimmunoprecipitate from insulin-treated but not from pervanadate-treated cells. Pervanadate-induced tyrosine phosphorylation of the insulin receptor led to an increase in insulin receptor tyrosine kinase activity toward IRS-1 in vivo and IRS-1 peptides in vitro equal to that induced by insulin treatment. Pervanadate-enhanced phosphorylation of IRS-1 led to a fifteenfold increase in IRS-1–associated phosphatidylinositol (PtdIns) 3-kinase activity. However, insulin receptor–associated PtdIns 3-kinase activity from pervanadate-treated cells was not detectable, while insulin receptor–associated PtdIns 3-kinase activity from insulin-treated cells was 20% of the IRS-1–associated activity. Thus, pervanadate but not H_2O_2 or vanadate alone under these conditions mimics many of insulin actions, but pervanadate treatment does not induce insulin receptor/IRS-1 association.

Key words: tyrosine kinase, PTPase inhibitor, pervanadate, insulin receptor, CHO cells

Vanadate is well documented to mimic some of insulin's actions, such as stimulating glucose uptake and oxidation [Dubyak and Kleinzeller, 1980] and activation of glycogen synthase [Tamura et al., 1984]. In addition, oral vanadate treatment of streptozotocin diabetic rats [Meyerovitch et al., 1987; Heyliger et al., 1985; Shechter, 1990] or mouse models of noninsulin-dependent diabetes mellitus [Brichard et al., 1990; Meyerovitch et al., 1991] induces a reduction in fed and fasted plasma glucose levels and in some cases a reduction in insulin levels. H_2O_2, like vanadate, is insulinomimetic with regard to glucose transport and oxidation [Czech et al., 1974], glycogen synthesis [Lawrence and Larner, 1978], hormone-stimulated lipolysis [Little and de Haen, 1980], and lipogenesis [Czech et al., 1974]. Treatment of cells with a combination of vanadate and H_2O_2 (pervanadate) produces a synergistic response [Kadota et al., 1993].

Treatment of cells with pervanadate is known to enhance the tyrosine phosphorylation of a number of cellular proteins by reducing the level of cellular protein tyrosine phosphatase (PTPase) activity [Shisheva and Shechter, 1993; Heffetz and Zick, 1989]. Several studies have suggested that the insulin receptor may be one of the proteins with enhanced levels of tyrosine phosphorylation [Fantus et al., 1989; Heffetz et al., 1992; Shisheva and Shechter, 1993; Hadari et al., 1992]. In addition, phostyrosyl-proteins of 180, 125, 100, 60, and 52 kDa have been detected. Some of these proteins may be tyrosine kinases with enhanced levels of tyrosine autophosphorylation, while others may be substrates

Abbreviations used: BSA, bovine serum albumin; CHO, Chinese hamster ovary; IRS-1, insulin receptor substrate-1; pervanadate, H_2O_2 (3 mM) and sodium vanadate (5 μM); PTPase, protein tyrosine phosphatase; PtdIns 3-kinase, phosphatidylinositol 3-kinase; SDS, sodium dodecyl sulfate.

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for tyrosine kinases including the insulin receptor whose tyrosine phosphorylation level is increased because of the reduced PTPase activity.

In these studies, we investigate the effect of pervanadate treatment of CHO cells that overexpress the insulin receptor and/or IRS-1 on insulin receptor tyrosine phosphorylation and tyrosine kinase activity, tyrosine phosphorylation of IRS-1, association of the receptor with IRS-1, and association of the phosphatidylinositol 3-kinase (PtdIns 3-kinase) with phosphotyrosyl-IRS-1. We report that pervanadate-enhanced tyrosine phosphorylation of the insulin receptor *is due to* receptor autophosphorylation and not because the receptor is a substrate for another cellular tyrosine kinase. This enhanced receptor autophosphorylation leads to an increase in insulin receptor kinase activity in vitro and an increase in phosphotyrosyl-IRS-1 in vivo. The increase in IRS-1 tyrosine phosphorylation leads to the association of IRS-1 and the PtdIns 3-kinase. However, pervanadate treatment does not lead to the association of the receptor with IRS-1. Thus, some of the insulinomimetic effects of pervanadate may be mediated via an increase in insulin receptor tyrosine phosphorylation due to a reduction in PTPase activity that counterbalances the intrinsic insulin receptor tyrosine kinase activity in the absence of insulin treatment.

MATERIALS AND METHODS

Expression of IRS-1 in CHO and CHO/IR Cells

Parental CHO cells and CHO cells that overexpress the wild-type insulin receptor have previously been described [Wilden et al., 1992a,b]. CHO/IR/IRS-1 cells that overexpress IRS-1 were prepared by calcium phosphate–mediated gene transfer of the rat IRS-1 cDNA in the CMV-his expression vector (M. Birnbaum, Harvard Medical School, Boston, MA) [Wigler et al., 1979] which confers histidinol resistance. Cells that overexpress IRS-1 were selected by culture in medium that contained 10 mM histidinol as previously described [Sun et al., 1991]. CHO cells were maintained weekly by splitting 1:25 and were fed every second day. All experiments were conducted on cells below passage 20.

In Vivo Tyrosine Phosphorylation Assay

Parental CHO cells or CHO cells that overexpress wild-type or mutant insulin receptor, IRS-1, or wild-type insulin receptor and IRS-1 were grown to ≈90% of confluence in six-well plates in Ham's F-12 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. The cells were serum deprived for 16 h in Ham's F-12 supplemented with 0.1% insulin-free bovine serum albumin (Arnel, New York, NY) prior to stimulation. Cells at 37°C were untreated or treated with hydrogen peroxide (3 mM, 20 min), sodium orthovanadate (5 μM, 20 min), pervanadate (hydrogen peroxide (3 mM) and sodium orthovanadate (5 μM) [20 min]), insulin (100 nM, 5 min), hydrogen peroxide (3 mM, 20 min) and insulin (100 nM, last 5 min), sodium orthovanadate (5 μM, 20 min) and insulin (100 nM, last 5 min), or hydrogen peroxide (3 mM, 20 min) and sodium orthovanadate (5 μM, 20 min) and insulin (100 nM, last 5 min). The plates were placed on ice, the medium was removed, and the plates were washed with ice-cold phosphate-buffered saline. The cells were solubilized in 0.3 ml of 2× Laemmli sample buffer which contained 100 mM DTT and boiled for 1 min. The samples were sonicated and the proteins separated on 7% SDS-PAGE gels. The proteins were transferred to nitrocellulose, blocked overnight at 4°C in 20 mM Tris, pH 7.4, 150 mM NaCl, 3% BSA, and 0.01% Tween 20. The transfers were probed with antiphosphotyrosine antibody in block buffer for 4 h at 23°C and washed four times in wash buffer (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.01% Tween 20). The bound antibody was detected by incubation with ^{125}I-protein A for 1 h at 23°C in block buffer (0.1 mCi/ml). The nitrocellulose was washed four times with wash buffer, dried, and autoradiographed.

Plates (10 cm) of CHO/IR/IRS-1 cells were untreated, treated with insulin (100 nM, 5 min), or treated with pervanadate (3 mM hydrogen peroxide, 5 μM sodium vanadate, 20 min) at 37°C. The plates were placed on ice, the medium was removed, and the plates were washed once with ice-cold phosphate-buffered saline and twice with buffer A (20 mM Tris, pH 7.5, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$). The cells were solubilized in 1 ml of buffer A that contained 1% NP-40, 10% glycerol, 0.034 mg/ml phenylmethylsulfonyl fluoride, and insoluble material removed by centrifugation at 13,000*g* for 10 min. The insulin receptor and IRS-1 were immunoprecipitated from the cleared supernatant with a monoclonal antiinsulin receptor antibody 83-14

[Wilden et al., 1992a], and a polyclonal anti-IRS-1 antibody complexed to protein A sepharose, respectively. The pellets were washed and eluted in Laemmli sample buffer which contained 100 mM DTT with boiling. Proteins were separated by SDS-PAGE and phosphotyrosyl-proteins detected by antiphosphotyrosine αP1 antibody blotting as described above.

Insulin and Pervanadate Stimulation of Insulin Receptor Tyrosine Kinase Activity

Plates (10 cm) of CHO/IR/IRS-1 cells were untreated, treated with insulin (100 nM, 5 min), or treated with pervanadate (3 mM hydrogen peroxide, 5 μM sodium vanadate, 20 min) at 37°C. The plates were placed on ice, the medium was removed, and the plates were washed once with ice-cold phosphate-buffered saline, and twice with buffer A (20 mM Tris, pH 7.5, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$). The cells were solubilized in 1 ml of buffer A that contained 1% Triton X-100, 10% glycerol, and 0.034 mg/ml phenylmethylsulfonyl fluoride, and insoluble material was removed by centrifugation at 13,000*g* for 10 min. The insulin receptor was immunoprecipitated from the cleared supernatant using a polyclonal antiinsulin receptor antibody raised to a peptide derived from the C-terminus of the receptor β-subunit in complex with protein A sepharose. The immunocomplexes were washed successively in phosphate-buffered saline which contained 1% NP-40 (three times), 100 mM Tris, pH 7.5, and 500 mM $LiCl_2$ (three times), and 10 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM EDTA. The pellets were resuspended in 50 μl of 10 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM EDTA, 10 μl 100 mM $MgCl_2$ and $MnCl_2$, 10 μl of 375 μM IRS-1 peptide that included the tyrosine phosphorylation site at amino acid 628 of rat IRS-1 (RKGNGDYMPM-SPKSV). The reaction was initiated by the addition of 5 μl of 880 μM ATP that contained 10 μCi of [γ-^{32}P]ATP (NEG-002A, New England Nuclear, Boston, MA). After 10 min at 23°C, the reaction was terminated by the addition of 65 μl of 20% trichloroacetic acid. Twenty microliters of 1% BSA was added as carrier, and proteins were precipitated on ice and microfuged. Two 75 μl aliquots of the reaction were spotted on P81 phosphocellulose paper and washed three times in 150 mM phosphoric acid, twice in water, and once in acetone and air dried. ^{32}P incorporated into peptide was determined by Cerenkov counting the papers.

Insulin and Pervanadate Stimulation of Insulin Receptor or IRS-1–Associated PtdIns 3-Kinase Activity

Parental CHO cells or CHO cells that overexpress wild-type or mutant insulin receptor, IRS-1 were grown to ≈50% of confluence in 10 cm dishes in Ham's F-12 (Gibco) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) at 37°C with 5% CO_2. The cells were serum deprived for 72 h in Ham's F-12 supplemented with 0.1% insulin-free bovine serum albumin (Arnel) prior to stimulation. Cells at 37°C were untreated or treated with hydrogen peroxide (3 mM, 20 min), sodium orthovanadate (5 μM, 20 min), hydrogen peroxide (3 mM) and sodium orthovanadate (5 μM, 20 min), insulin (100 nM, 5 min), hydrogen peroxide (3 mM, 20 min) and insulin (100 nM, last 5 min), sodium orthovanadate (5 μM, 20 min) and insulin (100 nM, last 5 min), or hydrogen peroxide (3 mM, 20 min) and sodium orthovanadate (5 μM, 20 min) and insulin (100 nM, last 5 min). The plates were placed on ice, the medium was removed, and the plates were washed once with ice-cold phosphate-buffered saline and twice with buffer A (20 mM Tris, pH 7.5, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$). The cells were solubilized in 1 ml of buffer A that contained 1% NP-40 (Sigma), 10% glycerol, and 0.034 mg/ml phenylmethylsulfonyl fluoride, and insoluble material was removed by centrifugation at 13,000*g* for 10 min. IRS-1–associated PtdIns 3-kinase activity was precipitated from the cleared supernatant using a polyclonal antibody raised to a peptide derived from the amino-terminal 12 amino acid of the rat IRS-1 protein (B. Cheatham and C.R. Kahn, Joslin Diabetes Center, Boston, MA), and insulin receptor–associated PtdIns 3-kinase activity was precipitated using a monoclonal anti-insulin receptor antibody (83-14) in complex with protein A-sepharose (Pharmacia, Gaithersburg, MD). The immunocomplexes were washed successively in phosphate-buffered saline that contained 1% NP-40 (three times), 100 mM Tris, pH 7.5, and 500 mM $LiCl_2$ (three times) and 10 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM EDTA. The pellets were resuspended in 50 μl of 10 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM EDTA and 10 μl of 100 mM $MgCl_2$ and 10 μl of phosphatidylinositol (2 μg/μl) in 10 mM Tris, pH 7.5, and 1 mM EGTA were added. The reac-

tion was initiated by the addition of 5 μl of 880 μM ATP that contained 10 μCi of [γ-^{32}P]ATP (NEG-002A, New England Nuclear). After 10 min at 23°C, the reaction was terminated by the addition of 20 μl of 8 N HCl and 180 μl of [$CHCl_3$:methanol] (1:1). The samples were centrifuged, and 50 μl of the organic phase was removed and applied to a silica gel TLC plate (Merck, Rahway, NJ) that had been coated with 1% potassium oxalate. TLC plates were run in $CHCl_3$:CH_3OH:H $_2O$:NH_4OH (60:47:11.3:2), dried, and visualized by autoradiography. The radioactivity in spots that comigrated with Ptd-Ins-4P standard (Sigma) was measured by Cerenkov counting.

RESULTS

Effect of H_2O_2, Vanadate, Pervanadate, and Insulin on Tyrosine Phosphorylation in Parental CHO Cells

Parental CHO cells in the absence of treatment had a tyrosine-phosphorylated protein of ≈125 kDa detected by antiphosphotyrosine (αPY) immunoblotting using ^{125}I-Protein A (Fig. 1). Insulin treatment of CHO cells stimulated tyrosine phosphorylation of a 185 kDa protein which has been previously purified [Rothenberg et al., 1991] and cloned [Sun et al., 1991]. This protein has been designated insulin receptor substrate-1 (IRS-1). At this level of exposure insulin-stimulated tyrosine phosphorylation of the 95 kDa insulin receptor β subunit was not detectable on this autoradiograph. Treatment of CHO cells with sodium vanadate in the absence or presence of insulin treatment had no apparent effect on tyrosine phosphorylation. Treatment of cells with H_2O_2 in the absence or presence of insulin treatment appeared to slightly enhance tyrosine phosphorylation of the 185 kDa protein in the presence of insulin. However, treatment of the CHO cells with pervanadate (combination of sodium orthovanadate and H_2O_2) dramatically enhanced tyrosine phosphorylation of a number of proteins with molecular weights of 185, 125, 95, 80, 75, 72, 65, and 40 kDa both in the presence and absence of insulin treatment (Fig. 1). Among these proteins there was a protein of 185 kDa that appeared to comigrate with the insulin-stimulated tyrosine-phosphorylated IRS-1 and a protein of ≈95 kDa which could be the tyrosine-phosphorylated insulin receptor β subunit.

Pervandate-Enhanced Tyrosine Phosphorylation of the Insulin Receptor and IRS-1

To determine if pervanadate treatment increased tyrosine phosphorylation of the insulin receptor and IRS-1, CHO cells that overexpress

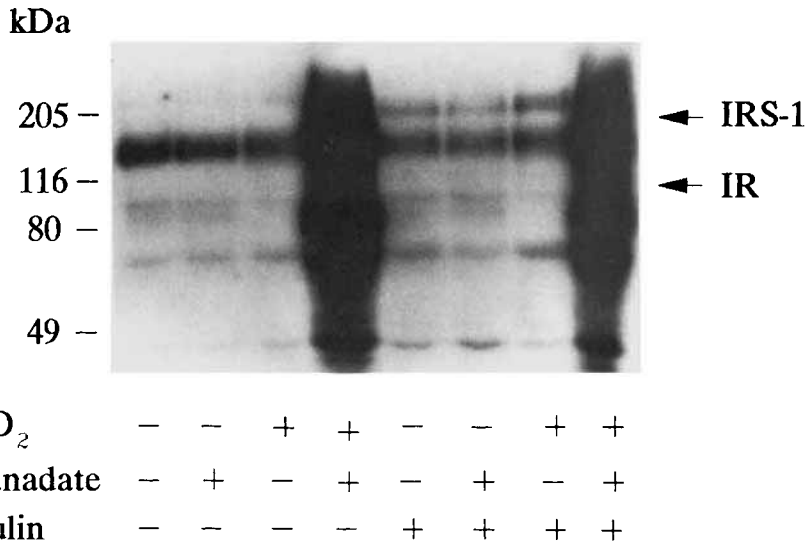

Fig. 1. Insulin, vanadate, H_2O_2, and pervanadate enhanced tyrosine phosphorylation in CHO cells. CHO cells were treated with sodium vanadate (5 μM), hydrogen peroxide (3 mM), and insulin (10^{-7} M) as noted in the figure and described in Materials and Methods. Following treatment, cells were lysed with Laemmli sample buffer, proteins separated by SDS-PAGE and transferred to nitrocellulose, and phosphotyrosyl-proteins detected by antiphosphotyrosine antibody, ^{125}I-Protein A, and autoradiography. This figure is representative of three similar experiments.

both the wild-type insulin receptor and IRS-1 were untreated or treated with insulin, or pervanadate and the insulin receptor immunoprecipitated with the monoclonal antiinsulin receptor antibody 83-14 or IRS-1 immunoprecipitated with a polyclonal anti-IRS-1 antibody (Fig. 2). The insulin receptor immunoprecipitated from untreated cells contained a low level of tyrosine phosphorylation detected by αPY blotting. The insulin receptor from insulin-treated cells showed a large increase in tyrosine phosphorylation of the β subunit. In addition, four phosphotyrosyl-proteins with molecular weights of 205 kDa, 185 kDa, 70 kDa, and 40 kDa were immunoprecipitated in complex with the insulin receptor. The 185 kDa protein (IRS-1) comigrates with the anti-IRS-1–immunoprecipitated 185 kDa phosphotyrosyl-protein. The insulin receptor from pervanadate-treated cells had a phosphotyrosine content equal to that observed in insulin-treated cells. Again, phosphotyrosyl-proteins with molecular weights of 205 kDa, 70 kDa, and 40 kDa were immunoprecipitated in complex with the insulin receptor following pervanadate treatment. However, the 185 kDa, IRS-1 protein was not coimmunoprecipitated with the insulin receptor following pervanadate treatment (Fig. 2). IRS-1 immunoprecipitated from untreated cells contained no detectable tyrosine phosphorylation. When IRS-1 was immunoprecipitated from insulin-treated cells, IRS-1 was tyrosine-phosphorylated, and the tyrosine-phosphorylated insulin receptor β subunit was coprecipitated. IRS-1 was tyrosine-phosphorylated in pervanadate-treated cells to a level equal to that seen in insulin-treated cells. However, the insulin receptor was not coprecipitated with IRS-1 from pervanadate-treated cells. Thus, pervanadate treatment of cells led to an increase in tyrosine phosphorylation of the insulin receptor and IRS-1; however, the insulin receptor and IRS-1 did not coprecipitate as readily from pervanadate-treated cells as from insulin-treated cells.

Effect of H_2O_2, Vanadate, Pervanadate, and Insulin on Tyrosine Phosphorylation in Parental CHO Cells and CHO Cells Which Overexpress Wild-Type or Mutant Insulin Receptor

We next wanted to determine if the enhanced tyrosine phosphorylation of the insulin receptor β subunit in pervanadate-treated cells was due to autophosphorylation of the receptor or if the receptor β subunit was simply a substrate for

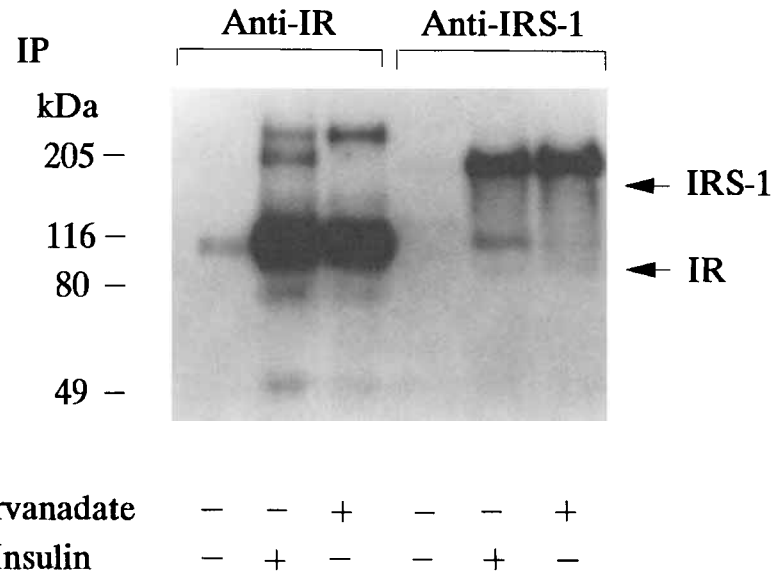

Fig. 2. Insulin and pervanadate enhanced tyrosine phosphorylation of the IR and IRS-1. CHO/IR/IRS-1 cells were untreated or treated with insulin (10^{-7} M) or pervanadate (sodium vanadate (5 μM) and hydrogen peroxide [3 mM]). Cells were lysed, and the insulin receptor immunoprecipitated with a monoclonal antiinsulin receptor antibody (83-14) and IRS-1 immunoprecipitated with a polyclonal anti-IRS-1 antibody. Proteins were eluted from the immunocomplex with 2× Laemmli sample buffer which contained 100 mM DTT and the proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose and phosphotyrosyl-proteins detected by antiphosphotyrosine antibody, ^{125}I-Protein A, and autoradiography. This figure is representative of three similar experiments.

another cellular tyrosine kinase. To address this point we used parental CHO cells or CHO cells which overexpress the wild-type insulin receptor (CHO/IR), a kinase-inactive insulin receptor (CHO/IR^{A1018}), or a kinase-impaired insulin receptor (CHO/IR^{3F}). These cell lines when untreated, treated with H_2O_2, or treated with sodium vanadate showed only tyrosine phosphorylation of the 125 kDa protein (Fig. 3, top). The parental CHO, CHO/IR^{A1018}, and CHO/IR^{3F} cell lines when treated with insulin, insulin and H_2O_2, or insulin and sodium vanadate showed only tyrosine phosphorylation of the 125 kDa protein; however, in the CHO/IR cells insulin treatment increased tyrosine phosphorylation of the 95 kDa insulin receptor β subunit and the 185 kDa IRS-1 protein (Fig. 3, bottom). Pervanadate treatment of parental CHO cells again led to a dramatic increase in phosphotyrosyl-proteins. When pervanadate-treated CHO/IR cells' phosphotyrosyl-proteins were compared to those of the parental CHO cells, there was large increase in tyrosine phosphorylation of the 95 kDa insulin receptor β subunit and an increase in tyrosine phosphorylation of the 185 kDa IRS-1 protein (Fig. 3, top). This was in contrast to the phosphotyrosyl-protein profile in pervanadate-treated CHO cells that overexpress an equal number of the kinase-inactive receptor (CHO/IR^{A1018}), where there was no enhancement of tyrosine phosphorylation of either the 95 kDa insulin receptor β subunit or the 185 kDa IRS-1 protein when compared to parental CHO cells. When the phosphotyrosyl-protein profile of pervanadate-treated CHO cells that overexpress an equal number of the kinase-impaired insulin receptor (CHO/IR^{3F}) was compared to the other cell lines, the level of tyrosine phosphorylation of the 95 kDa insulin receptor β subunit and the 185 kDa IRS-1 protein was enhanced above the level observed in parental CHO or CHO/IR^{A1018} but below the level observed in the CHO/IR cells. A similar pattern of tyrosine phosphorylation of the 95 kDa insulin receptor β subunit and the 185 kDa IRS-1 protein are seen when these cells are treated with insulin in combination with pervanadate (Fig. 3, bottom). Thus, enhanced tyrosine phosphorylation of the insulin receptor β subunit was due to an intrinsic activity of the receptor and not due to the larger amount of insulin receptor protein being used as a substrate by another cellular tyrosine kinase. In addition, the enhanced tyrosine phosphorylation of IRS-1 paralleled the tyrosine phosphorylation of the insulin receptor and may be due to activation of the insulin receptor tyrosine kinase by the increased tyrosine phosphorylation of the receptor.

Effect of Insulin and Pervanadate Treatment on Insulin Receptor Tyrosine Kinase Activity

To determine the activity state of the insulin receptor tyrosine kinase, CHO/IR cells were untreated, treated with insulin, or treated with pervanadate prior to immunoprecipitation of the insulin receptor and in vitro immunocomplex kinase assays using an IRS-1 peptide substrate. Insulin treatment of cells increased insulin receptor tyrosine kinase activity 1.9-fold above the activity in untreated cells (Fig. 4), similar to levels reported previously [Cheatham et al., 1993]. Pervanadate treatment led to an almost identical 1.9-fold increase in insulin receptor phosphorylation of the IRS-1 peptide. Thus, the increase in IR tyrosine autophosphorylation due to pervanadate treatment enhanced insulin receptor tyrosine kinase activity to a level similar to that caused by insulin-stimulated receptor autophosphorylation.

Effect of H_2O_2, Vanadate, Pervanadate, and Insulin on IRS-1–Associated PtdIns 3-Kinase Activity

Insulin-stimulated tyrosine phosphorylation of IRS-1 has been shown to cause association of phosphotyrosyl-IRS-1 with the PtdIns 3-kinase [Backer et al., 1992a,b]. To determine if the tyrosine phosphorylation of IRS-1 induced by pervanadate treatment affected IRS-1–associated PtdIns 3-kinase activity, cells were treated with H_2O_2, sodium vanadate, pervanadate, and/or insulin and assayed for IRS-1–associated PtdIns 3-kinase activity. Parental CHO cells display very low levels of IRS-1–associated PtdIns 3-kinase activity as assessed in in vitro immunocomplex assays (Fig. 5, top). Treatment of CHO cells with either sodium orthovanadate or H_2O_2 alone had no effect on IRS-1–associated PtdIns 3-kinase activity. However, treatment with pervanadate led to an eighteenfold increase in IRS-1–associated PtdIns 3-kinase activity. This increase in IRS-1–associated PtdIns 3-kinase was equal to that observed from insulin-treated cells. CHO cells when treated with sodium vanadate, H_2O_2, or pervanadate in combination with insulin showed no further

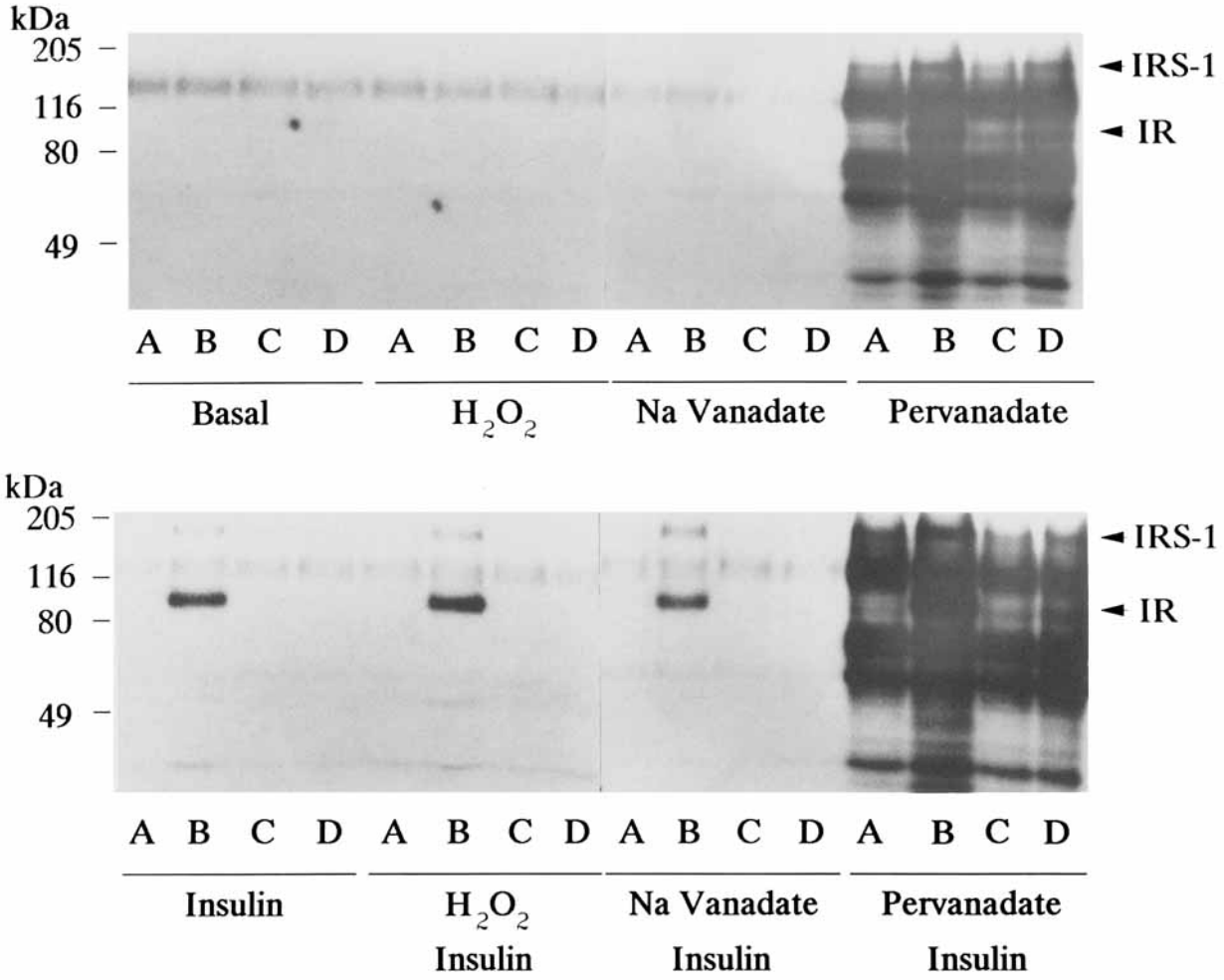

Fig. 3. Insulin, vanadate, H_2O_2, and pervanadate enhanced tyrosine phosphorylation in CHO/IR mutant cells. CHO cells were treated with sodium vanadate (5 μM), hydrogen peroxide (3 mM), and insulin (10^{-7} M) as noted in the figure and described in Materials and Methods. Following treatment cells were lysed with Laemmli sample buffer, proteins separated by SDS-PAGE and transferred to nitrocellulose, and phosphotyrosyl-proteins detected by antiphosphotyrosine antibody, ^{125}I-Protein A, and autoradiography. CHO cells (**lane A**), CHO/IR cells (**lane B**), CHO/IR^{A1018} cells (**lane C**), and CHO/IR^{3F} cells (**lane D**) are compared. This figure is representative of three similar experiments.

increase in IRS-1–associated PtdIns 3-kinase activity above that observed with insulin treatment alone. Thus, pervanadate treatment stimulated IRS-1 associated PtdIns 3-kinase activity in a manner and to an extent similar to that of insulin. When similar experiments were conducted with CHO cells which overexpress the wild-type insulin receptor (Fig. 5, bottom), a similar pattern of IRS-1–associated PtdIns 3-kinase responses was observed. Therefore, even though overexpression of the insulin receptor led to increases in IRS-1 tyrosine phosphorylation, there was no increase in IRS-1–associated PtdIns 3-kinase activity in these cell lines. Thus, it appeared that a maximum limit had been reached for IRS-1–associated PtdIns 3-kinase with pervanadate treatment or insulin treatment that was not increased by combination of the two treatments.

Effect of H_2O_2, Vanadate, Pervanadate, and Insulin on IRS-1–Associated PtdIns 3-Kinase Activity in Parental CHO Cells and CHO Cells That Overexpress Wild-Type or Mutant Insulin Receptor

The level of IRS-1–associated PtdIns 3-kinase activity in untreated cells was very low (Fig. 6). This low level of IRS-1–associated PtdIns 3-kinase activity was not enhanced in any of the cell lines by treatment with sodium vanadate or

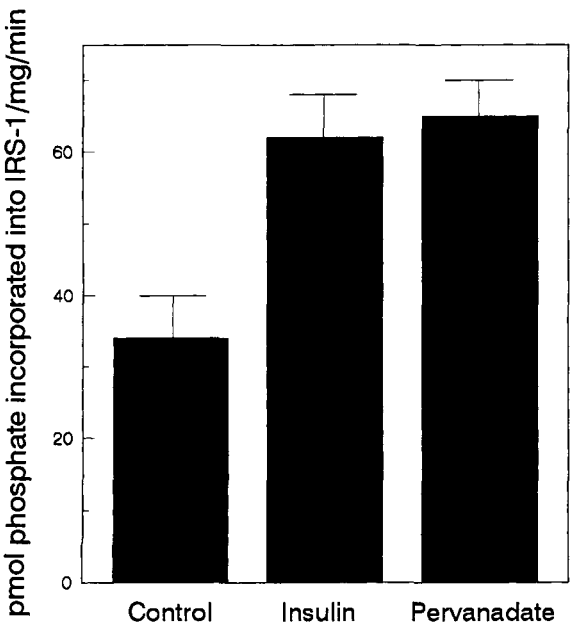

Fig. 4. Insulin and pervanadate treatment of CHO/IR cells activates insulin receptor tyrosine kinase activity. CHO/IR cells were untreated or treated with insulin (10^{-7} M) or pervanadate (sodium vanadate (5 μM) and hydrogen peroxide [3 mM]) as described in Materials and Methods. Cells were lysed and the insulin receptor immunoprecipitated with a polyclonal antiinsulin receptor antibody (αIR_{CT}). The immunocomplexes were washed as described in Materials and Methods and the insulin receptor tyrosine kinase activity assay with peptide derived from IRS-1. Insulin receptor tyrosine kinase activity toward the IRS-1 peptide substrate in untreated CHO/IR cells was 34.1 pmol phosphate incorporated/min/mg protein in the cell lysate. These results are the mean of three independent experiments ± SEM.

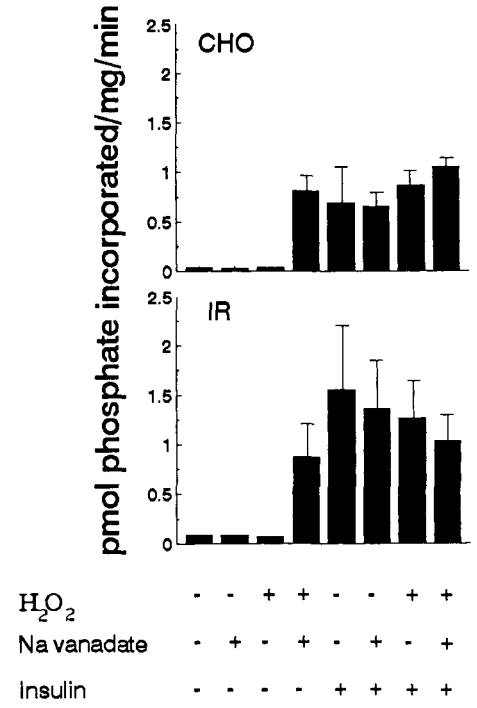

Fig. 5. IRS-1–associated PtdIns 3-kinase activity in CHO and CHO/IR cells. CHO cells were treated with sodium vanadate (5 μM), hydrogen peroxide (3 mM), and/or insulin (10^{-7} M) as noted in the figure (+) and described in Materials and Methods. Cells were lysed and IRS-1–associated PtdIns 3-kinase activity assayed as described in Materials and Methods. Values represent the mean of five experiments done in triplicate ± SEM.

H_2O_2. However, treatment of all four cell lines with pervanadate led to a thirteenfold increase in IRS-1–associated PtdIns 3-kinase activity. Insulin treatment of CHO, CHO/IR^{A1018}, and CHO/IR^{3F} cells stimulated IRS-1–associated PtdIns 3-kinase activity to comparable levels as pervanadate treatment; however, insulin treatment of CHO/IR cells showed a 1.8-fold increase in IRS-1–associated PtdIns 3-kinase activity above the level in pervanadate treated cells. This response was reproducible but not statistically significant. Treatment of the cell lines with sodium vanadate, H_2O_2, or pervanadate in combination with insulin led to no significant alterations in the insulin-stimulated IRS-1–associated PtdIns 3-kinase activity. Thus, it appeared that a maximum limit had been reached for IRS-1–associated PtdIns 3-kinase activity with pervanadate treatment or insulin treatment that was not increased by combination of the two treatments, and the increased amount of insulin receptor tyrosine phosphorylation and IRS-1 tyrosine phosphorylation in cells overexpressing the wild-type insulin receptor had little effect on enhancing IRS-1–associated PtdIns 3-kinase activity.

Effect of H_2O_2, Vanadate, Pervanadate, and Insulin on Insulin Receptor–Associated PtdIns 3-Kinase Activity in Parental CHO Cells and CHO Cells That Overexpress Wild-Type Insulin Receptor

It has previously been reported that the insulin receptor, IRS-1, and the PtdIns 3-kinase form a ternary complex with IRS-1 being the physical link between the insulin receptor and the PtdIns 3-kinase following insulin stimulation [Backer et al., 1993]. To assess the ability of vanadate, H_2O_2, and pervanadate treatment to induce the formation of this ternary complex, we have assayed PtdIns 3-kinase activity in insulin receptor immunoprecipitates. Parental CHO cells had very low levels of insulin receptor–associated PtdIns 3-kinase activity under all treatment conditions when assayed in insulin

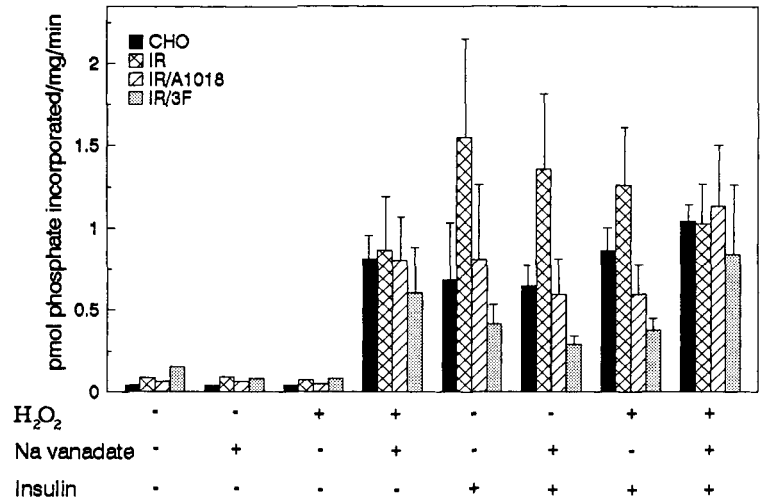

Fig. 6. IRS-1–associated PtdIns 3-kinase activity in CHO cells that overexpress IR mutants. CHO cells were treated with sodium vanadate (5 μM), hydrogen peroxide (3 mM), and/or insulin (10^{-7} M) as noted in the figure (+) and described in Materials and Methods. Cells were lysed and IRS-1–associated PtdIns 3-kinase activity assayed as described in Materials and Methods. Values represent the mean of five experiments done in triplicate ± SEM.

receptor immunoprecipitates (Fig. 7). Likewise, in untreated or sodium vanadate–, H_2O_2-, or pervanadate-treated CHO cells that overexpress wild-type insulin receptor, the receptor-associated PtdIns 3-kinase activity was equally low. Insulin treatment increased insulin receptor–associated PtdIns 3-kinase activity approximately sevenfold in these cells. Treatment of the cells with vanadate, H_2O_2, or pervanadate in combination with insulin had no further effect on insulin receptor–associated PtdIns 3-kinase activity. The insulin receptor–associated PtdIns 3-kinase results from pervanadate-treated cells differ from the results observed for IRS-1–associated PtdIns 3-kinase. Pervanadate treatment of cells stimulated association of PtdIns 3-kinase with IRS-1 but did not stimulate the formation of the ternary complex of the insulin receptor, IRS-1, and PtdIns 3-kinase. However, insulin treatment supported the association of the PtdIns 3-kinase with IRS-1 and the formation of the ternary insulin receptor, IRS-1, PtdIns 3-kinase complex. In addition, with insulin stimulation ≈20% of the IRS-1–associated PtdIns 3-kinase was in a ternary complex with the insulin receptor (Figs. 5, 7). This percentage is higher than that reported by others [Backer et al., 1993]. Thus, while pervanadate can mimic insulin-induced increases in insulin receptor autophosphorylation, receptor tyrosine kinase activity, tyrosine phosphorylation of IRS-1, and phosphotyrosyl-IRS-1 and PtdIns 3-kinase association, pervanadate treatment does not mimic insulin's ability to induce insulin receptor/IRS-1 interaction as assessed by coimmunoprecipitation of the insulin receptor and IRS-1 complex.

DISCUSSION

H_2O_2, vanadate, and pervanadate have been shown to be insulinomimetic agents in both cell culture systems and whole animal experimentation [Dubyak and Kleinzeller, 1980; Tamura et al., 1984; Shechter, 1990; Brichard et al., 1990; Czech et al., 1974; Lawrence and Larner, 1978; Little and de Haen, 1980; Hadari et al., 1992]. However, these agents do not appear to mimic all of insulin's actions in all systems. Therefore, these agents may share some but not all signal transmission pathways. Those signal transduction pathways activated should share some steps with insulin-stimulated signaling pathways. One of the potential mechanisms involved in vanadate and pervanadate insulinomimetic nature may be the inhibition of protein tyrosine phosphatase (PTPase). Both vanadate and pervanadate have been shown to be PTPase inhibitors [Shisheva and Shechter, 1993; Heffetz and Zick, 1989; Tonks et al., 1988; Chan et al., 1986]. We wanted to determine if the PTPase inhibitor activity of vanadate and pervanadate were directly or indirectly involved at the level of the insulin receptor in the insulinomimetic activity of these agents.

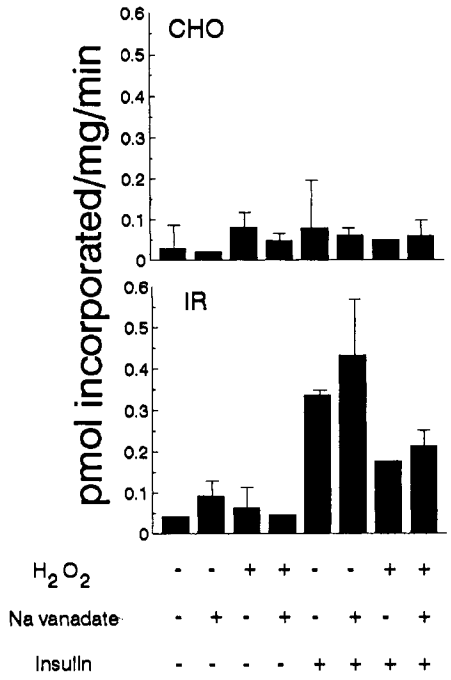

Fig. 7. IR-associated PtdIns 3-kinase activity in CHO cells that overexpress IRS-1 or the wild-type IR. CHO cells were treated with sodium vanadate (5 μM), hydrogen peroxide (3 mM), and/or insulin (10^{-7} M) as noted in the figure (+) and described in Materials and Methods. Cells were lysed and insulin receptor–associated PtdIns 3-kinase activity assayed as described in Materials and Methods. Values represent the mean of three experiments done in triplicate ± SEM.

Treatment of CHO with pervanadate but not with H_2O_2 or vanadate alone under our conditions leads to an increase in the tyrosine phosphorylation of a substantial number of proteins. Under the conditions used in these experiments, pervanadate appears to be a much more efficient PTPase inhibitor than vanadate. H_2O_2 also appears to have little or no PTPase inhibitor activity. As pervanadate decreases the activity of the PTPases, the level of protein tyrosine phosphorylation in the cell increases. These phosphotyrosyl-proteins may be proteins with intrinsic tyrosine kinase activity that autophosphorylate at a rate greater than the inhibited counterregulatory PTPase(s), or they may be substrates for tyrosine kinases and are now being tyrosine-phosphorylated at a rate faster than the pervanadate-inhibited dephosphorylation rate of the PTPases. Two of the proteins which have enhanced levels of tyrosine phosphorylation are the insulin receptor (an autophosphorylating tyrosine kinase) and IRS-1 (a tyrosine-phosphorylated substrate of the insulin receptor).

Insulin binding to the insulin receptor has been shown to increase the level of receptor autophosphorylation [Kasuga et al., 1982; Shia and Pilch, 1983; Roth and Cassell, 1993]. The phosphorylation of three critical tyrosine residues in a regulator domain of the receptor enhances the receptor tyrosine kinase activity toward substrates [Rosen et al., 1983; Yu and Czech, 1984; Wilden et al., 1992a; Zhang et al., 1991]. In pervanadate-treated cells the intrinsic basal autophosphorylating activity of the insulin receptor appears to exceed the pervanadate-inhibited PTPase activity which would dephosphorylate the receptor, thus increasing the level of receptor tyrosine phosphorylation. Pervanadate-induced tyrosine phosphorylation of the insulin receptor appears to have effects similar to those of insulin on the insulin receptor tyrosine kinase. It enhances the insulin receptor tyrosine kinase activity in immunoaffinity purified in vitro assays using an IRS-1–derived peptide substrate and enhances the receptor's ability to tyrosine-phosphorylate IRS-1 in the intact cell.

Upon insulin-stimulated tyrosine phosphorylation of IRS-1 the PtdIns 3-kinase binds to specific phosphotyrosyl residues of IRS-1 via two src homology 2 (SH2) domains in the 85 kDa subunit of the PtdIns 3-kinase [Backer et al., 1992a, 1993; Myers et al., 1992; Shoelson et al., 1993]. The binding of phosphotyrosyl-IRS-1 to the PtdIns 3-kinase increases the specific activity of the lipid kinase [Backer et al., 1992a; Myers et al., 1992; Shoelson et al., 1993]. Pervanadate treatment of cells also leads to an increase in tyrosine phosphorylation of IRS-1 and the association of IRS-1 with the PtdIns 3-kinase [Backer et al., 1993].

It has also been shown that insulin stimulation leads to the formation of a ternary complex of the insulin receptor, IRS-1, and PtdIns 3-kinase [Backer et al., 1993]. However, it appears that pervanadate treatment differs from insulin stimulation in this characteristic, because pervanadate treatment does not lead to formation of this ternary complex. The tyrosine-phosphorylated insulin receptor and tyrosine-phosphorylated IRS-1 do not coimmunoprecipitate from pervanadate-treated cells. Therefore, even though pervanadate treatment of cells leads to enhanced insulin receptor autophosphorylation, increased receptor tyrosine kinase activity, and IRS-1 tyrosine phosphorylation, there is some difference in the pervanadate-induced insulin receptor/IRS-1 interaction. This difference could be due to the insulin-induced conformational

change documented by many laboratories [Lipson et al., 1986; Harmon et al., 1981; Wilden et al., 1986; Sweet et al., 1986; Wilden and Pessin, 1987; Donner and Yonkers, 1983; Ginsberg et al., 1976; Krupp and Livingston, 1978; Maturo et al., 1983; Maturo and Hollenberg, 1985; Pilch and Czech, 1980]. This would appear to be the most obvious difference between insulin and pervanadate treatment.

Thus, the mechanism of the insulinomimetic action of pervanadate but not vanadate or H_2O_2 involves enhanced insulin receptor tyrosine phosphorylation by the intrinsic activity of the receptor which activates the tyrosine kinase activity of the receptor and leads to tyrosine phosphorylation of IRS-1. The tyrosine phosphorylation of IRS-1 is critical for insulin signaling through SH2 domain containing proteins such as, Grb 2, Nck, Syp, and the PtdIns 3-kinase [Myers and White, 1993; Kuhne et al., 1993; Skolnik et al., 1993a; Tobe et al., 1993; Baltensperger et al., 1993]. In addition, the activated insulin receptor tyrosine kinase has been shown to tyrosine-phosphorylate a number of other cellular substrates including another SH2 domain containing protein termed Shc, whose role in insulin signaling is undefined [Pronk et al., 1993; Ruff-Jamison et al., 1993; Skolnik et al., 1993b; Kovacina and Roth, 1993].

Insulin receptor tyrosine phosphorylation can be modulated in two manners. 1) Insulin stimulation leads to changes in the insulin receptor conformation and an increase in the intrinsic tyrosine kinase activity of the insulin receptor. This increased receptor autophosphorylating activity overcomes the basal level of cellular PTPase activity. This increase in the level of insulin receptor autophosphorylation leads to an increase in tyrosine phosphorylation of cellular substrates and generation of signal transmissions. 2) Treatment with the PTPase inhibitor pervanadate inhibits the basal PTPase activity below a level that will keep the insulin receptor dephosphorylated, thus allowing an increase in insulin receptor phosphorylation. This increase in the level of insulin receptor phosphorylation leads to an increase in tyrosine phosphorylation of cellular substrates and generation of signal transmissions. Thus, both insulin treatment and pervanadate treatment lead to an increase in insulin receptor phosphorylation; however, the mechanism used to achieve the increase is different. However, when the level of insulin receptor tyrosine phosphorylation increases by either mechanism, signaling appears to proceed in a similar manner. Thus, any mechanism which tips the balance between the basal tyrosine kinase activity of the receptor and the basal protein tyrosine phosphatase activity towards increasing the tyrosine phosphorylation of the insulin receptor should initiate insulin receptor signaling. This can be done by increasing the tyrosine kinase activity of the insulin receptor (insulin) or decreasing the insulin receptor targeted PTPase activity of the cell (pervanadate). However, the lack of insulin-induced receptor conformational change with pervanadate treatment may account for the lack of pervanadate's ability to mimic all of insulin's effects.

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