Vanadate and Rapamycin Synergistically Enhance Insulin-Stimulated Glucose Uptake

Jason C. O'Connor and Gregory G. Freund

Tyrosine dephosphorylation, serine phosphorylation, and proteasomal degradation of insulin receptor substrates (IRSs) are implicated in the negative regulation of insulin action. Here we show that simultaneous inhibition of IRS-1 tyrosine dephosphorylation and proteasomal degradation synergistically augments insulin-responsive glucose uptake. L6 skeletal muscle cells (L6 cells) were treated with inhibitors of protein-tyrosine phosphatases, proteasomal degradation, and mammalian target of rapamycin (mTOR), and the effects of insulin on glucose uptake, IRS-1 tyrosine phosphorylation, phosphatidylinositol (PI) 3-kinase activity, and IRS-1 mass were examined. Pretreatment of L6 cells with sodium orthovanadate (Na_3VO_4) plus the mTOR inhibitor rapamycin caused a 5-fold increase in insulin-responsive glucose uptake at 2 hours when compared to insulin alone. Evaluation of IRS-1 associated PI 3-kinase activity, IRS-1–associated p85 mass, and IRS-1 tyrosine phosphorylation showed that 2 hours after insulin addition they were reduced by 70% from maximal activity. Likewise, IRS-1 mass was reduced by 50%. When L6 cells were pretreated with Na_3VO_4 plus the proteasome inhibitor MG-132 or the mTOR inhibitor rapamycin prior to insulin addition, IRS-1 mass loss as well as IRS-1/PI-3 kinase complex decay was blocked at 2 hours and PI 3-kinase activity was increased 2.5-fold and 4-fold, respectively, over insulin alone. Finally, treatment of L6 cells with subtherapeutic amounts of vanadyl sulfate and rapamycin induced a synergistic 3-fold increase in insulin-induced glucose uptake at 2 hours. These findings indicate that vanadium and rapamycin synergize to enhance glucose uptake by preventing IRS-1 mass loss and IRS-1/PI 3-kinase complex decay and may offer a new approach to enhance glucose transport in diabetes.

INSULIN RECEPTOR substrate-1 (IRS-1) is linked to the activation of multiple signaling pathways required for insulin action, including those necessary for glycogen synthesis and glucose uptake.[1] Critical to IRS-1 function is its tyrosine phosphorylation and subsequent recruitment and association with Src homology 2 (SH2)-domain–containing proteins,[2] the best studied interaction being between IRS-1 and phosphatidylinositol (PI) 3-kinase.[3] PI 3-kinase is a heterodimeric protein comprised of a 110-kd catalytic subunit (p110) and an 85-kd regulatory subunit (p85).[4] The p85 subunit mediates the interaction between PI 3-kinase and IRS-1 through its N- and C- terminal SH2 domains by binding IRS-1 through phosphotyrosines contained within YXXM motifs.[5] This complexing of IRS-1 and PI 3-kinase increases p110 catalytic activity[6] and changes the subcellular location of PI 3-kinase, bringing it in close proximity to its lipid substrate at the cell membrane.[7]

Several potential mechanisms have been implicated in the counter-regulation of insulin signal transduction and insulin action. One of these is tyrosine dephosphorylation of the insulin receptor and/or receptor substrates by protein-tyrosine phosphatases (PTPases),[8] such as PTP-1B,[9-12] PTPalpha,[13] SHP2,[14] LAR,[15] and PTEN.[16] Vanadium-containing compounds like sodium orthovanadate (Na_3VO_4) and peroxovanadate complexes inhibit PTPases and mimic the biological effects of insulin and enhance insulin signal transduction.[17,18] Vanadium compounds adopt a trigonal bipyramidal structure that mimics the transition state of the phosphoryl transfer reaction, thereby acting as either a competitive inhibitor (Na_3VO_4) or as an oxidizer of the conserved cysteine residue in the PTP-loop (peroxovanadate complexes).[19] In combination with insulin, vanadium compounds can augment and prolong insulin receptor and IRS-1 tyrosine phosphorylation,[20] as well as enhance insulin-induced glucose transport.[21]

More recently, serine phosphorylation of IRSs, as a result of chronic insulin exposure in 3T3-L1 adipocytes, has been linked to attenuation of insulin signal transduction. Serine phosphorylation of IRS-1 renders it a less suitable substrate for tyrosine kinases like Jaks[22] and the insulin receptor,[23] thereby reducing insulin-induced IRS-1 tyrosine phosphorylation. Serine phosphorylation of IRSs has also been shown to target IRSs to the 26S proteasome for degradation.[24] Importantly, proteasomal degradation of IRSs can negatively regulate insulin signaling.[24-29] The proteasome was first shown to be critical to chronic insulin-dependent loss of IRS-1 in 3T3-L1 adipocytes, reducing the half-life of IRS from 25 hours to 2.5 hours.[25] Inhibition of the 26S proteasome with the peptide aldehyde transition state analog MG-132 blocked the effects of chronic insulin exposure on IRS-1 expression.[29] Targeting of IRS-1 for degradation by the 26S proteasome relies on ubiquitination.[28] In addition, serine phosphorylation and proline-rich motifs are likely mechanisms by which protein targets are recognized for ubiquitination by ubiquitin ligases.[30] A number of serine kinases are capable of phosphorylating IRS-1, including the mammalian target of rapamycin (mTOR).[22] Furthermore, blockage of mTOR kinase activity with rapamycin inhibits chronic insulin-induced IRS-1 serine phosphorylation and proteasomal degradation.[24,27] Here we show that rapamycin-sensitive proteasomal degradation is important in the negative regulation of acute insulin action in L6 skeletal muscle cells. Furthermore, PTPase inhibition and blockage of IRS-1 protea-

From the Departments of Animal Sciences and Pathology, University of Illinois at Urbana-Champaign, Urbana, IL.

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Address reprint requests to Gregory G. Freund, MD, Department of Pathology, College of Medicine, 506 South Mathews Ave, University of Illinois at Urbana Champaign, Urbana, IL 61801.

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somal degradation synergistically augments insulin-responsive glucose transport.

MATERIALS AND METHODS

Materials

L6 cells were purchased from American Type Culture Collection (Rockville, MD). Enhanced chemiluminescence (ECL) detection reagents, Hybond-ECL nitrocellulose, protein G-Sepharose, glutathione-Sepharose and (γ^{32}P)-adenosine triphosphate (ATP) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Minimum essential medium (Cat #61100-087) was purchased from Gibco Life Technologies (Grand Island, NY). Phosphotyrosine (pY) (Cat #05-321), IRS-1 (CAT #06-248C), and PI 3-kinase p85 (Cat #06-497) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). MG-132 and rapamycin were purchased from Calbiochem (San Diego, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Thin-layer chromatography plates were purchased from Analtech (Newark, DE). Liquid scintillation reagents and materials were purchased from National Diagnostics (Atlanta, GA). All other cell culture materials, chemicals, and (H^3) 2-deoxy-D-glucose were purchased from Sigma (St Louis, MO).

Cell Culture

L6 myoblasts were maintained in minimum essential medium supplemented with 10% fetal calf serum (FCS). L6 myotube generation was performed by culturing confluent myoblasts in minimum essential medium supplemented with 2% FBS, 100,000 U/L penicillin, and 100 mg/L streptomycin.

Glucose Uptake

2-Deoxyglucose uptake assays were performed as described.[31] L6 cells were treated as indicated then rinsed once with HEPES-buffered saline (20 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L KCL, 2.5 mmol/L $MgSO_4$, and 1 mmol/L $CaCL_2$, pH 7.4) and incubated for 8 minutes in HEPES-buffered saline containing 10 μmol/L (H^3) 2-deoxy-D-glucose (0.8 kBq/nmol). Cells were rinsed in ice-cold HEPES-buffered saline and lysed in ice-cold lysis buffer (1% Tritron X-100, 100 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L dithiothreitol [DTT], 25 mmol/L benzamidine, 1 mmol/L phenylmethyl sulfonyl fluoride [PMSF], 2 μg/mL aprotinin, 2 μg/mL leupeptin, 2 mmol/L sodium orthovanadate, 250 nmol/L okadaic acid, and 50 mmol/L Tris, pH 7.4). H^3 was detected by liquid scintillation counting.

PI 3-Kinase Activity

PI 3-kinase assays were performed as described.[32] Cells were treated as indicated and lysed in ice-cold lysis buffer. IRS-1 associated PI 3-kinase was immunoprecipitated from clarified lysates with anti–IRS-1. Kinase assays were performed in a buffer containing 0.33 mg/mL L-α-phosphatidylinositol, 7.5 mmol/L $MgCl_2$, 0.4 mmol/L EGTA, 0.4 mmol/L NaH_2PO_4, 7.5 μmol/L (γ^{32}P)-ATP (0.48 MBq/nmol), and 20 mmol/l HEPES, pH 7.1 for 15 minutes. Kinase reactions were stopped by addition of 15 μL 4N HCl. Phospholipids were extracted with 1:1 chloroform:methanol and resolved on silica gel plates by thin-layer chromatography in chloroform:methanol:ammonium hydroxide (4 mol/L) (75:58:17). γ^{32}P was detected by phosphorimaging on a Typhoon Phoshorimager System (Molecular Dynamics, Piscataway, NJ) using Molecular Dynamics Phosphorimage Software for analysis.

Western Analysis

Western analysis was performed as described previously.[33] L6 cells were lysed in ice-cold lysis buffer. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE; 250 μg per lane) under reducing conditions in 8% gels and electrotransfered to nitrocellulose. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) followed by autoradiography and densitometry. For blots requiring multiple analyses, nitrocellulose membranes were incubated at 100°C for 10 minutes in stripping buffer (2% SDS, 0.704% [vol/vol] β-mercaptoethanol, and 6.25 mmol/L Tris-HCl, pH 6.8). Membranes were washed in 0.05% Tween 20 and Tris-buffered saline, pH 7.4. Western analysis was again performed after membranes were reblocked with 5% bovine serum albumin (BSA) and Tris-buffered saline, pH 7.4, for 1 hour.

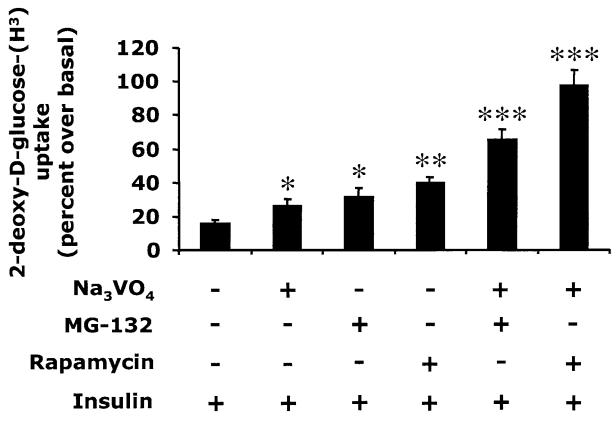

Fig 1. Na_3VO_4 and rapamycin synergistically enhance insulin-stimulated glucose uptake. L6 myotubes were pretreated with either 1 mmol/L Na_3VO_4 for 15 min, 10 μmol/l MG-132 for 30 min, 20 nmol/L rapamycin for 60 min, 1 mmol/L Na_3VO_4 for 15 min + 10 μmol/L MG-132 for 30 min, or 1 mmol/L Na_3VO_4 + 20 nmol/L rapamycin for 15 and 60 min, respectively, as indicated. Cells were then stimulated with 100 nmol/L insulin and (H^3) 2-deoxy-D-glucose was assayed at 2 h. Values represent means ± SEM from four independent experiments. *$P < .028$ *v* insulin-stimulated 2-deoxy-D-glucose uptake, **$P < .028$ *v* Na_3VO_4-pretreated insulin-stimulated 2-deoxy-D-glucose uptake, ***$P < .01$ *v* rapamycin-pretreated insulin-stimulated 2-deoxy-D-glucose uptake

Statistical Analysis

Where indicated experimental data were analyzed either by the Student's *t* test for comparison of means using Microsoft Excel (Redmond, WA) or by 2- or 3-factor factorial assuming unequal variances using Statistical Analysis Software (SAS Institute, Cary, NC).

RESULTS

Na_3VO_4 and Rapamycin Synergistically Enhance Insulin-Stimulated Glucose Uptake

Na_3VO_4[17,21] and rapamycin[24,34] have each been shown independently to augment insulin action in adipocytes. To determine their combined impact on glucose transport in muscle, L6 myotubes were examined for insulin-stimulated glucose uptake. Figure 1 shows that 2 hours after 100 nmol/L insulin exposure, glucose uptake was 16.23% ± 1.57% over basal glucose uptake. Pre-incubation of cells with either 1 mmol/L Na_3VO_4 for 15 minutes, 10 μmol/L MG-132 for 30 minutes, or 20 nmol/L rapamycin for 60 minutes prior to insulin addition increased glucose uptake to 26.42% ± 3.73%, 31.33% ± 5.33%, and 39.9% ± 3.39% over basal at 2 hours, respectively. When cells were pretreated with 1 mmol/L Na_3VO_4 for 15

minutes plus 10 μmol/L MG-132 for 30 minutes, or 1 mmol/l Na_3VO_4 for 15 minutes plus 20 nmol/L rapamycin for 60 minutes prior to 100 nmol/L insulin addition, glucose uptake at 2 hours was increased to 65.55% ± 5.72% and 97.83% ± 8.54% over basal glucose uptake. None of the inhibitor treatments had a significant effect on glucose uptake at 2 hours in the absence of insulin (data not shown). These findings indicate that Na_3VO_4 plus rapamycin synergistically enhance insulin-responsive glucose uptake.

Decay of Insulin-Induced IRS-1/PI 3-Kinase Complexes

Induction of IRS-1/PI 3-kinase complexes is required for insulin-dependent glucose transport in skeletal muscle. To examine decay of insulin-induced IRS-1/PI 3-kinase complexes, L6 myotubes were treated with 100 nmol/L insulin. Maximum IRS-1–associated PI 3-kinase activity was reached at 7.5 minutes and it declined 33% ± 4.6%, 43% ± 3.8%, 55% ± 4.5%, and 70% ± 4.3% from maximal activity at 15, 30, 60, and 120 minutes, respectively (Fig 2A). To determine the impact of insulin on IRS-1 tyrosine phosphorylation and IRS-1 mass, Western analysis of IRS-1 immunoprecipitates was performed. Consistent with Fig 2A, maximum tyrosine phosphorylation of IRS-1 was reached 7.5 minutes after 100 nmol/L insulin addition (Fig 2B). At 2 hours, IRS-1 tyrosine phosphorylation declined 70%. Analysis of IRS-1 mass demonstrated that it declined 50% from basal levels 2 hours after insulin addition whether measured in IRS-1 immunoprecipitates or in whole cell lysates. Like IRS-1 tyrosine phosphorylation, IRS-1–associated PI 3-kinase p85 mass showed peak IRS-1 binding at 7.5 minutes and declined 70% 2 hours after insulin addition. Importantly, PI 3-kinase p85 mass was unaffected by insulin addition in whole cell lysates. Taken together, these findings indicate that loss of insulin-induced IRS-1–associated PI 3-kinase activity is due to both IRS-1 tyrosine dephosphorylation and IRS-1 mass loss.

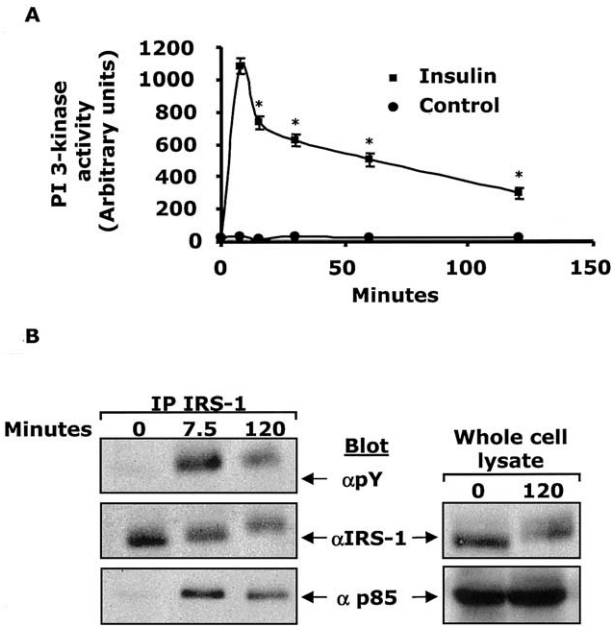

Fig 2. Decay of insulin-induced IRS-1/PI 3-kinase complexes. (A) L6 myotubes were treated with 100 nmol/L insulin for 0, 7.5, 30, 60, and 120 min and PI 3-kinase activity was measured in IRS-1 immunoprecipitates. Values represent means ± SEM from 3 independent experiments. *$P < .05$ *v* 7.5 min insulin-stimulated IRS-1–associated PI 3-kinase activity. (B) Cells were treated as in (A) for 0, 7.5, and 120 min, and IRS-1 tyrosine phosphorylation (upper left panel), IRS-1 mass (middle left panel), and IRS-1–associated p85 mass (lower left panel) were measured in IRS-1 immunoprecipitates by Western analysis. Cells were treated with insulin as in (A) for 0 or 120 min. IRS-1 (upper right panel) and p85 (lower right panel) mass were measured in whole cell lysates by Western analysis. Results are representative of 3 independent experiments.

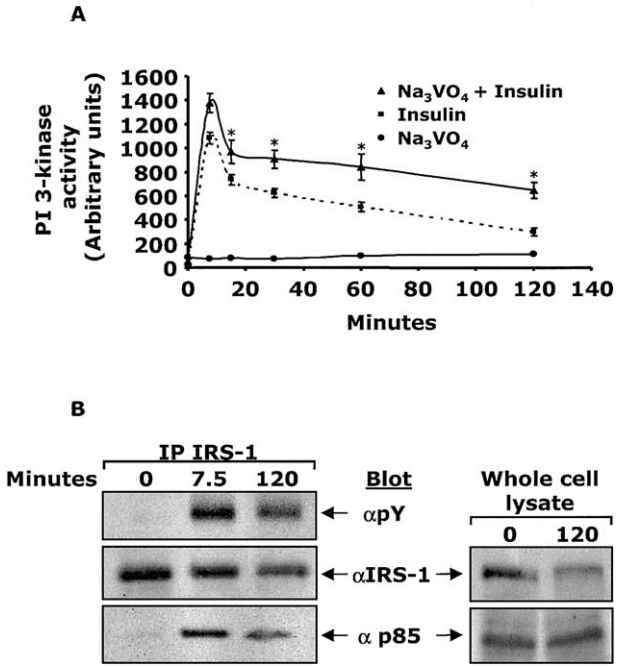

Fig 3. Na_3VO_4 does not fully block IRS-1/PI 3-kinase complex decay. (A) L6 myotubes were pretreated with 1 mmol/L Na_3VO_4 for 15 min prior to 100 nmol/L insulin addition. PI 3-kinase activity was measured in IRS-1 immunoprecipitates after 0, 7.5, 30, 60, and 120 min. Values represent means ± SEM from 3 independent experiments. *$P < .05$ *v* 7.5 min Na_3VO_4-pretreated insulin-stimulated IRS-1–associated PI 3-kinase activity. (B) Cells were treated as in (A) for 0, 7.5, and 120 min, and IRS-1 tyrosine phosphorylation (upper left panel), IRS-1 mass (middle left panel), and IRS-1–associated p85 mass (lower left panel) were measured in IRS-1 immunoprecipitates by Western analysis. Cells were treated as in (A) and IRS-1 (upper right panel) and p85 (lower right panel) mass were measured in whole cell lysates by Western analysis at 0 and 120 min. Results are representative of 3 independent experiments.

Na_3VO_4 Does Not Fully Block IRS-1/PI 3-Kinase Complex Decay

Sodium orthovanadate is a potent inhibitor of PTPases[35] and has been shown to block tyrosine dephosphorylation of IRS-1.[20] To determine if PTPase inhibition would block IRS-1/PI 3-kinase complex decay, L6 myotubes were pretreated with 1 mmol/L Na_3VO_4 for 15 minutes prior to 100 nmol/L insulin addition. Figure 3A shows that with Na_3VO_4 pretreatment, maximal insulin-induced IRS-1–associated PI 3-kinase activity

was achieved at 7.5 minutes and was 25% greater than with insulin alone. After 15, 30, 60, and 120 minutes, insulin-induced IRS-1–associated PI 3-kinase activity declined 30% ± 7.8%, 35% ± 6.2%, 40% ± 9.1%, and 50% ± 4.6%, respectively, in Na_3VO_4-pretreated cells. To determine the impact of Na_3VO_4 on IRS-1 tyrosine phosphorylation and IRS-1 mass loss, Western analysis was performed on IRS-1 immunopreciptates (Fig 3B). Consistent with Fig 3A, peak IRS-1 tyrosine phosphorylation after insulin addition occurred at 7.5 minutes in Na_3VO_4-pretreated cells, and by 2 hours, IRS-1 tyrosine phosphorylation had dropped 50%. In blots stripped and reprobed for IRS-1 and in whole cell lysates, IRS-1 mass declined 50% after 2 hours. Like IRS-1 tyrosine phosphorylation, IRS-1–associated PI 3-kinase p85 mass showed peak IRS-1 binding at 7.5 minutes and declined 50% 2 hours after insulin addition. PI 3-kinase p85 mass was unaffected by Na_3VO_4 and insulin addition. Taken together, these findings indicate that PTPase inhibition with Na_3VO_4 does not fully block insulin-induced IRS-1/PI 3-kinase complex decay in that insulin-dependent IRS-1 mass loss is not inhibited by Na_3VO_4.

MG-132 Fully Blocks Insulin-Dependent IRS-1 Mass Loss But Not IRS-1/PI 3-Kinase Complex Decay

MG-132 inhibits the rate-limiting step of proteasomal degradation[36] and has been shown to block chronic insulin-induced degradation of IRS-1.[29] To determine if acute insulin-induced IRS-1 mass loss via proteasomal degradation is critical to the downregulation of insulin signaling, L6 myotubes were pretreated with 10 μmol/L MG-132 for 30 minutes prior to 100 nmol/L insulin addition. Figure 4A shows that with MG-132 pretreatment, maximal insulin-induced IRS-1–associated PI 3-kinase activity was achieved at 7.5 minutes and was 25% greater than with insulin alone. After 15, 30, 60, and 120 minutes, insulin-induced IRS-1–associated PI 3-kinase activity declined 15% ± 5.1%, 30% ± 6.9%, 37% ± 3.3%, and 45% ± 9%, respectively, in MG-132–treated cells. To determine the impact of MG-132 on IRS-1 tyrosine phosphorylation and IRS-1 mass loss, Western analysis was performed on IRS-1 immunopreciptates (Fig 4B). Consistent with Fig 4A, peak IRS-1 tyrosine phosphorylation after insulin addition occurred at 7.5 minutes in MG-132–pretreated cells and, by 2 hours, IRS-1 tyrosine phosphorylation had dropped 45%. In blots stripped and reprobed for IRS-1 and in whole cell lysates, IRS-1 mass was unchanged at 0 and 2 hours. Like IRS-1 tyrosine phosphorylation, IRS-1–associated PI 3-kinase p85 mass showed peak IRS-1 binding at 7.5 minutes and declined 45% 2 hours after insulin addition. PI 3-kinase p85 mass was unaffected by MG-132 and insulin addition. Taken together, these findings indicate that 26S proteasome inhibition with MG-132 fully blocks insulin-induced IRS-1 mass loss but not IRS-1 tyrosine dephosphorylation and IRS-1/PI 3-kinase complex decay.

Rapamycin Parallels the Effects of MG-132 on IRS-1 Mass Loss

The mTOR inhibitor rapamycin has been shown to prevent degradation of IRS-1 induced by chronic insulin in 3T3-L1 adipocytes.[24,27] To determine if rapamycin would block acute

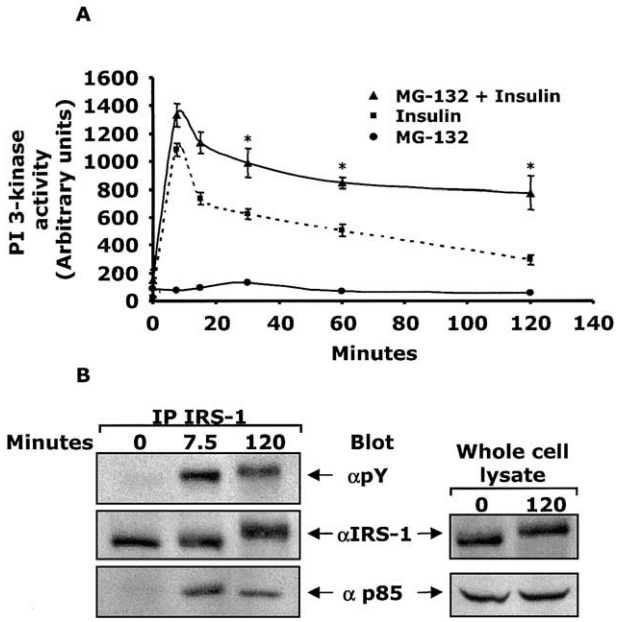

Fig 4. MG-132 fully blocks insulin-dependent IRS-1 mass loss but not IRS-1/PI 3-kinase complex decay. (A) L6 myotubes were pretreated with 10 μmol/L MG-132 for 30 min prior to 100 nmol/L insulin addition. PI 3-kinase activity was measured in IRS-1 immunoprecipitates after 0, 7.5, 30, 60, and 120 min. Values represent mean ± SEM from 3 independent experiments. *$P < .05$ *v* 7.5 min MG-132–pretreated insulin-stimulated IRS-1–associated PI 3-kinase activity. (B) Cells were treated as in (A) for 0, 7.5, or 120 min, and IRS-1 tyrosine phosphorylation (upper left panel), IRS-1 mass (middle left panel), and IRS-1– associated p85 mass (lower left panel) were measured in IRS-1 immunoprecipitates by Western analysis. Cells treated as in (A) and IRS-1 (upper panel) and p85 (lower panel) mass were measured in whole cell lysates by Western analysis at 0 and 120 min. Results are representative of 3 independent experiments.

insulin-induced IRS-1 mass loss in muscle, L6 myotubes were pretreated with 20 nmol/L rapamycin for 60 minutes prior to 100 nmol/L insulin addition. Figure 5A shows that with rapamycin pretreatment, maximal insulin-induced IRS-1–associated PI 3-kinase activity was achieved at 7.5 minutes and was 25% greater than with insulin alone. After 15, 30, 60, and 120 minutes, insulin-induced IRS-1–associated PI 3-kinase activity declined 33% ± 0.3%, 30% ± 7.3%, 35% ± 2%, and 35 % ÷ 2.3%, respectively, in rapamycin-treated cells. To determine the impact of rapamycin on IRS-1 tyrosine phosphorylation and IRS-1 mass loss, Western analysis was performed on IRS-1 immunopreciptates (Fig 5B). Consistent with Fig 5A, peak IRS-1 tyrosine phosphorylation after insulin addition occurred at 7.5 minutes in rapamycin-pretreated cells and, by 2 hours, IRS-1 tyrosine phosphorylation had dropped 40%. In blots stripped and reprobed for IRS-1 and in whole cell lysates, IRS-1 mass was unchanged at 0 and 2 hours. Like IRS-1 tyrosine phosphorylation, IRS-1–associated PI 3-kinase p85 mass showed peak IRS-1 binding at 7.5 minutes and declined 35% 2 hours after insulin addition. PI 3-kinase p85 mass was unaffected by rapamycin and insulin addition. Taken together, these findings indicate that rapamycin like MG-132 fully blocks insulin-induced IRS-1 mass loss but not IRS-1 tyrosine dephosphorylation and IRS-1/PI 3-kinase complex decay.

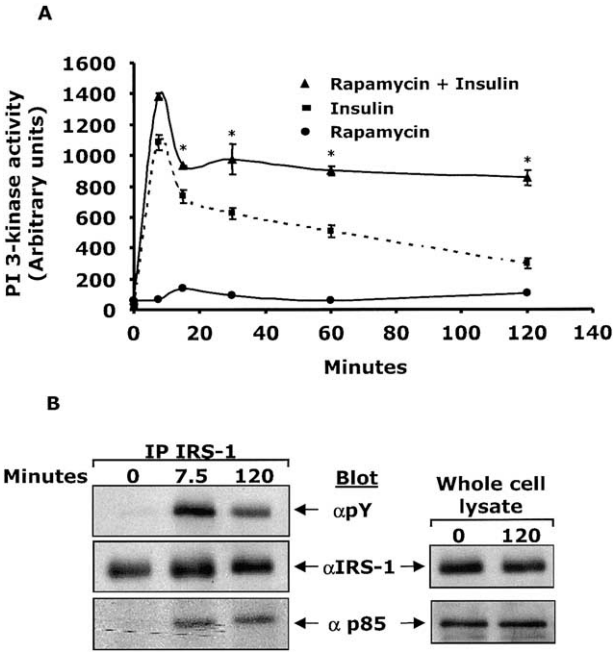

Fig 5. Rapamycin parallels the effects of MG-132 on IRS-1 mass loss. (A) L6 myotubes were pretreated with 20 nmol/L rapamycin for 60 min prior to 100 nmol/L insulin addition. PI 3-kinase activity was measured in IRS-1 immunoprecipitates at 0, 7.5, 30, 60, and 120 min. Values represent means ± SEM from 3 independent experiments. *$P < 0.05$ *v* 7.5 min rapamycin-pretreated insulin-stimulated IRS-1–associated PI 3-kinase activity. (B) Cells were treated as in (A) for 0, 7.5, or 120 min, and IRS-1 tyrosine phosphorylation (upper left panel), IRS-1 mass (middle left panel), and IRS-1–associated p85 mass (lower left panel) were measured in IRS-1 immunoprecipitates by Western analysis. Cells were treated as in (A) and IRS-1 (upper panel) and p85 (lower panel) mass were measured in whole cell lysates by Western analysis at 0 and 120 min. Results are representative of 3 independent experiments.

Insulin-Dependent IRS-1 Mass Loss and IRS-1/PI 3-Kinase Complex Decay Are Completely Blocked by Combined Na_3VO_4 Plus MG-132 Pretreatment

To determine if combined PTPase and proteasome inhibition would block insulin-induced IRS-1/PI 3-kinase complex decay, L6 myotubes were pretreated with 1 mmol/L Na_3VO_4 for 15 minutes and 10 μmol/L MG-132 for 30 minutes prior to 100 nmol/L insulin. Figure 6A shows that Na_3VO_4 plus MG-132 pretreatment increased PI 3-kinase activity 250% over insulin alone at 30 minutes and that at 120 minutes IRS-1–associated PI 3-kinase activity did not decline. To determine the impact of Na_3VO_4 plus MG-132 on IRS-1 tyrosine phosphorylation and IRS-1 mass loss, Western analysis was performed on IRS-1 immunopreciptates (Fig 6B). Consistent with Fig 6A, insulin-induced IRS-1 tyrosine phosphorylation in cells pretreated with Na_3VO_4 plus MG-132 was greater at 120 minutes than at 7.5 minutes. In blots stripped and reprobed for IRS-1 and in whole cell lysates, IRS-1 mass was unchanged at 0 and 2 hours. Like IRS-1 tyrosine phosphorylation, IRS-1–associated PI 3-kinase p85 mass was greater at 120 minutes than at 7.5 minutes. PI 3-kinase p85 mass was unaffected by Na_3VO_4 plus MG-132 addition. Taken together, these findings indicate that inhibition of PTPases and IRS-1 degradation completely prevents IRS-1:PI 3-kinase complex decay after insulin activation, signifying that downregulation of insulin signaling relies on both of these pathways.

Rapamycin in Combination With Na_3VO_4 Is More Effective Than MG-132 at Inhibiting IRS-1/PI 3-Kinase Complex Decay

To assess the impact of Na_3VO_4 plus rapamycin on IRS-1/PI 3-kinase complex decay, L6 myotubes were pretreated with 1 mmol/L Na_3VO_4 for 15 minutes and 20 nmol/l rapamycin for 60 minutes prior to 100 nmol/L insulin. Figure 7A shows that Na_3VO_4 plus rapamycin pretreatment increased PI 3-kinase activity 400% over insulin alone at 7.5 minutes and that at 120 minutes IRS-1–associated PI 3-kinase activity did not significantly decline. To determine the impact of Na_3VO_4 plus rapamycin on IRS-1 tyrosine phosphorylation and IRS-1 mass loss, Western analysis was performed on IRS-1 immunopreciptates (Fig 7B). Consistent with Fig 7A, insulin-induced IRS-1 tyrosine phosphorylation in cells pretreated with Na_3VO_4 plus

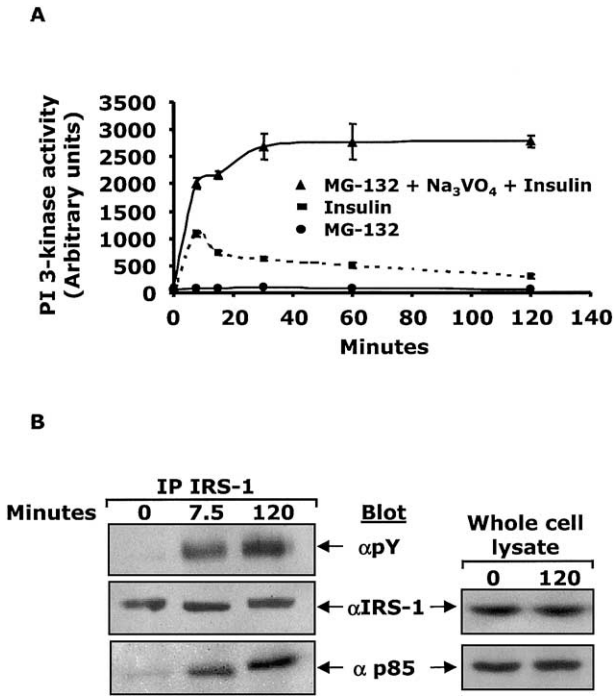

Fig 6. Insulin-dependent IRS-1 mass loss and IRS-1/PI 3-kinase complex decay are completely blocked by combined Na_3VO_4 + MG-132 pretreatment. (A) L6 myotubes were pretreated with 1 mmol/L Na_3VO_4 for 15 min + 10 μmol/L MG-132 for 30 min prior 100 nmol/L insulin addition. PI 3-kinase activity was measured in IRS-1 immunoprecipitates at 0, 7.5, 30, 60, and 120 min. Values represent means ± SEM from 3 independent experiments. No significant change from peak activation. (B) Cells were treated as in (A) for 0, 7.5, or 120 min, and IRS-1 tyrosine phosphorylation (upper left panel), IRS-1 mass (middle left panel), and IRS-1–associated p85 mass (lower left panel) were measured in IRS-1 immunoprecipitates by Western analysis. Cells were treated as in (A) and IRS-1 (upper panel) and p85 (lower panel) mass were measured in whole cell lysates by Western analysis at 0 and 120 min. Results are representative of 3 independent experiments.

rapamycin was equal at 7.5 and 120 minutes. In blots stripped and reprobed for IRS-1 and in whole cell lysates, IRS-1 mass was unchanged at 0 and 2 hours. Like IRS-1 tyrosine phosphorylation, IRS-1–associated PI 3-kinase p85 mass was equal at 7.5 and 120 minutes. PI 3-kinase p85 mass was unaffected by the addition of Na_3VO_4 plus rapamycin. Taken together, these findings indicate that Na_3VO_4 plus rapamycin is a potent augmenter of insulin-induced PI 3-kinase activation and an inhibitor of IRS-1/PI 3-kinase complex decay after insulin activation.

Comparison of Insulin-Dependent IRS-1 Tyrosine Phosphorylation and p85 Association Between the Inhibitors Na_3VO_4 MG-132, and Rapamycin

As demonstrated in Figs 3 through 7, when L6 cells are pretreated with Na_3VO_4, MG-132, rapamycin, MG-132 plus Na_3VO_4, or rapamycin plus Na_3VO_4, insulin-induced IRS-1–associated PI 3-kinase activity is increased when compared to cells treated with insulin alone. To directly compare inhibitor pretreatments to insulin after 7.5 minutes and 120 minutes of 100 nmol/L insulin addition, Western analysis was performed. As shown in Fig 8, Na_3VO_4, MG-132, rapamycin, MG-132 plus Na_3VO_4, and rapamycin plus Na_3VO_4, increased IRS-1 tyrosine phosphorylation at 7.5 minutes by 30%, 30%, 30%, 55%, and 45%, respectively, when compared to insulin alone at 7.5 minutes. In turn, insulin-associated PI 3-kinase p85 was increased 30%, 70%, 70%, 300%, and 300%, respectively. As expected, only MG-132 plus Na_3VO_4 and rapamycin plus Na_3VO_4 blocked the reduction in IRS-1 tyrosine phosphorylation and IRS-1–associated p85 mass loss at 120 minutes. Taken together, these findings show that the effect of inhibitor pretreatment on IRS-1 tyrosine phosphorylation and p85 association parallels the effect of these inhibitors on IRS-1–associated PI 3-kinase activity.

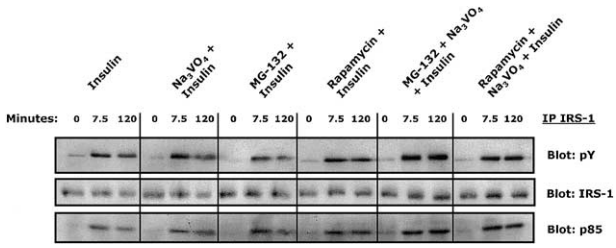

Fig 8. Comparison of insulin-dependent IRS-1 tyrosine phosphorylation and p85 association between the inhibitors Na_3VO_4, MG-132, and rapamycin. L6 myotubes were pretreated with the indicated inhibitors as described in Figs 3-7 prior to 100 nmol/L insulin addition. At the times indicated, IRS-1 tyrosine phosphorylation (upper panel), IRS- mass (middle panel), and IRS-1–associated p85 mass (lower panel) were measured in IRS-1 immunoprecipitates by Western analysis. Results are representative of 3 independent experiments.

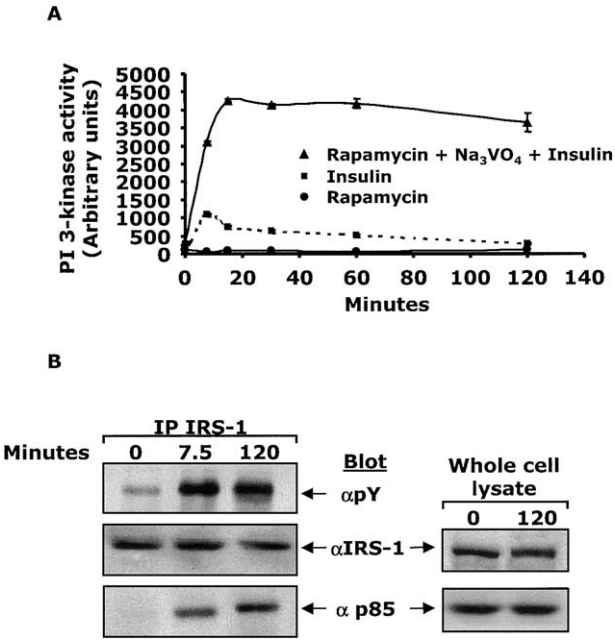

Fig 7. Rapamycin in combination with Na_3VO_4 is more effective than MG-132 at inhibiting IRS-1/PI 3-kinase complex decay. (A) L6 myotubes were pretreated with 20 nmol/L rapamycin for 60 min and 1 mmol/L Na_3VO_4 for 15 min prior to 100 nmol/L insulin addition. PI 3-kinase activity was measured in IRS-1 immunoprecipitates after 0, 7.5, 30, 60, and 120 min. Values represent means ± SEM from 3 independent experiments. No significant decline from peak activity. (B) Cells were treated as in (A) for 0, 7.5, or 120 min and IRS-1 tyrosine phosphorylation (upper left panel), IRS-1 mass (middle left panel), and IRS-1–associated p85 mass (lower left panel) were measured in IRS-1 immunoprecipitates by Western analysis. Cells were pretreated with rapamycin + Na_3VO_4 and as in (A) and IRS-1 (upper panel) and p85 (lower panel) mass were measured in whole cell lysates by Western analysis after 0 and 120 min. Results are representative of 3 independent experiments.

Low-Dose Vanadyl Sulfate Plus Rapamycin Synergistically Increases Insulin-Induced Glucose Uptake

At blood concentrations of 0.290 to 20 μmol/L, vanadyl sulfate has been shown to lower blood glucose in diabetic animals and humans.[37,38] To determine if vanadyl sulfate and rapamycin at potentially useful therapeutic concentrations synergize to augment glucose transport like Na_3VO_4 plus rapamycin, L6 myotubes were pretreated with 1 nmol/L vanadyl sulfate for 15 minutes, 10 nmol/L MG-132 for 30 minutes, 100 pmol/L rapamycin for 60 minutes, 1 nmol/L vanadyl sulfate plus 10 nmol/L MG-132 for 15 and 30 minutes, or 1 nmol/L vanadyl sulfate plus 100 pmol/L rapamycin for 15 and 60 minutes prior to 100 nmol/L insulin addition. Figure 9 shows that 2 hours after 100 nmol/L insulin exposure, glucose uptake was 21% ± 2.1% over basal glucose uptake. Cells pretreated with 1 nmol/L vanadyl sulfate plus 100 pmol/L rapamycin increased insulin-induced glucose uptake 3-fold (65.3% ± 18.4%) over insulin alone. Importantly, neither 1 nmol/L vanadyl sulfate nor 100 pmol/L rapamycin affected glucose uptake when used alone (data not shown). These findings indicate that subtherapeutic concentrations of vanadium and rapamycin synergize to markedly enhance insulin-responsive glucose uptake.

DISCUSSION

PTPases appear essential to the negative regulation of insulin signaling.[8] In mice, reduction of the G-protein subunit $G_{i\alpha 2}$ by

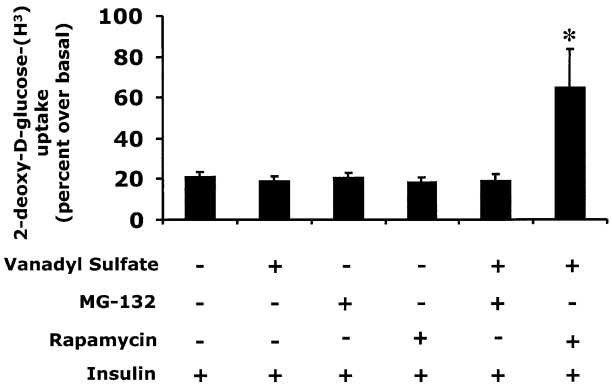

Fig 9. Low-dose vanadyl sulfate + rapamycin synergistically increases insulin-induced glucose uptake. L6 cells were pretreated with either 1 nmol/L vanadyl sulfate for 15 min, 10 nmol/L MG-132 for 30 min, 100 pmol/L rapamycin for 60 min, 1 nmol/L vanadyl sulfate for 15 min + 10 nmol/L MG-132 for 30 min, or 1 nmol/L vanadyl sulfate + 100 pmol/L rapamycin for 15 and 60 min, respectively as indicated. Cells were then stimulated with 100 nmol/L insulin and (H^3) 2-deoxy-D-glucose was assayed at 2 h. Values represent means ± SEM from 5 independent experiments. *$P < .05$ *v* all other treatments.

antisense mRNA expression induces a global increase in intracellular PTPase activity,[39] which subsequently causes decreased insulin-stimulated IRS-1 tyrosine phosphorylation, impaired glucose tolerance, and insulin resistance.[39] Overexpression of PTP-1B has been shown to decrease insulin-stimulated insulin receptor and IRS-1 tyrosine phosphorylation.[10] Likewise, PTP-1B knockout mice show increased insulin receptor autophosphorylation, IRS-1 tyrosine phosphorylation and PI 3-kinase activation, as well as augmented insulin-stimulated DNA synthesis.[9] While the physiologic role of vanadium has not been completely established, vanadate inhibits PTPases and exhibits insulin-mimetic effects in isolated adipocytes, hepatocytes, and muscle cells.[40] Sodium orthovanadate can also normalize hyperglycemia in streptozotocin-induced diabetic rats.[41] Our data support the importance of PTPases in negatively regulating insulin action. However, we found that Na_3VO_4 only partially blocked insulin signal downregulation. These findings are consistent with the existence of non–PTPase-requiring pathways for the counter-regulation of insulin action in skeletal muscle.

Proteasomal degradation of IRS-1 has emerged as an important mechanism by which chronic insulin exposure can lead to insulin resistance.[24-26] In 3T3-L1 adipocytes, chronic insulin targets IRS-1 to the proteasome but maintenance of IRS-1 tyrosine phosphorylation impairs its degradation.[24] Our data show that acute downregulation of insulin signaling in L6 myotubes utilized IRS-1 degradation and that inhibition of the 26S proteasome with MG-132 only partially blocked attenuation of insulin signal transduction. Importantly, inhibition of the proteasome with MG-132 did not inhibit the upward electromobility shift of IRS-1 after insulin stimulation. These results are consistent with what others have shown[24,26]: serine phosphorylation of IRS-1 is necessary for its degradation. In skeletal muscle cells treated chronically with insulin, inhibition of mTOR with rapamycin prevents IRS-1 elecromobility shift and IRS-1 degradation.[31] The specific mechanism by which mTOR regulates insulin-dependent IRS-1 degradation has not been fully delineated but mTOR-dependent IRS-1 serine phosphorylation on amino acid residues 636/639 appears essential.[42] Importantly, these IRS-1 serines are contained within SPXS motifs that are homologous to E3 ubiquitin-protein ligase bindings regions in other proteins.[30] Chronic insulin leads to IRS-1 ubiquitination and rapamycin prevents this process.[28] Our data support these findings and also show that acute insulin stimulation initiates IRS-1 degradation.

Insulin-dependent glucose transport in skeletal muscle is dependent on IRS-1–associated PI 3-kinase activation.[43] Our data show that when PTPase activity was inhibited with Na_3VO_4 and IRS-1 mass loss was blocked with either MG-132 or rapamycin, insulin-induced IRS-1/PI-3 kinase complexes were prevented from decaying. In addition, PI 3-kinase activity in these complexes was increased 2.5-fold and 4-fold, respectively. Without insulin, Na_3VO_4 in combination with either MG-132 or rapamycin did not significantly induce IRS-1–associated PI 3-kinase activity. When glucose uptake was examined, combinatorial treatment of L6 myotubes with Na_3VO_4 and rapamycin induced a synergistic increase that was 5-fold greater than that of insulin alone. These findings indicate that tyrosine dephosphorylation and IRS-1 mass loss are both necessary for decay of IRS-1/PI 3 kinase complexes and that augmentation of IRS-1 associated PI 3-kinase activity and prevention of complex decay increases glucose transport.

Unfortunately because of toxicity concerns, vanadate has little use as an antidiabetic agent in humans. Vanadyl sulfate, however, is 6 to 10 times less toxic than vanadate and elicits many of the same antidiabetic metabolic effects in rodents as vanadate.[44] Effective steady-state serum concentrations of vanadium in animal studies have ranged from 0.290 to 1.28 μmol/L[38] with peak values of 10 to 20 μmol/L in rats after receiving doses of 50 to 100 mg vanadium per day.[37] Importantly, toxicity did not appear important in long-term studies using streptozotocin (STZ)-treated rats.[38] In humans, vanadyl sulfate has been shown to improve hepatic and muscle insulin sensitivity[45] but results have been variable.[37] Consistent with these findings is that doses of vanadyl sulfate administered in humans have been lower than in rodents as have the resultant blood vanadium levels (2 to 10 times lower). Our data show that when 1 nmol/L vanadyl sulfate (the approximate serum concentration reached after taking a commercial multivitamin like Centrum [Wyeth, Madison, NJ]) is combined with 100 pmol/L rapamycin, insulin-dependent glucose uptake is increased 3-fold over insulin. While the synergistic effect on glucose uptake is apparent, the effect of low-dose vanadyl sulfate and rapamycin on protein turnover and tyrosine phosphorylation is not discernable by Western analysis (data not shown). In addition, neither vanadyl sulfate nor rapamycin at these concentrations had any effect on glucose transport by themselves. Insulin signaling proteins may be more sensitive to vanadate than to vanadyl in vitro,[46] which makes vanadate a valuable in vitro inhibitor in the study of signaling mechanisms involving PTPases; however, vanadyl sulfate appears to have more potential in the treatment of human disease due to its limited toxicity. Since this effect was seen at vanadium levels

100 times below human clinical trial levels and at rapamycin levels 50 times below its therapeutic immunosuppressive levels (rapamycin dispensed as the renal transplant rejection drug Rapamune [sirolimus; Wyeth][47]), these findings suggest that low dose vanadyl sulfate and rapamycin may offer a new approach to enhancing glucose transport in type 2 diabetes.

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