Cellular Chromium Enhances Activation of Insulin Receptor Kinase†

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Received December 21, 2004; Revised Manuscript Received March 17, 2005

ABSTRACT: Chromium has been recognized for decades as a nutritional factor that improves glucose tolerance by enhancing in vivo insulin action, but the molecular mechanism is unknown. Here we report pretreatment of CHO-IR cells with chromium enhances tyrosine phosphorylation of the insulin receptor. Different chromium(III) compounds were effective at enhancing insulin receptor phosphorylation in intact cells, but did not directly activate recombinant insulin receptor kinase. The level of insulin receptor phosphorylation in cells can be increased by inhibition of the opposing protein tyrosine phosphatase (PTP1B), a target for drug development. However, chromium did not inhibit recombinant human PTP1B using either *p*-nitrophenyl phosphate or the tyrosine-phosphorylated insulin receptor as the substrate. Chromium also did not alter reversible redox regulation of PTP1B. Purified plasma membranes exhibited insulin-dependent kinase activity in assays using substrate peptides mimicking sites of Tyr phosphorylation in the endogenous substrate IRS-1. Plasma membranes prepared from chromium-treated cells had higher specific activity of insulin-dependent kinase relative to controls. We conclude that cellular chromium potentiates insulin signaling by increasing insulin receptor kinase activity, separate from inhibition of PTPase. Our results suggest that nutritional and pharmacological therapies may complement one another to combat insulin resistance, a hallmark of type 2 diabetes.

Chromium(III) is considered an essential element for normal carbohydrate and lipid metabolism and has an established recommended daily administration (RDA). Chromium has been studied in defined diets as a micronutrient for a variety of animals for decades (*1*). A landmark discovery for chromium in human nutrition was reported in 1977 when a female patient on long-term total parenteral nutrition (TPN)[1] developed severe diabetic-like symptoms. The patient lost weight, accompanied by glucose intolerance and neuropathy, even with 45 units of exogenous insulin per day. When 250 μg of chromium chloride was added to the TPN solution for 2 weeks, the diabetic-like symptoms were alleviated (*2*). The beneficial effects of chromium on patients on TPN have been independently confirmed (*3*−*5*), and chromium is now routinely added to TPN solutions at a daily administration for adults of 10−15 μg/kg and 0.14−0.20 μg/kg for pediatric TPN patients (*6*). The signs of chromium deficiency include elevated blood glucose levels, increased insulin, cholesterol, and triglyceride levels, and decreased high-density lipoprotein (HDL) levels (*7*).

Studies over the past several years suggest a role for chromium in adjuvant therapy for type 2 diabetes (*7*, *8*). Anderson et al. (*9*) demonstrated that in type 2 diabetics supplemental chromium improved blood glucose levels and reduced blood insulin, cholesterol, and hemoglobin HbA1c levels in a dose-dependent manner. Follow-up studies confirmed these results (*10*). Other researchers report chromium supplementation alleviates gestational diabetes (*11*), steroid-induced diabetes (*12*), and glucose intolerance (*13*). Though patients with diabetes appear to benefit from supplemental chromium, conflicting results have clouded the issue (*14*−*18*). Multiple studies report that chromium would not lower blood glucose levels in healthy individuals (*19*−*22*). Various forms of chromium have been used in these clinical studies, including chromium chloride, nicotinate, and picolinate. Chief among these supplements is chromium picolinate, $Cr(Pic)_3$. Supplemental chromium has been suggested as an alternative or supplemental medication for patients with type 2 diabetes. This may or may not complement other existing and new pharmacological therapies for type 2 diabetes, an important issue.

How chromium produces biological effects is not well understood. More than 40 years ago, Mertz first showed dietary chromium improved glucose tolerance (*23*). A natural "glucose tolerance factor" (GTF) containing chromium, nicotinate, and amino acids was isolated from yeast (*23*−*26*). Other preparations of chromium complexes were isolated from liver (*27*, *28*), and called low-molecular weight chromium-binding substance (LMWCr) or chromodulin (*29*, *30*), and these have been proposed as candidates for the biologically active forms of chromium. However, the exact composition and structure of GTF or LMWCr have not been

† This work was supported by Grant AT01304 from National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS), National Institutes of Health, U.S. Public Health Service, Department of Health and Human Services.

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[1] Abbreviations: IR, insulin receptor; $CrCl_3$, chromium chloride; $Cr(Pic)_3$, chromium picolinate; $Cr(His)_3$, chromium histidine complex; $Cr_3O(Prop)_6$, trichromium propionate compound $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3{\cdot}H_2O$; TPN, total parenteral nutrition; GTF, glucose tolerance factor; PTP1B, protein tyrosine phosphatase 1B; pNPP, *p*-nitrophenyl phosphate.

10.1021/bi0473152 CCC: $30.25
Published on Web 05/13/2005

determined. Chromium picolinate (U.S. Patent 4,315,927) is a relatively insoluble complex produced to mimic GTF, and its absorption rate may contribute to its reported biological activity. This patented formulation is added to many consumer products, including daily multivitamins. The chromium histidine complex developed by scientists at the U.S. Department of Agriculture (USDA) and patented (U.S. Patent 6,689,383) is absorbed at least 50% better than Cr(Pic)$_3$, according to the patent. A synthetic chromium(III) oxo-hexapropionate cation is reported to mimic the ability of LMWCr, and when administered to rats, it lowered plasma insulin, cholesterol, and triglyceride levels (*31*, *32*).

There is compelling evidence that protein tyrosine phosphatase 1B (PTP1B) dephosphorylates and inactivates the insulin receptor and therefore downregulates insulin signaling (*33*−*38*). The most intriguing data come from knockout mice reported by two different labs (*36*, *37*). PTP1B-deficient mice displayed enhanced insulin sensitivity in muscle and resistance to diet-induced obesity. Antisense PTP1B oligonucleotide administration to selectively deplete this phosphatase in diabetic mice normalized blood glucose levels and improved insulin sensitivity (*38*), consistent with the results of PTP1B gene deletion. These results have spurred intense efforts at drug development of new PTP1B inhibitors for type 2 diabetes (*39*−*42*). As opposed to PTP inhibition that would enhance insulin action, chromium was suggested to activate membrane PTP activity (*43*). Whether chromium inhibits or activates PTP1B is an important open question.

Chromium enhances multiple actions of insulin, such as glucose and lipid metabolism, consistent with effects at an early step in signaling. Further, chromium has been reported to directly activate the IR (*44*), directing our attention to the IR. In this study, we found that chromium enhanced IR Tyr phosphorylation and IR kinase activity when added to intact cells, but not when it was added to the purified recombinant IR kinase domain. We show the effects were not due to inhibition of PTP1B activity, a reduced level of PTP1B dephosphorylation of the insulin receptor, or redox regulation of PTP1B. There was a stable increase in the specific activity of the IR kinase isolated from chromium-treated cells, suggesting some modification of the IR itself. Therefore, chromium supplementation remains a possible complementary therapy for type 2 diabetes, in conjunction with PTP1B inhibition.

EXPERIMENTAL PROCEDURES

Materials. The anti-phosphotyrosine (4G10), anti-insulin receptor (β-subunit), and anti-PTP1B antibodies were purchased from Upstate Biotechnology, Inc. The phospho-IGF (Tyr1131)−insulin receptor (Tyr1146) antibody was obtained from Cell Signaling Technology. The GST fusion protein of β-insulin receptor kinase (GST−IRTK) was obtained from Calbiochem Biosciences, Inc. pGEX-PTP1B was constructed by inserting the truncated catalytic domain of human PTP1B (residues 1−321 amplified by PCR) into the pGEX vector, and the GST−PTP1B protein was purified with glutathione−Sepharose. Chromium(III) histidine [Cr(His)$_3$] and the chromium(III) oxo-hexapropionate complex [$Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3 \cdot H_2O$ [$Cr_3O(Prop)_6$] were kindly provided by R. Anderson and J. Vincent, respectively. Chromium(III) chloride, $CrCl_3$, *p*-nitrophenyl phosphate (pNPP), and insulin were from Sigma. Chromium(III) picolinate [Cr(Pic)$_3$] was provided by Nutrition 21. Solutions of chromium compounds were standardized for elemental chromium content by graphite furnace atomic absorption (*45*).

Cell Culture, Immunoprecipitation, and Immunoblotting. CHO-IR cells (fewer than 20 passages) were maintained in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS) and 2 mM glutamine. CHO-IR cells were seeded into 100 mm plastic dishes and grown to 50% confluency, and then treated with different chromium compounds overnight (16−24 h). The treated cells were incubated in media without serum for 3 h, and then treated with human insulin for 5 min at 4 °C. The cells were washed with ice-cold PBS and lysed in 0.5 mL of RIPA lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM DTT, and protease inhibitors]. The lysates were centrifuged at 15000*g* for 10 min. The supernatants were removed and assayed for total protein amount by the Coomassie blue dye binding method (Bio-Rad). After normalization of protein, equal amounts (20 μg) of total proteins were subjected to SDS−PAGE and immunoblotted with anti-phospho-Tyr (4G10) antibody. For immunoprecipitation, CHO-IR cell lysates were incubated with 4 μg of anti-IRβ for 2 h, and then protein A beads were added for overnight incubation. The beads were washed three times with lysis buffer, and then used in the phosphatase assay.

pNPP Assay. Protein phosphatase activity was assayed in 96-well plates using *p*-nitrophenyl phosphate (pNPP) as a substrate. Enzyme was incubated with 10 mM pNPP in a 50 μL reaction solution [50 mM Tris-HCl (pH 7.5) and 50 mM 2-mercaptoethanol] for 30 min at 37 °C, and then reaction was stopped by adding 200 μL of 1 M Na_2CO_3. PTPase activity was measured by monitoring the increased absorbance at 410 nm on a microplate reader (EL_X800, Bio-Tek Instruments Inc.).

Inactivation of PTP1B by Hydrogen Peroxide (H_2O_2) and Reactivation by a Reducing Agent (DTT). For inactivation of PTP1B by H_2O_2, the GST−PTP1B fusion protein was incubated with increasing concentrations of H_2O_2 in 50 μL of reaction buffer [50 mM degassed MOPS (pH 7.2)] for 10 min at room temperature. The inactivation of PTP1B was assayed as the decrease in phosphatase activity against pNPP. To reactivate H_2O_2-inactivated PTP1B, the H_2O_2 was removed by adding catalase, and the solution was then incubated with the reducing agent DTT for 15 min. Chromium compound was added before adding DTT.

Dephosphorylation of the Insulin Receptor by Protein Tyrosine Phosphatase. Insulin receptor (IR) from different sources was used as the substrate: GST−IRTK fusion protein (Calbiochem), IR from immunoprecipitation, and IR in isolated CHO-IR cell membranes. The IR was autophosphorylated by incubation with 100 μM ATP in kinase reaction buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM β-glycerophosphate, 0.1% 2-mercaptoethanol, and 200 μM DTT]. Dephosphorylation of IR was carried out with either added GST−PTP1B or endogenous tyrosine phosphatases with or without the chromium compound.

Preparation of CHO-IR Plasma Membranes. CHO-IR membranes were prepared by the previously described method (*46*, *47*). Briefly, CHO-IR cells were grown to subconfluency on 150 mm plates, and incubated without serum for 3 h prior to preparation of the membranes. The cells were scraped from the plates and collected by centrifugation. The pellets were resuspended in 2 pellet volumes of harvesting solution [0.05 M boric acid (pH 7.2), 0.15 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$], and the suspension was added slowly with stirring to the extraction solution [0.02 M boric acid (pH 10.2) and 0.2 mM EDTA]. After the mixture had been stirred for 10 min, borate solution [0.5 M boric acid (pH 10.2)] was added and stirred for an additional 5 min. The majority of nuclei were pelleted by centrifugation at 1000*g*. The supernatants were removed and centrifuged for 30 min at 24000*g* to pellet membranes. The membrane-rich pellet was suspended in PBS and layered on top of a 35% sucrose solution, and centrifuged for 30 min at 24000*g*. The plasma membrane fraction at the sucrose−PBS interface was removed and diluted with PBS. The membrane was collected by centrifugation at 100000*g* and dissolved in 0.5% NP-40 lysis buffer, and the concentration of the protein was measured.

IR Kinase Assay. Plasma membranes were purified from CHO-IR cells pretreated with $Cr(His)_3$ or histidine (as control) overnight, and different amounts of membrane protein were incubated with 25 μM Axl-tide (KKSRGD**YMTM**QIG) (Upstate) or IRS-1(Y608) peptide (KKHTDDG**YMPM**SPGVA) (BioMol) as substrates and 100 μM [γ-^{32}P]-ATP with or without 100 nM insulin in 25 μL of kinase reaction buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM β-glycerophosphate, 0.1% 2-mercaptoethanol, 200 μM DTT, and 200 μM Na_3VO_4] for 20 min at 30 °C. After the reaction, 20 μL of the solution was spotted onto P81 paper (Whatman) squares, washed three times with a 0.75% phosphoric acid solution and once with acetone, and then dried. The kinase activity was calculated on the basis of the level of ^{32}P incorporation determined by scintillation counting, and reactions without added kinase were used as blanks.

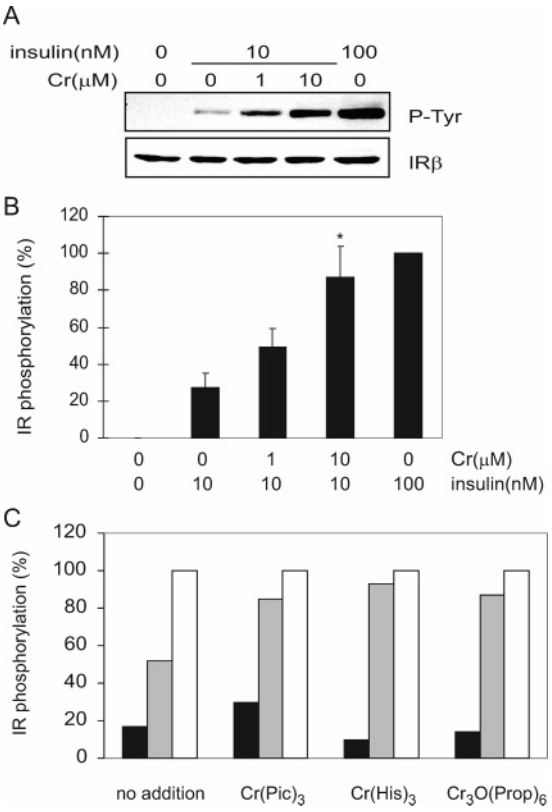

FIGURE 1: Chromium(III) enhances insulin receptor (IR) Tyr phosphorylation in intact CHO-IR cells. (A and B) CHO-IR cells were pretreated with $Cr(His)_3$ at the indicated concentration overnight. IR phosphorylation was analyzed by immunoblotting the IR β-subunit with the anti-phospho-Tyr (4G10) antibody (top portion of panel A). Phosphorylation levels were quantitated by densitometry in three independent experiments. The graphs in panel B show the relative IR phosphorylation level as the mean ± the standard error (the asterisk indicates $p < 0.05$). (C) CHO-IR cells were pretreated with or without different chromium compounds (1 μM chromium) overnight, followed the next day by insulin treatment (black bars for no insulin, gray bars for 5 nM insulin, and white bars for 100 nM insulin). IR phosphorylation was analyzed by immunoblotting with the anti-phospho-Tyr (4G10) antibody, and IR β-subunit phosphorylation levels were quantitated by densitometry.

RESULTS

Chromium(III) Enhances Insulin Receptor Tyrosine Phosphorylation in Living Cells. We examined activation of the insulin receptor (IR) in cultures of CHO-IR cells, which stably overexpress human IR. Insulin was added to intact cells, and phosphorylation of IR was detected by Western blotting whole cell lysates using a specific P-Tyr antibody (4G10). The 95 kDa β-subunit of the IR was the major P-Tyr protein detected in insulin-treated CHO-IR cells (Figure 1A). The IR was partially phosphorylated with low doses of insulin (1−10 nM) and maximally phosphorylated in response to 100 nM insulin. Insulin receptor Tyr phosphorylation with a low dose of insulin was examined over a range of chromium concentrations (from nano- to micromolar), and pretreatment of cells with a micromolar level of chromium enhanced IR Tyr phosphorylation. The level of insulin receptor Tyr phosphorylation was almost doubled in cells pretreated with 1 μM $Cr(His)_3$ and was further significantly enhanced by pretreatment with 10 μM $Cr(His)_3$ (Figure 1A and 1B). This showed chromium produced a dose-dependent enhancement of IR activation by insulin. As control histidine pretreatment of CHO-IR cells showed no dose-dependent increase in the level of IR Tyr phosphorylation (not shown). Concentrations of elemental chromium were verified by graphite furnace atomic absorption spectroscopy of the stock solutions. Live cells also were treated with different chromium(III) compounds, including chromium picolinate [$Cr(Pic)_3$], chromium histidine [$Cr(His)_3$], and chromium oxohexapropionate [$Cr_3O(Prop)_6$], compared to no addition as a control. Each of these different formulations produced enhanced Tyr phosphorylation of the IR at a low dose of insulin (Figure 1C). The results have been independently replicated with different stocks of cells and by different individuals. Chromium(III) compounds added to living CHO-IR cells enhanced insulin receptor Tyr phosphorylation.

Chromium Uptake by CHO-IR Cells. Overnight (16−20 h) incubation of cells with $Cr(His)_3$ or other chromium(III) compounds enhanced insulin-stimulated IR phosphorylation, compared to short-term (<1 h) chromium pretreatment that had little effect. Because cells required pretreatment with chromium for many hours, our interpretation was that chromium exerted its effects from inside the cell, not on the

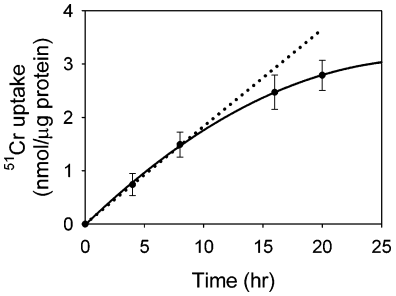

FIGURE 2: Chromium(III) uptake by CHO-IR cells. Cells at 50% confluence in six-well plates were incubated with radioactive [^{51}Cr]-chromium chloride (10 μCi/mL) in DMEM with 0.5% serum for various times, washed three times with cold PBS, and solubilized with 200 μL of buffer containing 50 mM Tris-HCl (pH 8.0), 2% Triton X-100, and 2 mM EDTA, and radioactivity was measured by gamma counting (Gamma Counter, LKB Compugamma model 1282). The data are plotted with a best fit curve and a dotted line added to indicate linear uptake. The graph shows the mean ± the standard error from three independent assays.

extracellular surface. We assayed the uptake of tracer levels of ^{51}Cr (as chromium chloride) by CHO-IR cells. Uptake was linear for 6−8 h, and the rate progressively slowed thereafter, approaching a plateau after 24 h (Figure 2). The accumulation of chromium by cells increased ~4-fold between 4 and 20 h. The results showed cells took up chromium from the medium relatively slowly. Consistent with the hypothesis of intracellular chromium(III) producing the effects, we observed no difference in specific binding of [^{125}I]insulin to intact CHO-IR cells in the presence or absence of chromium(III), using competition with various concentrations of unlabeled insulin (not shown).

Chromium Does Not Directly Activate the Recombinant IR Kinase Domain. We tested whether chromium would enhance the auto-Tyr phosphorylation of recombinant GST−IRTK. This glutathione *S*-transferase fusion protein comprises the cytoplasmic portion of the β-subunit of the IR, including the kinase domain and multiple autophosphorylation sites. GST−IRTK was preincubated with and without different concentrations of chromium chloride or chromium histidine, and then MgATP was added to assay autophosphorylation. The extent of Tyr phosphorylation was measured by Western blotting with anti-P-Tyr antibodies, and the amount of GST−IRTK in the assay was verified by anti-GST immunoblotting. Chromium compounds added at 1 or 10 μM did not enhance the autophosphorylation of the insulin receptor kinase domain (not shown). Further, the kinase activity of GST−IRTK was assayed with peptides as added exogenous substrate and micromolar concentrations of chromium had no effect on activity (not shown). We concluded that the effects of chromium on IR activation in cells occurred via an indirect mechanism, not by direct interaction with the kinase domain.

Chromium(III) and PTP1B. PTP1B is known as a major negative regulator of insulin signaling (*36*−*38*), and in animals, knockout or knockdown of PTP1B produces an elevated level of Tyr phosphorylation of IR. Inhibition of PTP1B by chromium could enhance IR Tyr phosphorylation in cells. To test this idea, we constructed a pGEX-PTP1B vector, expressed it in bacteria, and purified the GST−PTP1B protein. The GST−PTP1B protein was assayed using the chromogenic substrate *p*-nitrophenyl phosphate as a substrate in the presence of various concentrations of $CrCl_3$ and, as a

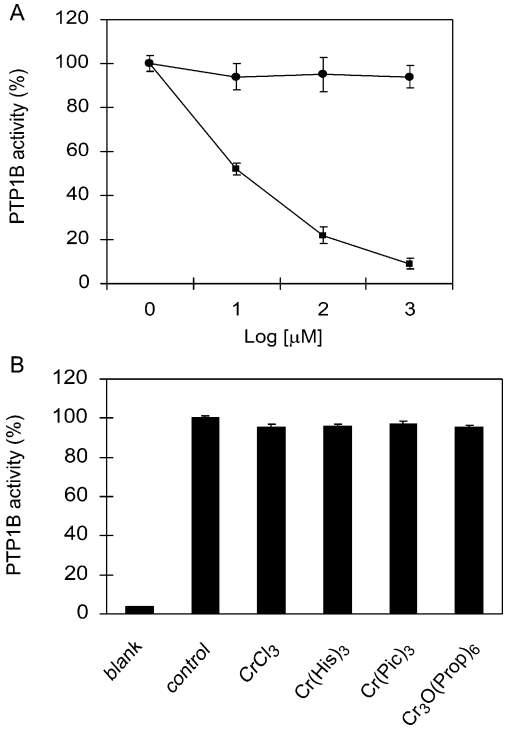

FIGURE 3: Effects of chromium(III) and vanadate on PTP1B activity. (A) Different concentrations of $CrCl_3$ (●) or Na_3VO_4 (■) were incubated with purified GST−PTP1B at room temperature for 10 min, and phosphatase activity was assayed by hydrolysis of *p*-nitrophenyl phosphate. (B) Different Cr(III) compounds (100 μM) were incubated with GST−PTP1B for 10 min, and then PTP1B activity was assayed as for panel A. The blank was buffer alone in the reaction. The positive control was GST−PTP1B with no chromium in the assay. The graphs show the relative PTP1B activity as the mean ± the standard error from three independent assays.

control, with *o*-vanadate, a known inhibitor of PTPases (Figure 3A). $CrCl_3$ did not inhibit GST−PTP1B at concentrations of up to 1 mM. In contrast, Na_3VO_4 inhibited PTP1B in a dose-dependent manner, with an IC_{50} of 10 μM. We also tested the effects of different chromium(III) compounds on GST−PTP1B activity, but none of them inhibited GST−PTP1B at 100 μM (Figure 3B). These results demonstrate that various forms of chromium(III) did not inhibit PTP1B phosphatase activity.

Chromium(III) and PTP1B Dephosphorylation of the Insulin Receptor. Chromium could inhibit dephosphorylation of IR in cells by interaction with IR (substrate) even if it did not directly inhibit PTP1B (enzyme). Therefore, we assayed dephosphorylation of a recombinant fusion protein of IR Tyr kinase (GST−IRTK) (Figure 4A), and also the intact IR immunoprecipitated from CHO-IR cells (Figure 4B). These IR preparations were autophosphorylated by incubation with MgATP, and dephosphorylation by added GST−PTP1B was tested in the presence of various concentrations of chromium, or the inhibitor Na_3VO_4 as a control. Immunoblotting confirmed that reaction mixtures all contained the same amounts of IR and added PTP1B (Figure 4). Dephosphorylation of GST−IRTK (Figure 4A) and immunoprecipitated IR (Figure 4B) were inhibited effectively by Na_3VO_4 (far right lanes), confirming that the diminished anti-P-Tyr immunoreactivity was due to PTP activity. However, chromium did not inhibit dephosphorylation of either of these Tyr-phosphorylated forms of IR. We con-

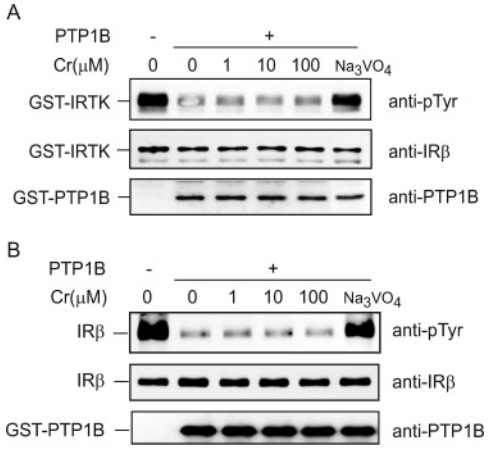

FIGURE 4: Dephosphorylation of IR by PTP1B with and without added chromium(III). GST−IRTK (A) or IR immunoprecipitated from CHO-IR cells with the anti-IRβ antibody (B) was autophosphorylated by incubation with ATP and Mg^{2+} for 1 h at 30 °C. Phosphorylated IR preparations were incubated with different concentrations of $CrCl_3$ on ice for 10 min, followed by dephosphorylation by addition of GST−PTP1B for 20 min at 30 °C. The level of phosphorylation of GST−IRTK and IR was analyzed by immunoblotting with the anti-phospho-Tyr (4G10) antibody. An anti-IRβ blot was used as loading control, and identical amounts of added GST−PTP1B were shown by immunoblotting.

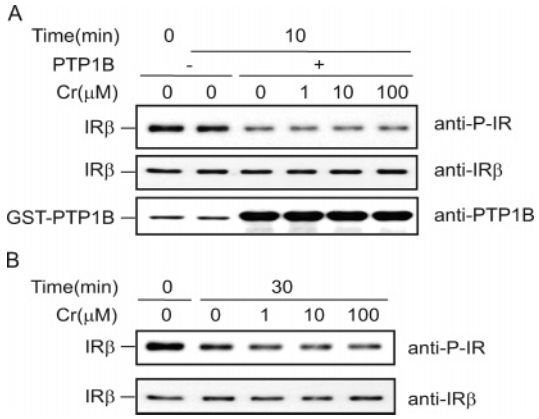

FIGURE 5: Dephosphorylation of IR in purified CHO-IR cell membranes. CHO-IR cells were incubated without serum for 3 h, and membranes were prepared by ultracentrifugation. IR was activated by incubation in kinase assay buffer with or without 100 nM insulin for 1 h at 30 °C. Dephosphorylation was performed with either added GST−PTP1B (A) or endogenous PTPases (B) in the presence of different concentrations of $Cr(His)_3$ at room temperature, for the indicated times. The Tyr phosphorylation level of IR was analyzed by immunoblotting with the anti-phospho-InsR antiboby. The amounts of IR present and added PTP1B were determined as controls by immunoblotting with anti-IRβ and anti-PTP1B antibodies.

cluded that dephosphorylation of P-Tyr sites in the β-subunit of the IR by PTP1B was not inhibited by chromium.

Another alternative is that endogenous PTPases besides PTP1B (such as LAR) might be involved in dephosphorylation of IR in cells, and these PTPs could be inhibited by cellular chromium. To test this possibility, we assayed the dephosphorylation of IR in CHO-IR cell membranes by endogenous PTPases as well as by added GST−PTP1B (Figure 5). The IR in membranes was phosphorylated by incubation with MgATP and the kinase reaction stopped by addition of EDTA to chelate divalent cations. After incubation for 10 min at room temperature, the Tyr phosphorylation of IR was relatively unchanged (Figure 5A, left two lanes),

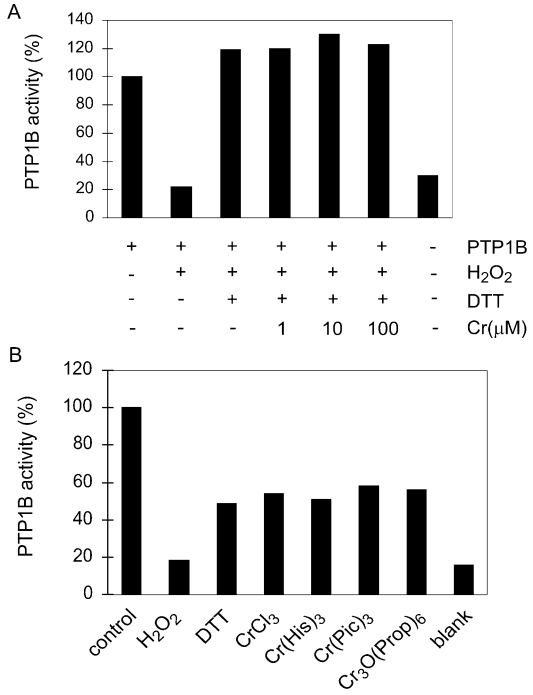

FIGURE 6: Redox regulation of PTP1B is unaffected by chromium-(III). GST−PTP1B was inactivated by incubation with H_2O_2 at room temperature for 10 min and incubated with catalase to remove H_2O_2 after inactivation. Reactivation of H_2O_2-inactivated PTP1B was achieved by incubation with DTT for 15 min. PTP1B activity was measured with the pNPP assay. (A) Increasing concentrations of $CrCl_3$ or (B) different Cr(III) compounds at 100 μM were added to the reaction mixture (100 μM H_2O_2 and 1 mM DTT were used) and incubated for 10 min before reduction with DTT. Control and blank were the same as for Figure 3.

consistent with a low level of endogenous PTP activity in these preparations of CHO-IR membranes. Added GST−PTP1B partially dephosphorylated the IR in these samples during the 10 min incubation, but addition of increasing concentrations of chromium together with the GST−PTP1B had no effect on the extent of dephosphorylation (Figure 5A). During longer incubation (30 min), IR was dephosphorylated by endogenous PTPases, but addition of various concentrations chromium did not inhibit this reaction (Figure 5B). The results showed that chromium did not inhibit dephosphorylation of the IR in CHO-IR membranes by either endogenous PTPases or added GST−PTP1B.

Chromium(III) and Redox Regulation of PTP1B. Insulin stimulation of cells produces reactive oxygen species (ROS) that reversibly inhibit PTPase activity (*48*, *49*), and cellular chromium(III) might reduce PTP1B activity in cells by affecting this regulation of oxidation and reduction. Incubation of GST−PTP1B with increasing concentrations of H_2O_2 showed dose-dependent inactivation of PTP activity (not shown). GST−PTP1B was fully inactivated with 100 μM H_2O_2, and this concentration was used in experiments described below. This oxidized, inactive PTP1B could be reactivated by reduction with DTT (Figure 6). The reduction−reactivation reaction mixture was assayed with 1, 10, and 100 μM added $CrCl_3$, without an obvious effect on the recovery of PTP1B activity (Figure 6A). Further, different chromium(III) compounds did not alter redox regulation of PTP1B (Figure 6B). Chromium apparently did not reduce PTP1B activity by trapping the oxidized, inactive form or by preventing its reduction and reactivation.

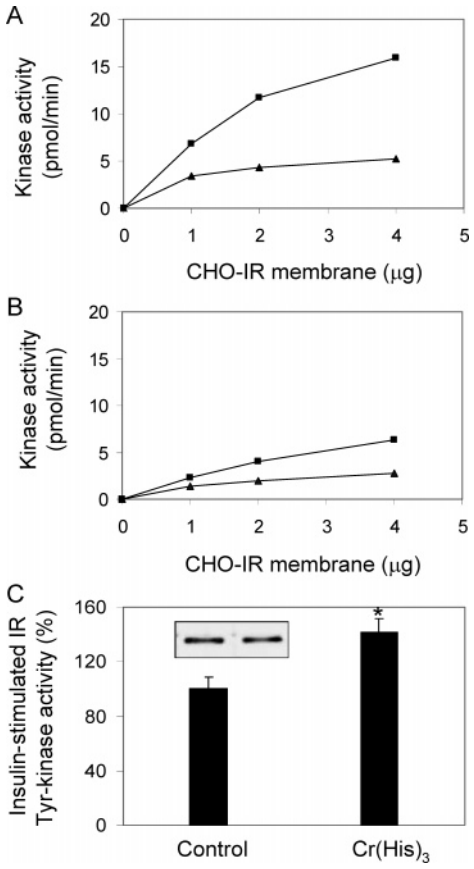

FIGURE 7: Cellular chromium(III) enhances insulin receptor (IR) tyrosine kinase activity. CHO-IR cells were treated with $Cr(His)_3$ or histidine overnight, and plasma membranes were purified as described in Experimental Procedures. Various amounts of membrane total protein were used in the kinase assays using as substrates the synthetic peptides Axl-tide (A) or IRS-1(Y608) (B) with 100 nM insulin (■) or no added insulin (▲). (C) Insulin-stimulated Tyr-kinase activity of membrane preparations is compared as the mean ± standard error of duplicate assays from four independent preparations of plasma membranes (the asterisk indicates $p = 0.05$). The inset in panel C is an immunoblot of the IR β-subunit in aliquots of the membrane preparations.

IR Kinase Activation by Cellular Cromium(III). We purified plasma membranes from CHO-IR cells using alkaline lysis and sucrose density gradient ultracentifugation and assayed the insulin-stimulated kinase activity of the IR (Figure 7). We tested as substrates two different peptides that contained a common YMXM motif that is preferred by the insulin receptor (*50*). These peptides mimicked different known phosphorylation sites in IRS-1, namely, Y612 and Y989 (human IRS-1 numbering). The level of peptide phosphorylation was increased by addition of 100 nM insulin to the membranes, and the difference (with or without insulin) was taken as the IR kinase activity. With the peptide corresponding to the Y989 site in IRS-1, there was a more than 3-fold increase with added insulin (Figure 7A), whereas with the peptide corresponding to the Y612 (Y608 in rat) site in IRS-1, there was a lower level of activity and only an ~2-fold increase in kinase activity with added insulin (Figure 7B). Plasma membranes from chromium-pretreated cells had increased levels of peptide kinase activity both with and without added insulin. The extent of insulin-dependent peptide phosphorylation (using the Y989 site) was significantly higher (by 40%) in membranes from chromium-pretreated cells, compared to that in membranes made in parallel from control cells (Figure 7C). This increase in IR kinase activity was assayed under conditions linear with respect to the amount of added membrane protein (1−2 μg) and time of incubation. Note that even greater positive and negative insulin differences were seen with larger amounts of membrane. The membranes were immunoblotted for the IR β-subunit, showing that identical amounts of IR were present in the assays (inset in Figure 7C). This revealed there was an increase in the specific activity of the IR kinase in plasma membranes purified from cells that were pretreated with chromium(III).

DISCUSSION

Nutritional studies over several decades starting with the pioneering work of W. Mertz have established that chromium enhances the actions of insulin. As a result, chromium(III) is considered an essential nutrient, and it is incorporated into multivitamins and animal feeds and appears as a supplement in a variety of consumer products. Supplemental chromium benefits patients with symptoms of type 2 diabetes and has been suggested as an adjuvant therapy to assist in regulating blood glucose levels. However, no protein or enzyme has a demonstrated specific requirement for chromium as a co-factor, and few studies have addressed the mechanisms for chromium action. Understanding the molecular basis for chromium enhancement of insulin signaling is key in deciding whether chromium supplementation can be expected to be an effective complementary approach for other therapies for type 2 diabetes.

In this work, we examined the effects of adding chromium to intact living cells on the responses to low and high doses of insulin, compared to no insulin added as a control. This experimental design is to assay for enhancement of insulin signaling under conditions of partial receptor activation, at submaximal doses of insulin. These conditions were intended to simulate the partial responses encountered in insulin resistant states, such as type 2 diabetes or related metabolic syndromes. We found that cellular chromium enhanced the insulin-stimulated Tyr phosphorylation of the human insulin receptor in CHO-IR cells. The stably overexpressed human insulin receptor in CHO-IR cells gave a pronounced dose-dependent response to chromium. In other preliminary experiments with CHO-IR cells, we observed by immunoblotting that cellular chromium enhanced Tyr phosphorylation of IRS-1 and SHC at submaximal doses of insulin (not shown). Our observations are consistent with reports that chromium enhanced membrane-associated GLUT4, PI3K activity, and Akt phosphorylation in response to insulin administration to JCR-LA rats (a model for insulin resistance) (*51*, *52*) or human subjects with type 2 diabetes (*53*). Molecular and cellular assays support the conclusion that chromium enhances signals emanating from the insulin receptor.

Various results indicated that chromium exerted its effects from inside cells rather than on the exterior surface of the cell. Chromium compounds did not produce effects when added to cells together with insulin or minutes before insulin, and experiments showed preincubation with cells for hours was required. This suggested that chromium was not enhancing binding of insulin to its receptor, or activating the receptor

or other signaling proteins by binding to extracellular domains. This by itself is an important conclusion in light of the recent discovery of a second hormone that activates the insulin receptor at a separate binding site, called visfatin (*54*). Furthermore, we found that chromium at micromolar concentrations did not alter the specific binding of [^{125}I]-insulin to intact CHO-IR cells in a competitive binding assay. This is consistent with chromium not altering insulin dose−response curves for glucose oxidation (*55*). We did observe a progressive increase in the cellular uptake of [^{51}Cr]-chromium chloride by CHO-IR cells over 24 h and routinely incubated cells overnight with chromium compounds prior to insulin stimulation. Apparently, chromium had to be taken into the cells to produce its effects. However, effects of cellular chromium appeared to be indirect because our assays with purified recombinant IR kinase indicated that chromium chloride or chromium histidine complex did not directly activate either the Tyr autophosphorylation of the β-subunit or Tyr phosphorylation of added peptide substrates. These results appear to contradict a previous publication in which the insulin-dependent receptor kinase activity with peptide substrates was increased 3−7-fold in response to picomolar concentrations of a chromium−peptide complex (called LMWCr) (*44*). We did not test LMWCr, so the results are not necessarily mutually exclusive; however, our chromium-(III) formulations were bioactive with cells without producing direct activation of the IR kinase.

One attractive hypothesis for the intracellular action of chromium is inhibition of the protein Tyr phosphatase (PTP1B) that dephosphorylates and inactivates the insulin receptor. Indeed, compelling evidence for PTP1B control of insulin receptor comes from gene deletion; PTP1B knockout mice exhibit enhanced sensitivity to insulin, and in vivo, there was an elevated level of Tyr phosphorylation of the insulin receptor. These mice also have improved glucose tolerance and are resistant to weight gain on high-fat diets. Intense efforts have been devoted to developing new PTP1B inhibitors as therapeutics to boost insulin sensitivity and treat type 2 diabetes. PTP1B and other PTPs employ a common mechanism involving an active site thiol that forms a covalent phosphoenzyme intermediate and are sensitive to inhibition by vanadate. We thought different chromium compounds might inhibit PTP1B, but observed no decrease in activity with the small molecule substrate *p*-nitrophenyl phosphate over the same range of concentrations where vanadate produced complete inhibition. Dephosphorylation reactions can be blocked by inhibiting the phosphatase active site, or by interfering with binding of the phosphoprotein substrate. To test this possibility, we assayed the dephosphorylation of different preparations of the insulin receptor by PTP1B in the presence and absence of chromium compounds. Again, no inhibition of dephosphorylation by purified PTP1B was observed, with either the recombinant insulin receptor cytoplasmic domain, the immunoprecipitated insulin receptor, or CHO-IR cell membranes as sources of the Tyr-phosphorylated receptor as a substrate. A previous publication claimed that a chromium complex (LMWCr) increased PTP activity in membranes (*43*), curious because such activation would be predicted to inhibit rather than enhance insulin signaling. Our data would exclude PTP1B dephosphorylation of the insulin receptor as the site of action of chromium.

Reversible oxidation of the active site cysteine residue in various PTPs offers a mechanism for regulation of phosphatase activity that could thereby affect Tyr phosphorylation of the insulin receptor. Recent studies demonstrated the structural basis for inactivation of PTP1B by hydrogen peroxide oxidation and reactivation by thiol-mediated reduction (*56*, *57*). We reproduced dose-dependent inactivation of PTP1B by hydrogen peroxide and reactivation by dithiothreitol. However, various chromium compounds did not affect these reactions, making it unlikely that in cells chromium diminished PTP1B activity by altering the redox cycle of regulation.

How does chromium enhance activation of the insulin receptor in cells if it does not inhibit or affect regulation of PTP1B? An alternative is that chromium produces changes in the insulin receptor itself. To test this possibility, we prepared plasma membranes from control and chromium-treated cells using alkaline cell hydrolysis. This method of preparation strips away most peripheral membrane proteins and yields highly purified membranes that exhibit insulin-dependent kinase activity, attributed to the receptor itself. The synthetic peptide substrates replicated sites of Tyr phosphorylation found in IRS-1 and had a common YMXM motif. Membranes from chromium-treated cells consistently exhibited a higher level of insulin-stimulated kinase activity. There was a significant 40% increase in the specific activity of IR kinase in the plasma membrane preparation. This suggested to us that the insulin receptor had been modified. The insulin receptor was long ago reported to undergo Ser phosphorylation by PKC (*58*, *59*). Further, Ser phosphorylation reportedly inhibited hormone activation of the insulin receptor, in effect partially uncoupling hormone binding from kinase activation. However, we do not detect any difference in PKC phosphorylation of IR using a specific PKC phosphosite antibody in immunoblotting side-by-side samples of IR immunoprecipitated from control and chromium-treated CHO-IR cells (not shown). Other recent studies have implicated multiple other kinases in stress signaling as a mechanism for eliciting insulin resistance (*60*−*64*), though attention has been directed mostly to Ser phosphorylation of IRS-1 and other downstream components of insulin signaling pathways (*63*, *64*). It is possible that the mechanism for chromium to enhance insulin receptor activation and insulin signaling is by reducing Ser phosphorylation, inhibiting the relevant Ser/Thr kinase(s), or promoting dephosphorylation by Ser/Thr phosphatases. Another possibility is that chromium alters the activity of G proteins that are reported to promote insulin-dependent autophosphorylation of the IR receptor (*65*). Chromium activation of a G protein by direct binding or indirectly by inhibition of GAP action could be involved.

An important conclusion from our studies is that cellular chromium enhances the action of insulin via changes in the insulin receptor, and accomplishes this without inhibition of PTP1B or membrane PTPs that regulate insulin receptor Tyr phosphorylation. As a consequence, chromium nutrition, especially of type 2 diabetics who have shown responsiveness to supplementation, should be an effective adjuvant treatment to act in concert with new therapeutic targeting of PTP1B. Nutritional and pharmacological approaches should offer a complementary means of enhancing insulin action in the face of insulin resistance.

ACKNOWLEDGMENT

We thank Richard Anderson of the USDA for the chromium histidine complex and for graphite furnace atomic absorption measurements of chromium content. John Vincent of the University of Alabama (Tuscaloosa, AL) provided the trichromium oxo-hexapropionate complex. Preliminary studies on chromium effects were supported by a grant from Kemin Foods and Kemin Industries.

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BI0473152