Reversible Change in Thiol Redox Status of the Insulin Receptor α-Subunit in Intact Cells

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ABSTRACT: In this study, we used maleimidobutyrylbiocytin to examine possible alteration that may occur in the redox state of the insulin receptor (IR) sulfhydryl groups in response to reduced glutathione (GSH) or *N*-acetyl-L-cysteine (NAC). Short-term treatment of intact cells expressing large numbers of IR with GSH or NAC led to a rapid and reversible reduction of IR α-subunit disulfides, without affecting the receptor β-subunit thiol reactivity. The overall integrity of the oligomeric structure of IR was maintained, indicating that neither class I nor class II disulfides were targeted by these agents. Similar findings were obtained in cells transfected with IR mutants lacking cysteine^{524}, one of the class I disulfides that link the two IR α-subunits. Membrane-associated thiols did not participate in GSH- or NAC-mediated reduction of IR α-subunit disulfides. No difference in insulin binding was observed in GSH-treated cells; however, ligand-mediated increases in IR autophosphorylation, tyrosine phosphorylation of cellular substrates, and dual phosphorylation of the downstream target mitogen-activated protein kinase were inhibited at concentrations of GSH (10 mM or greater) that yielded a significant increase in IR α-subunit thiol reactivity. GSH did not affect IR signaling in the absence of insulin. Our results provide the first evidence that the IR α-subunit contains a select group of disulfides whose redox status can be rapidly altered by the reducing agents GSH and NAC.

The insulin receptor is a member of the protein tyrosine kinase family that plays an important role in diverse physiological processes, including modulation of glucose homeostasis, gene expression, and cell survival (*1*, *2*). The insulin receptor is a heterotetrameric glycoprotein consisting of two α-β dimers linked by disulfide bonds (*3*). The α-chain has a total of 37 cysteine (Cys) residues, including a Cys-rich sequence that separates two homologous domains (L1 and L2), whereas the β-chain possesses 4 extracellular and 6 intracellular Cys residues (*4*, *5*). The disulfides that link the insulin receptor subunits have been placed in two categories based on location and sensitivity to reduction. Class I disulfides are characterized by their sensitivity to reduction with low concentrations of dithiothreitol (DTT)[1] and are responsible for the covalent linkage between the two α-subunits, giving rise to disulfide-linked α−β monomeric receptor species that can still bind insulin and undergo a ligand-mediated increase in receptor autophosphorylation and activation of the tyrosine kinase activity toward exogenous substrates (*3*, *6*, *7*). Two Cys residues per monomer involved in class I disulfide bonds have been identified either by microsequencing (*8*), by labeling with *N*-ethylmaleimide (*9*, *10*), or by site-directed mutagenesis (*7*). These include the α−α dimer bond in Cys^{524} and Cys^{682}. A second class of disulfide bonds in the insulin receptor, termed class II disulfide, has been described which requires stronger reducing conditions and/or denaturation prior to reduction (*3*). A single intramonomeric α−β disulfide linkage has been identified between the α-chain Cys^{647} and the β-chain Cys^{872} (*8*, *11*), whose substitutions resulted in impaired insulin signal transduction (*11*, *12*). Previous studies have demonstrated that insulin binding properties were altered following treatment of isolated rat adipocytes with low concentrations of DTT, culminating in modulation of cellular responses (*13*, *14*). It follows that a change in the thiol redox status of the insulin receptor's extracellular sulfhydryls may alter its intrinsic properties.

In this report we show that short-term treatment of cultured cells with the tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) but not the oxidized GSSG derivative led to a rapid and reversible reduction of select insulin receptor α-subunit disulfides, thereby allowing their conjugation with the thiol-reactive reagent maleimidobutyrylbiocytin (MBB) (*15*). *N*-Acetyl-L-cysteine (NAC) is widely used as modulator of thiol levels as a means of increasing the intracellular GSH pool (*16*, *17*). However, NAC itself also functions effectively as a free radical scavenger and reducing agent independent of GSH synthesis (*16*−*19*). As with the charged monothiol GSH, we observed that treatment of cells with NAC caused a reversible reduction of receptor α-subunit

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[1] Abbreviations: DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized product of GSH; NAC, *N*-acetyl-L-cysteine; MBB, maleimidobutyrylbiocytin; NEM, *N*-ethylmaleimide; CHO, Chinese hamster ovary cells; HIRc, CHO cells overexpressing the wild-type human insulin receptor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; IRS, insulin receptor substrate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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disulfides other than the previously reported class I and class II disulfides. The effect of GSH on insulin receptor activation and signaling was then examined.

EXPERIMENTAL PROCEDURES

Materials. Monoclonal anti-insulin receptor antibody (clone 29B4) and protein G-Plus/protein A−agarose were purchased from Oncogene Science (Manhasset, NY). Horseradish peroxidase (HRP)-conjugated monoclonal anti-phosphotyrosine antibody and polyclonal antibody against insulin receptor α-subunit (sc-710) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), MBB and insulin were from Calbiochem (La Jolla, CA), and polyclonal anti-rat carboxy-terminal IRS-1 antibody (06-248) was from Upstate Biotechnology (Lake Placid, NY). Sodium orthovanadate, L-cysteine, 2-mercaptoethanol, iodoacetamide, *N*-ethylmaleimide (NEM), NAC, GSH, oxidized glutathione (GSSG), and polyvinylpyrrolidone (PVP) were purchased from Sigma Chemical Corp. (St. Louis, MO); 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was from Pierce (Rockford, IL); blotting grade blocker nonfat dry milk was from Bio-Rad Laboratories (Hercules, CA); precasted 4−12% Tris−glycine gels, Tris−glycine SDS sample buffer, and polyvinylidene difluoride (PVDF) membranes were from Novex (San Diego, CA); HRP-conjugated streptavidin was from Vector Laboratories, Inc. (Burlingame, CA); PefablocSC was from Boehringer-Mannheim (Indianapolis, IN); and HRP-modified donkey anti-rabbit antibody and the Enhanced Chemiluminescent (ECL) detection system were from Amersham Corp. (Arlington Heights, IL).

Cell Culture and Treatments. Chinese hamster ovary (CHO) cells that overexpress the wild-type human insulin receptors (HIRc) and HTC rat hepatoma cells expressing either a large number of the wild-type human insulin receptors (IR^{wt}) or a mutated receptor with a Cys → Ser substitution at position 524 (IR^{S524}) were previously described (*20*, *21*). Both HTC cell lines were generously provided by Dr. Cecil C. Yip (University of Toronto, Canada). Cells were grown attached on tissue culture plates in Ham's F-12 medium (CHO cells) or DMEM (HTC cells) containing 100 units/mL penicillin, 100 μg/mL streptomycin, and either 10% (CHO cells) or 8% (HTC cells) fetal bovine serum. Three hours before the start of the experiment, the confluent cell monolayers were washed 3 times in phosphate-buffered saline (PBS), and serum-free medium was added. Cells were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Solutions (200 mM) of GSH, GSSG, and NAC were prepared in 1 M Hepes, pH 7.5, and added to the cells for the indicated times and concentrations.

Analysis of the Free Sulfhydryl Groups of the Insulin Receptor. Treated cells were washed with ice-cold PBS and immediately maintained in incubation buffer I containing 20 mM Hepes, pH 7.5, 125 mM NaCl, 5 mM KCl, and 11.1 mM glucose. After incubation at 6 °C for 5 min, 100 μM MBB was added, and the cells were incubated for another 10 min. L-Cysteine (4 mM) was subsequently added to remove unreacted MBB; the fluid was removed, and the dishes were rapidly immersed in liquid nitrogen. Cells were lysed in ice-cold immune precipitation buffer [25 mM Hepes, pH 7.5, 135 mM NaCl, 1% (w/v) NP-40, 0.1% (w/v) SDS, 1 mM orthovanadate, 0.5% (w/v) deoxycholate, 100 mM NaF, 0.01% NaN_3, 1 mM benzamidine, 8 μg/mL aprotinin, 2 μg/mL leupeptin, and 0.25 mM PefablocSC], and the crude lysates were clarified at 12000*g* for 20 min at 4 °C. The lysates were incubated with anti-insulin receptor antibody 29B4 in the presence of protein G-plus/protein A−agarose beads for 16 h at 4 °C. Immunocomplexes were washed twice in immune precipitation buffer and twice in washing buffer (50 mM Hepes, pH 7.4, 0.1% TX-100) followed by the addition of Laemmli sample buffer (*22*) in the presence of 7.5% 2-mercaptoethanol or 20 mM NEM. Proteins were resolved by SDS−PAGE on a 4−12% polyacrylamide gradient gel and electrotransferred onto PVDF membranes. Blots were blocked in Tris-buffered saline (TBS)−0.1% (w/v) Tween-20 (TBS-T) containing 1% PVP for 1 h at room temperature. Then the blots were incubated with a 1:100 000 dilution of HRP-conjugated streptavidin in 1% PVP/TBS-T for 1 h at room temperature. Membranes were washed extensively and developed using the Amersham ECL detection system. Unless otherwise indicated, the membranes were then incubated in TBS-T containing 5% nonfat dry milk and reprobed with a polyclonal antibody against the insulin receptor α-subunit in the presence of donkey anti-rabbit antibody coupled to HRP. The signals were quantified by laser densitometry (ImageQuant, Molecular Dynamics, Sunnyvale, CA).

Thiol-Biotinylation of Immunoprecipitated Insulin Receptors. Serum-starved CHO/HIRc cells were lysed in immune precipitation buffer, and the insulin receptors were immunoprecipitated with antibody 29B4 as described above. After a series of washes in washing buffer, aliquots of the immune pellet were incubated either with vehicle (100 mM Hepes, pH 7.5), with 20 mM NAC, or with 10 mM GSH for 1 h at 37 °C, followed by rapid washes in ice-cold washing buffer and addition of 100 μM MBB for 30 min at 4 °C. L-Cysteine (6 mM) was then added to quench unreacted MBB. The samples were washed, and prepared for SDS−PAGE and Western blot analysis.

Insulin Binding. Confluent monolayers grown in 12-well dishes were washed twice with PBS and then incubated with serum-free medium for 3−5 h prior to addition of GSH, NAC, or vehicle for 1 h at 37 °C. The cells were then washed twice in ice-cold binding buffer (100 mM Hepes, pH 7.4, 120 mM NaCl, 0.2 mM $MgSO_4$, 1 mM EDTA, 10 mM glucose, and 2 mg/mL sodium acetate) containing 5 mM NEM. Therefore, any disulfide bonds on the insulin receptor that were reduced with GSH or NAC were blocked by alkylation with NEM. ^{125}I-Insulin binding (10 pM) was carried out overnight at 4 °C with different concentrations of unlabeled insulin (0.6−30 nM) in binding buffer and 1% bovine serum albumin. Nonspecific binding was measured in the presence of 0.5 μM unlabeled insulin. Cells were washed 3 times with ice-cold TBS and solubilized in 0.5 N NaOH/0.1% SDS, and the lysates were counted in an ICN APEX 10/600 γ-counter.

Ligand-Stimulated Insulin Receptor Autophosphorylation in Intact Cells. Confluent cell monolayers were incubated for 3 h in serum-free F-12 medium, followed by the addition of 200 μM orthovanadate in the presence of GSH (0−20 mM) for 1 h. Cells were washed twice in PBS containing

2.5 mM DTNB, and were maintained in incubation buffer I with 2.5 mM DTNB for 15 min at 37 °C prior to quick PBS washes and a 5 min stimulation with 100 nM insulin. Hence, any free sulfhydryl groups on the insulin receptor α-subunit that were generated by GSH were blocked with DTNB. The cells were solubilized in immune precipitation buffer and insulin receptors immunoprecipitated with antibody 29B4 as described (*20*). Proteins were resolved by SDS−PAGE under reducing conditions and electrotransferred onto a PVDF membrane, and the membrane was incubated for 1 h in blocking buffer containing TBS-T with 5% (w/v) nonfat dried milk. Finally, the membrane was probed with a 1:48 000 dilution of HRP-conjugated anti-phosphotyrosine antibody in blocking buffer. The changes in tyrosine phosphorylation were detected by ECL. Membranes were reprobed with polyclonal anti-insulin receptor α-subunit antibody as described above.

Determination of Insulin Receptor Signaling in Intact Cells. Following treatment, cells were lysed directly in Laemmli sample buffer containing 7.5% 2-mercaptoethanol and 1 mM orthovanadate. After heating at 70 °C for 10 min, aliquots from each sample were separated by SDS−PAGE under reducing conditions on a 4−12% polyacrylamide gradient gel and electrotransferred onto PVDF membranes. The extent of IRS-1 and Shc tyrosine phosphorylation was detected by Western blotting with anti-phosphotyrosine antibodies. Activation of ERK 1/2 was assessed with phospho-specific anti-ERK 1/2 antibodies. In many instances, membranes were reprobed with polyclonal antibodies raised against IRS-1, Shc, and ERK 1/2 proteins.

Measurement of GSH Consumption. Empty dishes or dishes containing confluent CHO/HIRc cells were incubated in Ham's F12 medium supplemented with 200 μM orthovanadate and 100 mM Hepes, pH 7.5, in the presence of 1 or 10 mM GSH. At the indicated times, aliquots of the culture medium were collected and immediately frozen at −70 °C. The reduced form of GSH was determined using Ellman's DTNB method (*23*).

RESULTS

Time-Dependent Depletion in GSH Levels in Culture Medium. As shown in Figure 1, the addition of culture medium supplemented with GSH (1 or 10 mM) in empty dishes in a CO_2 incubator led to a rapid loss in the amount of reduced GSH. Less than 50% GSH remained after 0.5 and 4 h incubation with 1 and 10 mM GSH, respectively. By 2 h incubation, more than 90% of the 1 mM GSH initially added to the dishes was lost. Similar results were obtained using dishes containing CHO/HIRc cells. This finding is of special relevance because the response of the insulin receptor to low millimolar concentrations of GSH in cultured cells may be markedly attenuated due to GSH autoxidation (*24*).

Treatment with GSH or NAC Alters Insulin Receptor Thiol Reactivity in Intact Cells. To determine whether the insulin receptor in intact cells is subject to regulation by glutathione, we exposed CHO/HIRc cells to 0−40 mM GSH for 1 h at 37 °C, and quantitated the levels of free sulfhydryl groups in the insulin receptor using the thiol-biotinylating reagent MBB in conjunction with a sensitive immunoblot technique with enzyme-linked streptavidin. In control cells, only the receptor β-subunit was reactive to MBB, indicating that free

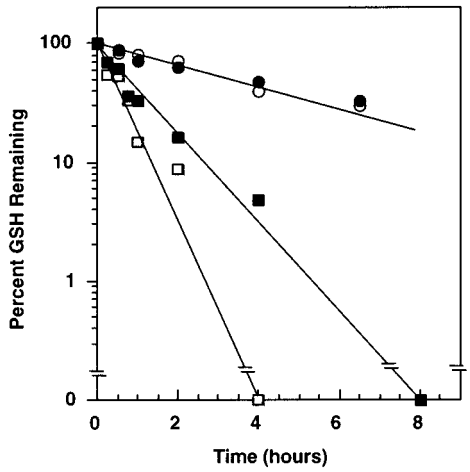

FIGURE 1: Loss in GSH levels in culture medium. Empty dishes (open symbols) or dishes containing CHO/HIRc cells (filled symbols) were incubated in culture medium supplemented with GSH at 1 (□, ■) or 10 mM (○, ●). At the indicated times, aliquots of the medium were collected and immediately frozen in liquid nitrogen. GSH levels remaining in the culture medium were quantitated using Ellman's method. Results are expressed as a percentage of the initial values. Data are the means ± range of two separate experiments.

thiol group(s) is (are) present on the β-chain. Our earlier work has demonstrated that this reactive Cys residue was located in the intracellular domain of the receptor β-subunit (*15*), in agreement with the work of others (*10*, *25*). Thiol reactivity of the α-chain but not that of the β-subunit increased in response to GSH in a dose- and time-dependent manner (Figure 2). Treatment of cells with 10 mM GSH led to reduction of the α-subunit thiols, achieving maximal reactivity at ≥20−30 mM GSH (Figure 2A). The ratio of thiol-biotinylated receptor subunits (α/β) passed from 0.6 to 4.0 in cells incubated with 10 and 20 mM GSH, respectively. In a second set of experiments, the preincubation step with 20 mM GSH varied from 15 min to 2 h. Within 15 min of exposure to GSH, the thiol reactivity in the α-chain increased, achieving 50% labeling at 30−45 min and maximal reaction at 1 h (Figure 2B). A change of glutathione redox status resulted in the attenuation of this GSH-mediated response, as the decrease in extracellular GSH/GSSG ratio caused a progressive loss in α-chain thiol reactivity without affecting the levels of thiol-alkylation of the β-subunit (Figure 3). Pretreatment of cells with 20 mM GSSG failed to modify receptor thiol reactivity.

We next assessed whether removal of the GSH from the incubation medium restores the native thiol redox status in the receptor α-chain. Evidence of recovery from thiol reduction due to GSH was readily detected after GSH washout (Figure 4). After a 15 min recovery, MBB labeling of the α-chain was reduced by more than 50%, and by 2 h incubation, there were no reactive α-subunit thiols detectable. The reactivity of the β-subunit thiols toward MBB remained unabated under these experimental conditions. Because GSH does not penetrate cells readily (*26*), these results indicate that extracellular GSH might act by affecting the redox state of membrane-associated protein thiols, including those of the insulin receptor α-subunit.

N-Acetyl-L-cysteine (NAC), a widely used modulator of thiol levels, acts as a cytoprotective drug with multiple therapeutic applications (reviewed in ref *27*), in part, by

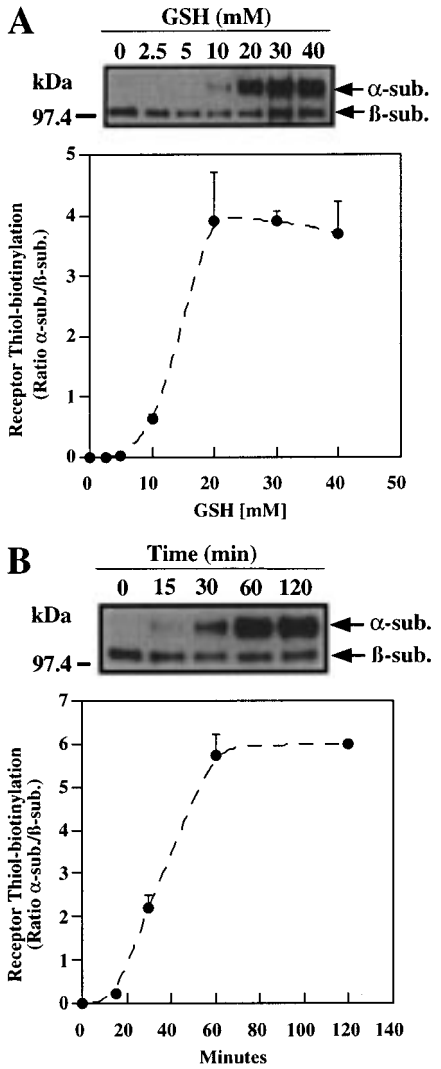

FIGURE 2: Addition of glutathione increases reactivity of the insulin receptor α-subunit thiols in intact cells. (A) CHO/HIRc cells grown to confluence were serum-starved for 3−5 h before experiments. Cells were then treated with GSH (0−40 mM) for 1 h at 37 °C, washed, and then incubated with 100 μM MBB at 6 °C. After the removal of unreacted MBB, cells were lysed and insulin receptors immunoprecipitated. Proteins were resolved in SDS−PAGE under reducing conditions, transferred to a PVDF membrane, and immunoblotted with HRP-conjugated streptavidin. Upper panel: A representative blot is presented. The positions of α- and β-subunits are indicated. Lower panel: Thiol-biotinylation of insulin receptor subunits was quantitated by scanning densitometry and expressed as a ratio of α-subunit/β-subunit. Data are the means ± SE of three independent experiments. (*B*) Cells were treated with 20 mM GSH for the times indicated and then labeled with MBB. Upper panel: A representative immunoblot is presented. Lower panel: Results are the means ± range of two separate experiments.

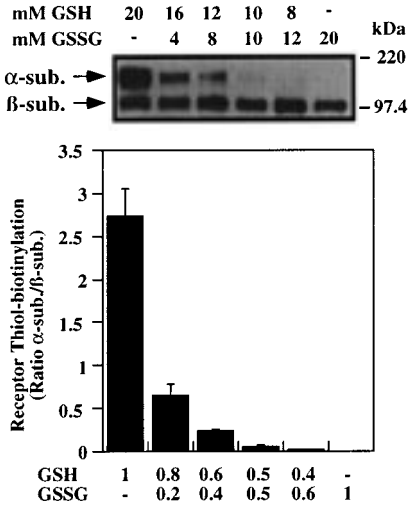

FIGURE 3: Insulin receptor α-subunit thiol reactivity is altered by the glutathione redox status. Serum-starved cells were pretreated with GSH and GSSG at the concentrations indicated for 1 h at 37 °C, washed, and then incubated with 100 μM MBB at 6 °C. Ten minutes later, cells were lysed and insulin receptors immunoprecipitated. Proteins were resolved in reducing SDS−PAGE and immunoblotted with HRP-conjugated streptavidin. Upper panel: A representative blot is presented. Lower panel: The level of thiol-biotinylated insulin receptor subunits was quantitated by scanning densitometry and expressed as a ratio of α-subunit/β-subunit. Data are the means ± SE of three independent experiments.

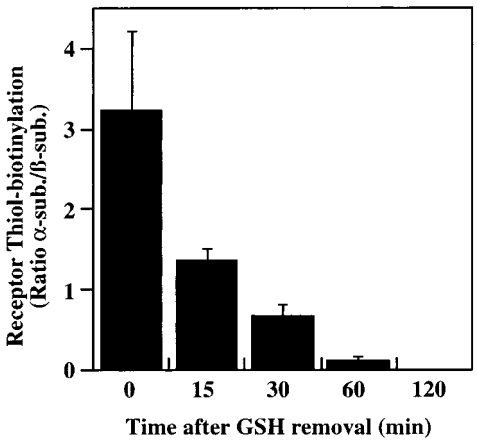

FIGURE 4: Reduction of insulin receptor α-chain thiols by glutathione is reversible. Cells were pretreated with 15 mM GSH for 1 h and labeled with MBB either immediately or after GSH washout for the times indicated. After cell lysis, receptors were immunoprecipitated and subjected to SDS−PAGE under reducing conditions and Western blotting. Thiol-biotinylated receptor subunits were detected by ECL as described above. Results are the means ± range of densitometric readings from two separate experiments.

acting directly as a reducing agent. Figure 5 shows that when CHO/HIRc cells were pretreated with NAC, the level of thiol reactivity in the insulin receptor α-chain increased in a concentration- and time-dependent fashion. The results also indicate that α-subunit thiols were less sensitive to NAC-mediated reduction than with GSH, as treatment of cells with 30 mM NAC gave rise to a ratio of thiol-biotinylation of receptor subunits (α/β) of only ~0.6 compared to a ratio of 4.0 with 20 mM GSH (Figure 5A vs Figure 2A). There was a rapid decline in the α-chain thiol reactivity in response to a 30 min NAC washout at 37 °C (Figure 5C). This perturbation in the redox state of the insulin receptor α-subunit thiols by NAC or GSH quickly fades after their removal.

Alteration of Insulin Receptor Thiol Reactivity by GSH and NAC Treatment in Vitro. It has been previously reported that a membrane-associated thiol component copurifies with the insulin receptor and can reduce specific disulfide bonds of the insulin receptor (*28*). To determine whether the action of GSH or NAC on the insulin receptor α-subunit thiols in intact cells results from the activation of a similar component, we assessed the reactivity of the insulin receptor sulfhydryls to thiols in vitro. In control experiments, incubation of immunoprecipitated insulin receptors with MBB led to selective thiol-biotinylation of the receptor β-subunit (Figure

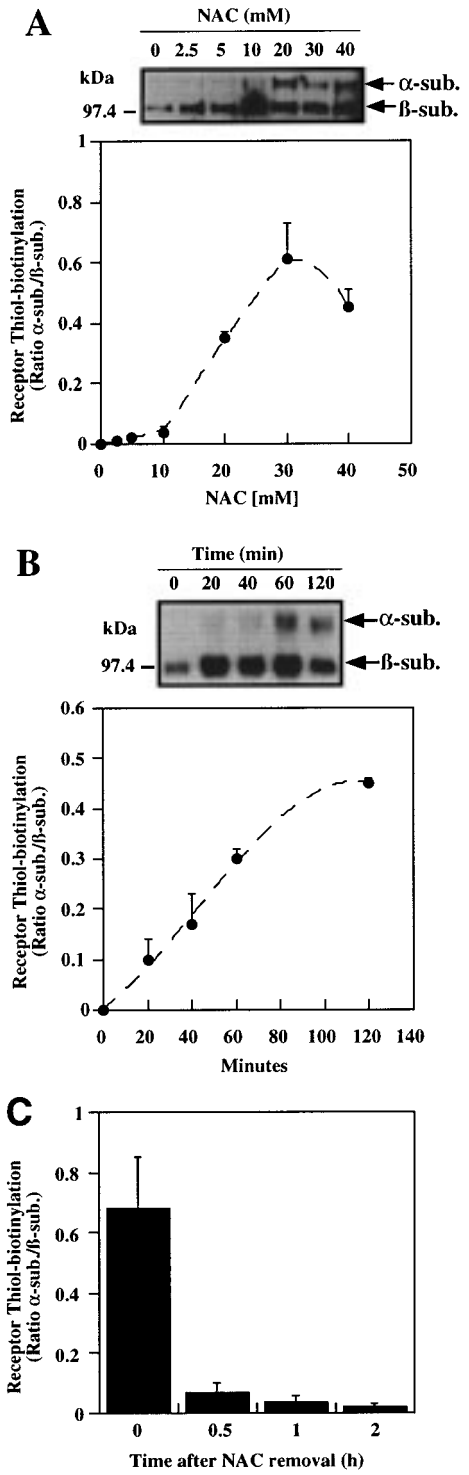

FIGURE 5: Reversible effect of NAC on insulin receptor α-subunit thiol reactivity in intact cells. (A) The NAC response is concentration (panel A) and time (panel B) dependent. The experimental conditions used are the same as in the legend of Figure 2. *(A)* Data are the means ± SE of three independent experiments. (B) Results are the means ± range of two separate experiments. (C) Cells were pretreated with 20 mM NAC for 1 h at 37 °C and labeled with MBB either immediately or after NAC washout for the times indicated. Insulin receptors were immunoprecipitated and subjected to reducing SDS−PAGE. Thiol-biotinylated receptor α- and β subunits were detected with streptavidin·HRP. Bars represent the means ± SE of four independent experiments.

6). The treatment of these purified receptors with GSH or NAC resulted in a marked increase in the reactivity of the insulin receptor α-subunit thiols to MBB.

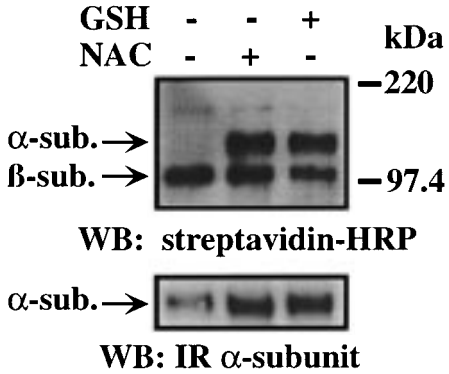

FIGURE 6: Effect of GSH and NAC on insulin receptor thiol reactivity in vitro. Insulin receptors were immunoprecipitated and then treated with 10 mM GSH or 20 mM NAC for 1 h at 37 °C, followed by a 30 min incubation with 100 μM MBB at 4 °C. After extensive washing, the samples were subjected to reducing SDS−PAGE and immunoblot analysis with enzyme-linked streptavidin (upper panel). A second membrane was probed with an antibody against the insulin receptor α-subunit (lower panel).

Oligomeric Structure of the Insulin Receptor and Thiol Reactivity. The possibility exists that perturbation in the redox status present in the extracellular milieu is associated with structural and/or functional changes in the insulin receptor, whereby GSH or NAC may selectively trigger reversible cleavage of class I disulfides, thus affecting the oligomeric structure of the insulin receptor. To determine whether treatment with GSH or NAC results in the appearance of $\alpha\beta$ heterodimeric receptor species, we used CHO/HIRc cells and HTC hepatoma cells expressing either the wild-type insulin receptor (IR^{WT}) or the mutant insulin receptor with a Cys→Ser substitution in one of the two class I disulfide bonds (IR^{S524}) (*21*). Following MBB labeling and cell lysis, the insulin receptors were immunoprecipitated, and then the immune complexes were eluted in Laemmli sample buffer containing 25 mM NEM or 7.5% 2-mercaptoethanol for 2 h at room temperature. Western blot analysis was performed both with enzyme-linked streptavidin (Figure 7A) and with anti-insulin receptor α-subunit antibody (Figure 7B). Under nonreducing conditions, the predominant receptor species migrated as intact thiol-biotinylated heterotetrameric molecules, indicating that the native insulin receptors are resistant to cleavage of class I thiols following treatment of CHO/HIRc cells with GSH or NAC. Similar results were obtained with HTC cells expressing IR^{WT} or IR^{S524} (Figure 7). These data support the hypothesis that class I or II disulfide bonds are not likely targets of GSH or NAC action.

Effect of GSH or NAC on Insulin Binding and Insulin Receptor Functions. Intact cells were incubated for 1 h at 37 °C in medium containing NAC or GSH at 20 mM, followed by alkylation of any free sulfhydryl groups with NEM. Following removal of excess NEM, ^{125}I-insulin binding was carried out overnight at 4 °C. Cell treatment with GSH or NAC resulted in a small 15% increase in insulin binding; however, the K_d values for insulin were similar to untreated cells, as measured by Scatchard analysis (data not shown). Thus, the enhancement in reactivity of the insulin receptor α-subunit thiols by GSH or NAC had little impact on insulin binding.

In this study, cell treatment with GSH is followed by a reversible reduction of disulfide bond(s) in the insulin receptor α-subunit. Addition of 5,5′-dithiobis(2-nitrobenzoic

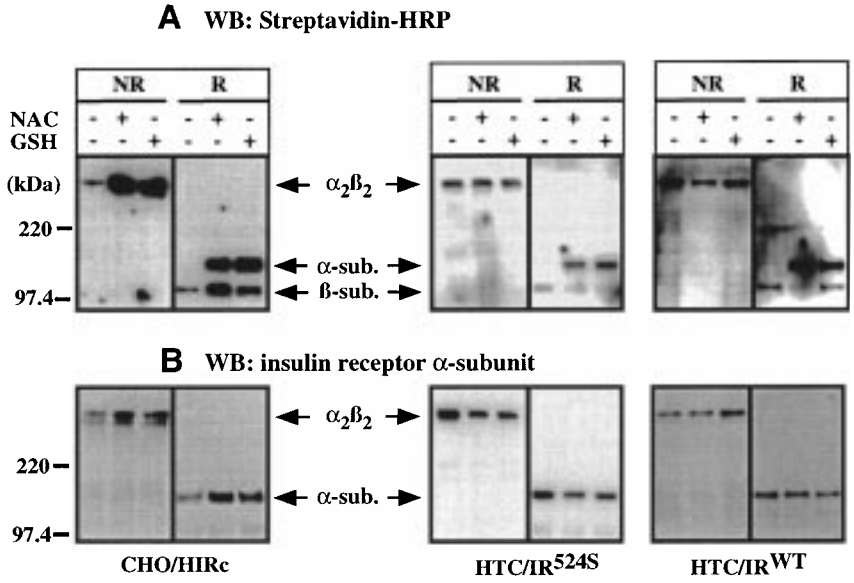

FIGURE 7: Oligomeric structure of the insulin receptor following cell treatment with GSH or NAC. CHO/HIRc cells and HTC cells expressing IR^{wt} or IR^{S524} were pretreated with GSH (15 mM) or NAC (20 mM) for 1 h and then thiol-biotinylated with MBB as described above. The cells were solubilized and the insulin receptors immunoprecipitated. The immunoprecipitates were denatured in Laemmli sample buffer, separated by SDS−PAGE under nonreducing (NR) or reducing (R) conditions, and subjected to immunoblotting with HRP-conjugated streptavidin and anti-insulin receptor α-subunit antibody.

acid) immediately following the removal of GSH was performed in order to create a disulfide bond between free sulfhydryl groups on the receptor α-subunit and DTNB. Under these conditions, MBB-mediated thiol-biotinylation of the receptor α-subunit was blocked while having no effect on *β*-subunit thiol reactivity in CHO/HIRc cells (Figure 8A). Hence, this approach provided a way to assess whether modification of sulfhydryl groups on the receptor α-subunit by GSH culminated with alteration in insulin action. As depicted in Figure 8B,C, insulin receptor-mediated responses including ligand-stimulated autophosphorylation of the receptor *β*-subunit (*29*), tyrosine phosphorylation of a 185 kDa cellular protein and Shc proteins (*30*), and activation of mitogen-activated protein kinase ERK 1/2 by dual phosphorylation (*31*) were inhibited by a 1 h treatment of CHO/HIRc cells with concentrations of GSH greater than 10 mM. Note that in the absence of insulin, GSH did not induce ligand-independent activation of insulin receptor autophosphorylation and downstream signaling.

DISCUSSION

Redox regulation through reactive sulfhydryls is emerging as an important mechanism by which the activities of receptor tyrosine kinases and their associated protein tyrosine phosphatases are modulated by oxidants and thiol-alkylating agents in vivo (*32*). Earlier studies indicated that the insulin receptor *β*-subunit contains one or more accessible cysteine residues in the cytoplasmic domain whose derivatization by sulfhydryl-modifying reagents (*15*, *33*) or their oxidative modification by alteration of intracellular redox state (*34*−*36*) can modulate the intrinsic tyrosine kinase activity of the receptor. In this study, we presented evidence for a rapid and reversible reduction of disulfide bond(s) in the insulin receptor α-subunit by reduced GSH or NAC that occurred without affecting the insulin receptor *β*-subunit thiols or the receptor oligomeric structure. This alteration in the redox state of the receptor sulfhydryl groups was accompanied by a decrease in insulin signal transduction in GSH-treated cells.

We determined that GSH or NAC mediates reduction of select insulin receptor α-subunit disulfide(s) by analyzing receptor thiol reactivity with MBB, a thiol-specific biotinylation reagent. GSH is a cell-impermeant monothiol that has the property of forming mixed disulfides with proteins (*37*, *38*). The possibility of *S*-thiolation of the insulin receptor in GSH-treated cells is highly unlikely due to the fact that the receptor α-subunit thiols reacted avidly with MBB. Moreover, a marked attenuation in the ability of MBB to thiol-biotinylate insulin receptor α-subunit thiols was achieved by incubating GSH-treated cells with the disulfide exchange reagent DTNB prior to the addition of MBB. There was no reduction of the receptor α-subunit disulfides when cells were treated with 20 mM oxidized glutathione (GSSG), indicating the absence of extracellular glutathione metabolism in which GSSG is converted to its reduced form. We found that an increase in exogenous GSSG levels resulted in marked attenuation of GSH-mediated reaction with the insulin receptor α-subunit disulfides.

When added to intact CHO/HIRc cells, GSH had no effect on insulin binding nor on insulin receptor structure, as denoted by the maintenance of the $\alpha_2\beta_2$ oligomeric receptor species. This indicates that the insulin receptor class I disulfides are resistant to reduction by GSH, in agreement with the findings of Chiacchia (*39*). An independent line of evidence that class I disulfides are not the target of GSH action is provided by the observation that treatment of HTC-IR^{524S} cells (expressing a mutant insulin receptor lacking one of the two class I disulfides) with GSH failed to disrupt the heterotetrameric structure of the receptor mutant while retaining a MBB-induced thiol-biotinylated signal in the α-subunit of these mutated receptors. GSH and NAC both induced the reduction of select insulin receptor α-subunit disulfides, suggesting that a structural feature common for both reagents contributed to their action. However, the more

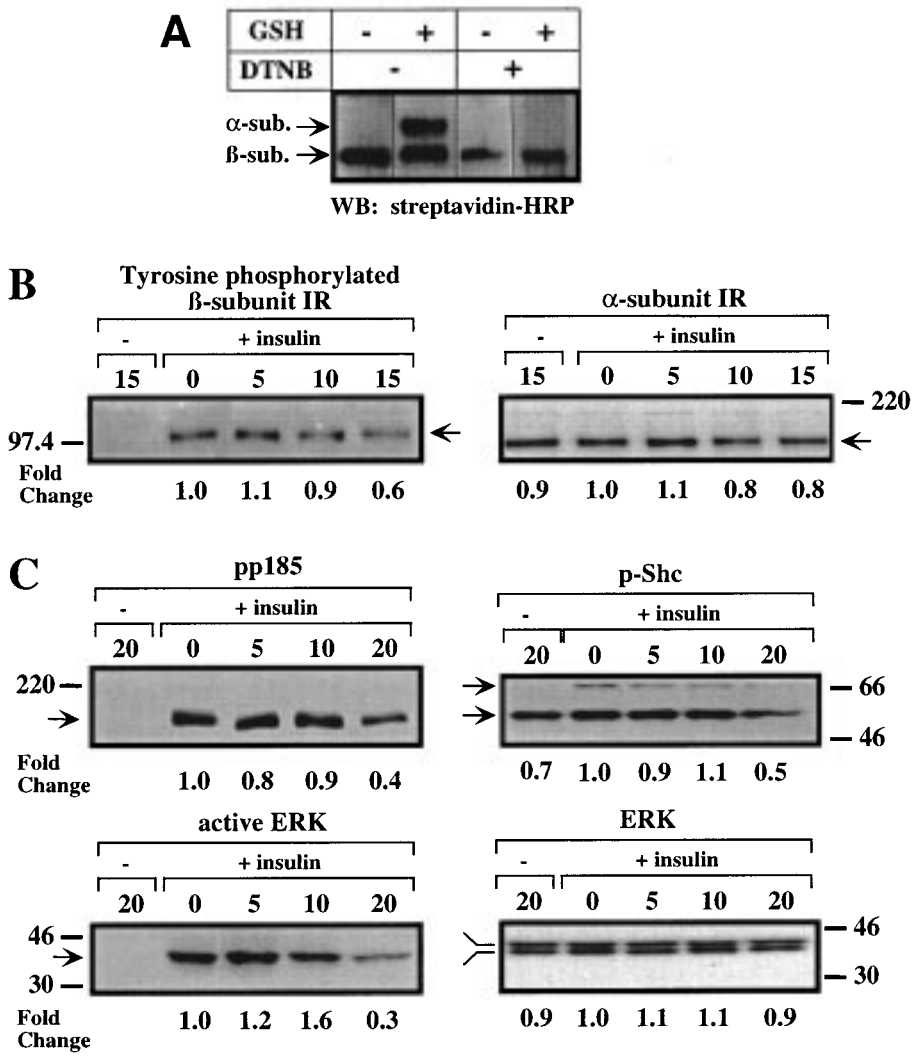

FIGURE 8: Effect of GSH on insulin receptor-mediated signaling events. (A) Formation of mixed disulfides between DTNB and the GSH-responsive insulin receptor α-subunit thiols. Serum-starved CHO/HIRc cells were treated with 200 μM orthovanadate in the absence (−) or presence (+) of 15 mM GSH for 1 h at 37 °C and then washed in PBS prior to the addition of incubation buffer containing or not containing 2.5 mM DTNB. After a 15 min incubation, cells were washed, and thiol biotinylation with MBB was performed. **(**B) GSH and insulin receptor autophosphorylation. Confluent cells were pretreated with the indicated concentrations of GSH (in mM) for 1 h, washed, and treated with 2.5 mM DTNB for 15 min at 37 °C. Cells were washed and then incubated without (−) or with (+) 100 nM insulin for 5 min at 37 °C. The cells were solubilized; the insulin receptors immunoprecipitated and subjected to immunoblotting with anti-phosphotyrosine (left panel) and anti-insulin receptor α-subunit (right panel) antibodies. (C) Whole cell extracts were loaded on gels, and the proteins were separated by SDS−PAGE under reducing conditions. Blots were probed with phosphotyrosine (upper panel), phosphoERK (lower left panel) and ERK (lower right panel) antibodies. The signal associated with each band was quantitated as described under Experimental Procedures. The fold change in each protein of GSH-treated cells relative to that of control cells was calculated from several independent experiments. Molecular mass markers (in kilodaltons) are shown.

potent effect of GSH may be due to the difference in oxidation potential of the two reagents (*16*). Of interest, we report here that GSH- and NAC-dependent reduction of the insulin receptor α-subunit disulfides is a rapid and reversible process, which contrasts sharply with the irreversible cleavage of the receptor extracellular disulfides by DTT (*3*). The dithiol DTT (at millimolar concentrations) causes an irreversible reduction of class I disulfides, thereby affecting the oligomeric structure of the insulin receptor.

The mechanism of action of GSH or NAC upon the insulin receptor α-subunit is unclear. We determined that cell permeabilization with digitonin did not lead to reduction of the receptor α-subunit disulfides, indicating that the release of intracellular low-molecular-mass thiols per se did not influence the reactivity of the receptor α-subunit sulfhydryls (*15*). Previous studies have reported that membrane-associated thiol components can trigger reduction of insulin receptor α-subunit disulfides (*40*, *41*). Although poorly characterized, this activity was shown to be inhibited by alkylating agents. Thus, the possibility existed whereby activation of membrane thiol(s) by GSH or NAC could subsequently act on the insulin receptor in intact cells. Our observation that the immunoprecipitated insulin receptors were also reactive with GSH or NAC supports the notion of a direct interaction between these reducing agents and the receptor α-subunit disulfides.

Exposing cells to GSH (10 mM or greater) for 1 h resulted in an attenuation of insulin receptor autophosphorylation and signaling in response to insulin, but not in its absence. This result suggests a possible association between GSH-mediated reduction in insulin receptor α-subunit thiols and alteration in insulin receptor signal transduction. Despite the attractive-

ness of these findings, the possibility exists that changes in redox state may affect the steady-state phosphorylation levels of the insulin receptor, IRS-1, and Shc proteins through a mechanism that is independent of GSH-induced effects on insulin receptor α-subunit disulfides. GSH may not need to enter the cells to alter insulin receptor activation and signaling, as several cell lines, including CHO cells, have been shown to be impermeable to the charged reducing agent GSH (*26*, *42*). Moreover, under conditions where normal cellular GSH levels are present, no increase in GSH intracellular levels is measured when it is added to the culture medium (*43*). Our results contrast with those of Clark and Konstantopoulos (*42*), who showed that addition of GSH up to 50 mM did not affect insulin receptor activation in CHO/T cells. These authors measured receptor activation after cell stimulation with insulin in the presence or absence of GSH followed by receptor immunoprecipitation and autophosphorylation in vitro. Considering that the reoxidation of the receptor α-subunit thiols occurs very rapidly if they are not blocked (see above), we believe that their findings could be explained by the fact that no sulfhydryl blocking reagent was added prior to the recovery and analysis of the insulin receptor autophosphorylation. From our data, it is evident that GSH did not alter insulin binding nor did it produce insulin-like activities in intact CHO/HIRc cells and fully differentiated 3T3-L1 adipocytes (unpublished results). The lack of ligand-independent responses by GSH contrasts with the direct cause−effect relationship that has been previously established between the insulin-mimicking actions of DTT and its interaction with insulin receptors both in isolated adipocytes (*13*, *14*) and in vitro (*6*, *44*). An activation of basal autophosphorylation by GSH was demonstrated only with the isolated insulin receptors (*42*), illustrating the existence of fundamental differences between the receptor in its native environment and purified insulin receptor preparations, which may be lacking crucial interaction with cellular proteins, including membrane-bound protein tyrosine phosphatases whose activities depend on thiol-sensitive moieties (*32*).

Another finding of this study was that GSH concentrations of 10 mM or greater were required in order to inhibit insulin action. It is noteworthy that the concentration of GSH in plasma is ∼10 μM and that of GSSG ∼1 μM (*45*, *46*). This indicates that the concentration of GSH required to induce detectable changes in insulin receptor structure and function in intact cells exceeds plasma concentrations by more than 3 orders of magnitude. Interestingly, the present work showed also that addition of GSH to culture medium (either alone or in the presence of cells) led to a rapid decrease in its concentration, possibly as a result of autoxidation (*24*). Our observation indicated that less than 10% of the initial 1 mM GSH remained after a 2 h incubation in vitro. This spontaneous loss of the reduced form of GSH cannot be prevented and, thus, has hampered our effort to assess the biological effect of physiological levels of GSH. Nevertheless, our data provide the first evidence that the insulin receptor α-subunit contains a select group of disulfides, other than the classical class I and class II disulfides, whose redox status can be rapidly and reversibly altered by reduced GSH or *N*-acetyl-L-cysteine in intact cells.

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