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Role of insulin receptor phosphorylation in the insulinomimetic effects of hydrogen peroxide

(insulin action/hexose transport/tyrosine kinase)

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ABSTRACT **The oxidant H_2O_2 has many insulin-like effects in rat adipocytes. To determine whether these effects could be mediated by the tyrosine kinase activity of the insulin receptor, the ability of H_2O_2 to stimulate receptor phosphorylation in intact adipocytes and partially purified insulin receptors has been examined. Phosphorylation of the β subunit of the insulin receptor was increased ≈2-fold by treatment of intact cells with 3 mM H_2O_2, a concentration that maximally stimulates 2-deoxyglucose uptake. Stimulation of receptor phosphorylation was rapid, reaching maximal levels within 5 min, and preceded activation of glucose transport. Phosphoamino acid analysis of insulin receptors from H_2O_2-treated adipocytes showed that ^{32}P incorporation into phosphotyrosine and phosphoserine residues of the β subunit was enhanced. Furthermore, partially purified receptors from H_2O_2-treated cells exhibit increased tyrosine kinase activity, as measured by phosphorylation of the peptide $Glu_{80}Tyr_{20}$. In contrast, the direct addition of H_2O_2 to partially purified insulin receptors did not stimulate tyrosine kinase activity or insulin receptor autophosphorylation. This was not due to breakdown of H_2O_2 or oxidation of ATP or the required divalent cations. To define the factors involved in H_2O_2's effect, we have examined receptor phosphorylation in fat cell homogenates and purified plasma membranes. Although insulin stimulated receptor phosphorylation in both of these systems, H_2O_2 was only effective in the cell homogenates. These data demonstrate that, under certain conditions, H_2O_2 stimulates insulin receptor phosphorylation and tyrosine kinase activity, suggesting that the insulin-like effects of H_2O_2 may be mediated by stimulation of insulin receptor phosphorylation. This does not appear to be a direct effect of H_2O_2 on the insulin receptor and requires nonplasma membrane cellular constituents.**

In intact cells (1) and cell-free systems (2) the insulin receptor is rapidly phosphorylated subsequent to insulin binding. In cell-free systems phosphorylation occurs exclusively on tyrosine residues (2), whereas in intact cells serine is also phosphorylated (1). The β subunit of the receptor contains an ATP binding site (3) and evidence indicates that the insulin receptor itself is a tyrosine-specific protein kinase that undergoes autophosphorylation in an insulin-dependent manner (4). Receptor autophosphorylation enhances the tyrosine kinase activity toward exogenous substrates (5). The intrinsic nature of this kinase activity, as well as the insulin concentration dependence (6) and time course of autophosphorylation (7), has led to the proposal that receptor phosphorylation is an early step in coupling hormone binding to insulin action.

The oxidant H_2O_2 has many insulin-like effects in rat adipocytes, including stimulation of glucose transport (8, 9), stimulation of glucose utilization (10), and inhibition of hormone-stimulated lipolysis (11). However, H_2O_2 has no effect on insulin binding (12). This has led to its use as a tool to evaluate postreceptor alterations in insulin action (13). Since tyrosine kinase activity is intrinsic to the insulin receptor, we have approached the question of the role of this activity in insulin action by using this oxidant, which apparently does not interact with the insulin-binding subunit of the insulin receptor. In this report we have asked whether the insulin-like effects of H_2O_2 are mediated by phosphorylation of the insulin receptor. To this end, the action of H_2O_2 on insulin receptor phosphorylation and tyrosine kinase activity in intact adipocytes, cell homogenates, plasma membranes, and partially purified receptors has been examined.

MATERIALS AND METHODS

Preparation of Adipocytes. Adipocytes from epididymal fat pads of male Sprague–Dawley rats (180–220 g, Charles River Breeding Laboratories) were prepared by collagenase digestion (14) in Krebs/Ringer phosphate buffer (pH 7.4) containing 3% (wt/vol) bovine serum albumin (Sigma) and 1 mg of collagenase per ml (Cooper Biomedical, Malverne, PA). Cells were washed and suspended in phosphate-free Krebs/Ringer bicarbonate buffer supplemented with 25 mM Hepes (pH 7.4) containing 1% bovine serum albumin.

^{125}I-Labeled Insulin (^{125}I-Insulin)-Binding Studies. ^{125}I-insulin (1 Ci/μmol; 1 Ci = 37 GBq) was prepared by the chloramine-T method and purified by chromatography on Sephadex G-50 (15). Partially purified receptors were incubated with ^{125}I-insulin (0.1 nM) at 4°C overnight (16). Binding was terminated by addition of bovine gamma globulin and receptor-bound insulin was separated from free hormone by precipitation with 10% polyethylene glycol (16). Nonspecific binding of ^{125}I-insulin was defined as the amount of radioactivity not displaced by 0.83 μM native insulin.

2-Deoxyglucose Transport Assay. 2-Deoxyglucose transport was measured using 0.1 mM D-2-deoxy[1-^{3}H]glucose (10.4 mCi/mmol) and the indicated concentrations of insulin or H_2O_2 as described (13), except that incubation was for 90 sec and cytochalasin B (50 μM) was used to correct for extracellular trapping.

***In Situ* Phosphorylation.** Isolated adipocytes (1 ml; ≈3 × 10^6 cells) were incubated for 2 hr at 37°C with 0.5 mCi of $^{32}PO_4$. Cells were then incubated at 37°C in the absence or presence of insulin (0.1 μM; 5 min) or H_2O_2 (3 mM; 10 min). The buffer was removed and the cells were solubilized in 50 mM Tris·HCl (pH 7.4) containing 1% Triton X-100, phosphatase, and protease inhibitors (17). Insulin receptors were partially purified on wheat germ agglutinin (WGA)-Sepharose in the presence of phosphatase inhibitors (17), immunoprecipitated, and analyzed by NaDodSO$_4$/PAGE as described below.

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Abbreviation: WGA, wheat germ agglutinin.

Receptor Autophosphorylation. Insulin receptors, partially purified by chromotography on WGA-Sepharose (17), were incubated in the absence or presence of insulin (100 nM) or H_2O_2 (0.03–3 mM) for 15 min at 24°C. The phosphorylation assay was initiated by addition of [γ-^{32}P]ATP (final concentration, 50 μM; 2.5 μCi/nmol), $MgCl_2$ (10 mM), and $MnCl_2$ (2 mM). After 10 min at 4°C the reaction was terminated (17) and insulin receptors were immunoprecipitated and analyzed by $NaDodSO_4$/PAGE as described below. Phosphorylation of a polymer of glutamic acid and tyrosine ($Glu_{80}Tyr_{20}$, Sigma) was performed using partially purified insulin receptor by the procedure of Braun *et al.* (18) as described (17). In experiments determining activation of tyrosine kinase activity in intact cells, insulin receptors were isolated in the absence of ATP but in the presence of the phosphatase inhibitors described above for *in situ* phosphorylation.

Phosphorylation in Adipocyte Homogenates and Isolated Plasma Membranes. Isolated plasma membranes were prepared by the method of McKeel and Jarret (19) as modified by Cushman and Wardzala (20) except that the homogenization buffer contained 100 mM NaF, 0.1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. The final pellet was suspended in 50 mM Tris·HCl (pH 7.4) containing 0.1 mM sodium vanadate. To prepare cell homogenates, adipocytes were suspended in 50 mM Tris·HCl (pH 7.4) containing 100 mM NaF, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 mg of bacitracin per ml and homogenized in a glass homogenizer using a Teflon pestle (10 strokes, 2000 rpm). The homogenate was centrifuged at 100,000 × *g* for 60 min at 4°C. The fat cake was discarded and the pellet was suspended in the infranatant. Plasma membranes or homogenates were incubated for 5 min at 20°C with 10 mM $MgCl_2$ and 2 mM $MnCl_2$ in the absence or presence of insulin (1 μM) or H_2O_2 (0.3–3 mM). Phosphorylation was initiated by addition of [^{32}P]ATP (100 μM; 2–8 μCi/nmol). After 10 min at 20°C reactions were terminated by solubilization in 50 mM Tris·HCl (pH 7.4) containing 1% Triton X-100 and phosphatase inhibitors and insulin receptors were partially purified on WGA-Sepharose. Insulin receptor phosphorylation was analyzed by immunoprecipitation and gel electrophoresis.

Immunoprecipitation and Gel Electrophoresis. Phosphorylated insulin receptors were immunoprecipitated by incubation with a 1:25 dilution of antibody to rat liver insulin receptor for 16 hr at 4°C; this was followed by precipitation with Pansorbin (Calbiochem). Immunoprecipitates were solubilized at 100°C in 80 mM Tris·HCl (pH 6.8) containing 3.8% $NaDodSO_4$, 5% 2-mercaptoethanol, 7.5% glycerol, and 0.0025% bromophenol blue. $NaDodSO_4$ gel electrophoresis was carried out using the discontinuous buffer system described by Laemmli (21) with a 4.5% acrylamide stacking gel and a 7.5% acrylamide resolving gel. Molecular weight standards, obtained from Bio-Rad, were myosin (M_r 200,000), β-galactosidase (M_r 116,250), phosphorylase b (M_r 92,500), bovine serum albumin (M_r 66,200), and ovalbumin (M_r 45,000). Radioactive bands were detected by autoradiography of fixed and stained gels using intensifying screens. Densitometric scanning of autoradiograms was performed on an LKB model 2202 Ultrascan laser densitometer (LKB).

RESULTS

Addition of 3 mM H_2O_2, a maximally effective concentration that stimulates glucose transport ≈50% as effectively as insulin (9), to intact adipocytes equilibrated with $^{32}PO_4$ resulted in a 2-fold increase in phosphorylation of the β subunit of the insulin receptor (Fig. 1*A*, lane 2). Similar to H_2O_2's effect on glucose transport, this increase was ≈50% of the stimulation seen when cells are treated with insulin at maximally effective concentrations (lane 3). Although only phosphotyrosine residues are found when partially purified insulin receptors undergo insulin-stimulated autophosphorylation, insulin-stimulated receptor phosphorylation in intact cells occurs on tyrosine, serine, and, to a lesser extent, threonine residues (1). Therefore, phosphoamino acid analysis of ^{32}P-labeled insulin receptors from H_2O_2-treated adipocytes was performed (Fig. 1*B*). As has been shown in other cell types (1), insulin receptors from nonstimulated adipocytes were phosphorylated only on serine residues (data not shown). Similar to insulin, stimulation of fat cells with 3 mM H_2O_2 resulted in the phosphorylation of tyrosine residues as well as increased serine phosphorylation.

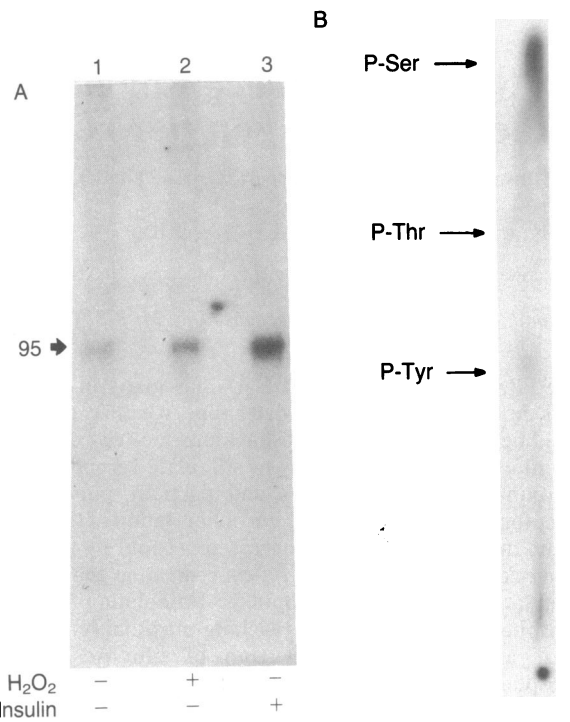

FIG. 1. Effects of H_2O_2 on *in situ* receptor phosphorylation. (*A*) Rat adipocytes were equilibrated with $^{32}PO_4$ for 2 hr at 37°C. Insulin (100 nM) or H_2O_2 (3 mM) was then added and incubation was continued for 5 or 10 min, respectively. The buffer was removed and cells were solubilized in the presence of 5 mM ATP and phosphatase inhibitors. Insulin receptors were partially purified on WGA-Sepharose, immunoprecipitated, and analyzed by $NaDodSO_4$/PAGE and autoradiography. The arrow indicates the receptor β subunit (shown as $M_r \times 10^{-3}$). (*B*) Phosphoamino acid analysis of H_2O_2-stimulated receptor phosphorylation. ^{32}P-labeled β subunit from H_2O_2-treated cells was eluted from the gel and hydrolyzed in 6 M HCl for 1 hr at 110°C. Acid was removed by repeated drying under vacuum. Unlabeled phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) were added and phosphoamino acids were separated by high-voltage thin-layer electrophoresis at pH 3.5 (6).

Since H_2O_2 stimulated tyrosine phosphorylation of the insulin receptor in intact adipocytes, the relationship between insulin receptor phosphorylation and stimulation of glucose transport by this agent was examined further. We first examined the time dependence of H_2O_2's effects (Fig. 2*A*). In agreement with previous studies (9), there was a short lag (1–3 min) before H_2O_2 stimulated uptake of 2-deoxyglucose. Hexose transport then reached maximal levels at 30 min of treatment. In contrast, stimulation of receptor phosphorylation by H_2O_2 was more rapid (Fig. 2 *A* and *B*). Insulin receptor phosphorylation was enhanced within 1 min of exposure to H_2O_2 and reached maximal levels within 5 min. Stimulation of receptor phosphorylation by insulin was even more rapid, being essentially complete within 1 min (Fig. 2*B*).

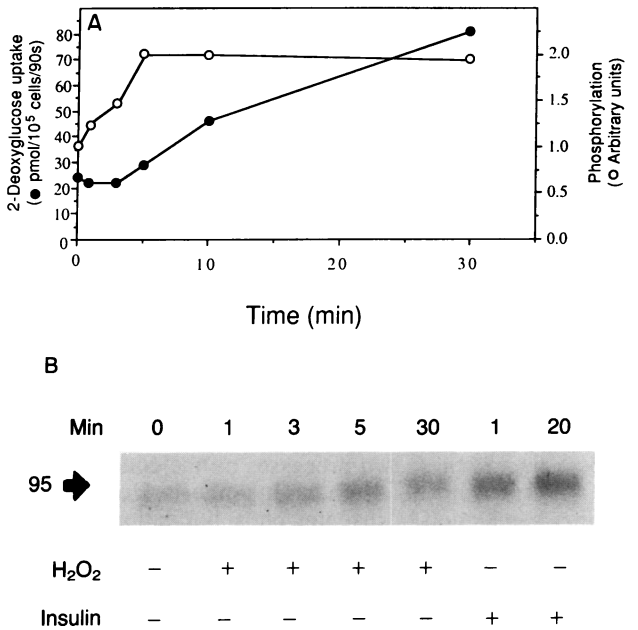

FIG. 2. Comparison of the time dependence of H_2O_2's effects on insulin receptor phosphorylation and hexose transport. (*A*) Adipocytes, equilibrated with $^{32}PO_4$, were incubated for the indicated times with H_2O_2 (3 mM) or insulin (100 nM). Cells were then immediately solubilized with Triton X-100 in the presence of 5 mM ATP and phosphatase inhibitors. Insulin receptors were partially purified, immunoprecipitated, and analyzed by $NaDodSO_4$/PAGE. Phosphorylation was quantitated by densitometric scanning of autoradiograms. In parallel incubations, hexose transport activity was measured by uptake of 2-deoxy[^{3}H]glucose (0.1 mM) for 90 sec at 37°C after preincubation for the indicated times with 3 mM H_2O_2. (*B*) Portion of the autoradiogram showing β-subunit phosphorylation.

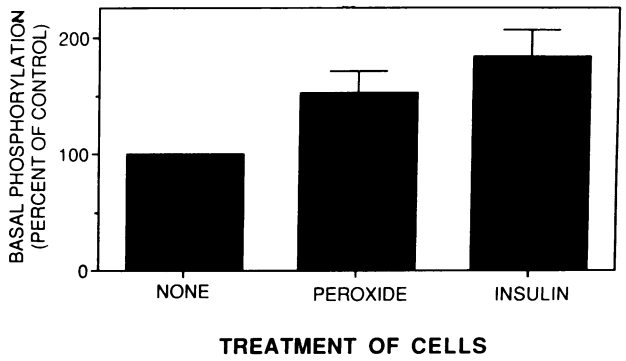

FIG. 4. *In situ* activation of insulin receptor tyrosine kinase by H_2O_2. Adipocytes were incubated for 30 min at 37°C with no additions (none), 3 mM H_2O_2 (peroxide), or 7 nM insulin (data for H_2O_2 and insulin are expressed as mean ± SEM). Cells were then acid washed (pH 6.3, 10°C) and solubilized in the presence of phosphatase inhibitors. Insulin receptors were partially purified and kinase activity was determined by phosphorylation of $Glu_{80}Tyr_{20}$ in the absence of insulin.

As is shown in Fig. 3, the concentration dependence of H_2O_2-stimulated receptor phosphorylation and 2-deoxyglucose transport were identical. Maximal stimulation of both activities occurred between 0.3 and 3 mM H_2O_2. Neither transport nor receptor phosphorylation was enhanced by treatment of adipocytes with 0.03 mM H_2O_2.

To assess whether H_2O_2-stimulated receptor phosphorylation activated tyrosine kinase activity, insulin receptors were isolated from adipocytes incubated in the absence or presence of H_2O_2 (3 mM) or insulin (7 nM). Tyrosine kinase activity was determined by phosphorylation of the synthetic peptide $Glu_{80}Tyr_{20}$ in the absence of insulin. As is shown in Fig. 4, tyrosine kinase activity in preparations from H_2O_2-treated cells was 50% greater than from untreated adipocytes, whereas insulin treatment of cells increased this activity 80%. This enhanced kinase activity could be attributed to the kinase activity of the insulin receptor since incubation of partially purified receptors with anti-insulin receptor antibodies reduced substrate phosphorylation to the same level in preparations from untreated, H_2O_2-treated, and insulin-treated cells (data not shown).

The effect of H_2O_2 on exogenous substrate phosphorylation and autophosphorylation of partially purified insulin receptor was determined. Although insulin stimulated the receptor kinase activity 5-fold in these preparations, concentrations of H_2O_2 (0.03–3 mM) that stimulated 2-deoxyglucose uptake and receptor phosphorylation *in situ* (Fig. 3) had no effect on tyrosine kinase activity when added directly to partially purified insulin receptors (data not shown). Similarly, insulin stimulated autophosphorylation of the β subunit of the insulin receptor (Fig. 5, lane 2), whereas 3 mM H_2O_2 had no effect (lane 3). Importantly, H_2O_2 did not inhibit insulin-stimulated receptor phosphorylation (lane 4), indicating that the lack of stimulation with H_2O_2 was not due to oxidative destruction of ATP or required cofactors. Although breakdown of H_2O_2 occurred in these preparations this could not account for the lack of an effect since, at 3 mM H_2O_2, degradation did not lower the concentrations below that effective in stimulating insulin action and receptor phosphorylation *in situ*. Furthermore, the use of peroxide-free Triton X-100 or the inclusion of dithiothreitol, as suggested by the work of Chan *et al.* (22), did not alter these results.

To examine the cellular location of the factors involved in H_2O_2 stimulation of receptor phosphorylation, our initial experiments have employed total cellular homogenates and isolated plasma membranes. Similar to the intact cell, insulin

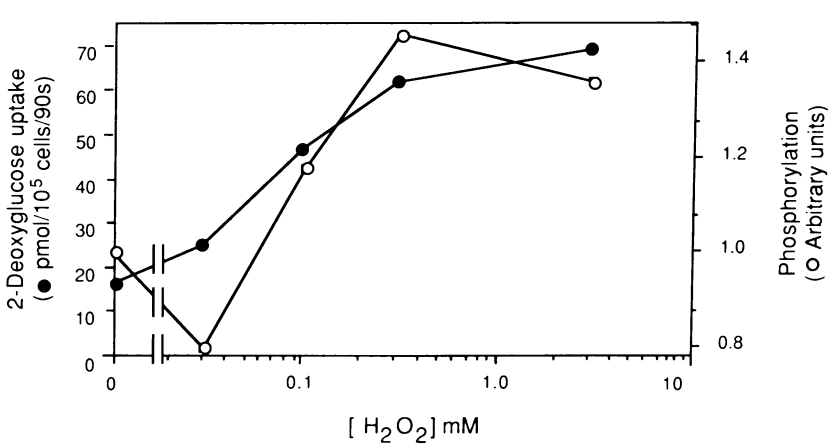

FIG. 3. Concentration dependence of H_2O_2's effect on receptor phosphorylation and hexose transport. Adipocytes, equilibrated with $^{32}PO_4$, were incubated for 30 min at 37°C with the indicated concentrations of H_2O_2. In parallel, hexose transport activity was determined by measurement of 2-deoxyglucose uptake. Receptor phosphorylation was quantitated by densitometric scanning of autoradiograms.

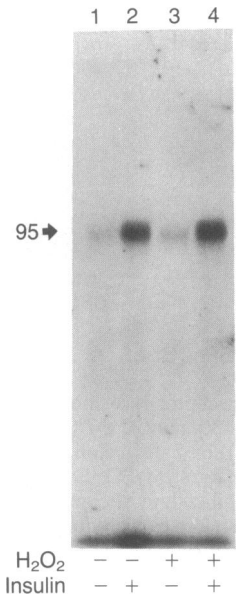

FIG. 5. Effect of H_2O_2 on *in vitro* insulin receptor autophosphorylation. Partially purified insulin receptors were incubated for 20 min at 24°C with no additions, 100 nM insulin, 3 mM H_2O_2, or insulin and H_2O_2. Phosphorylation was initiated by the addition of [^{32}P]ATP. After 20 min at 24°C, the reaction was terminated by addition of unlabeled ATP and phosphatase inhibitors. Insulin receptors were immunoprecipitated and analyzed by $NaDodSO_4$ gel electrophoresis and autoradiography. The arrow indicates the β subunit of the insulin receptor (shown as $M_r \times 10^{-3}$).

and H_2O_2 stimulate receptor phosphorylation in adipocyte homogenates (Fig. 6*A*). In contrast, although insulin increased receptor phosphorylation in purified plasma membranes, H_2O_2 had no effect (Fig. 6*B*).

DISCUSSION

Our data demonstrate that addition of H_2O_2 to intact adipocytes stimulates phosphorylation of the insulin receptor on tyrosine and serine residues. Additionally, partially purified insulin receptor preparations from H_2O_2- or insulin-treated cells exhibit increased tyrosine kinase activity. This increase can be attributed to activation of the intrinsic kinase activity of the insulin receptor since immunoprecipitation with anti-receptor antibodies returned the kinase activity to the level seen with preparations from untreated cells.

Several lines of evidence indicate that insulin receptor phosphorylation and activation of its intrinsic tyrosine kinase activity is an early step linking hormone binding to action—e.g., glucose transport. The time course of receptor phosphorylation and dephosphorylation in intact cells is rapid and precedes insulin action (7). Microinjection of monoclonal antibodies that block receptor kinase activity inhibit insulin action (23). Replacement of the tyrosines that become phosphorylated (24) or a lysine residue in the ATP-binding region (25, 26) by site-directed mutagenesis impairs insulin action. Also several insulinomimetic agents stimulate receptor phosphorylation (27–29).

Our data suggest that the insulin-like effects of H_2O_2 on glucose transport may be mediated by stimulation of receptor phosphorylation. As is the case with insulin, stimulation of receptor phosphorylation by H_2O_2 occurs more rapidly than activation of the glucose transport system. The parallel dependence of these two processes on the concentration of H_2O_2 is also compatible with this hypothesis. However, since neither stimulation of receptor phosphorylation nor activation of glucose transport reaches the levels seen with maximal insulin concentrations, receptor phosphorylation may not be the rate-limiting step for H_2O_2's insulin-like effects. In this regard, Lane and co-workers (30) have suggested that, in 3T3-L1 adipocytes, tyrosine phosphorylation of a small (15 kDa) protein may be the rate-limiting step in insulin activation of hexose uptake. To date, neither the presence of this protein nor the effects of insulin or H_2O_2 on its phosphorylation have been established in other cell types.

H_2O_2 may not require the entire insulin receptor to exert insulin-like effects. Several insulinomimetic agents that act by extracellular generation of H_2O_2 retain their action in adipocytes rendered insulin insensitive by trypsin treatment, which destroys insulin binding (12, 31). Furthermore, H_2O_2 has easily demonstrable insulin-like effects in Madin–Darby canine kidney cells, which have no insulin-binding activity (32), and in fibroblasts from an insulin-resistant patient with leprechaunism, which have markedly reduced insulin-binding capacity (33). In all of the above examples, the status of the insulin receptor was established by insulin binding and

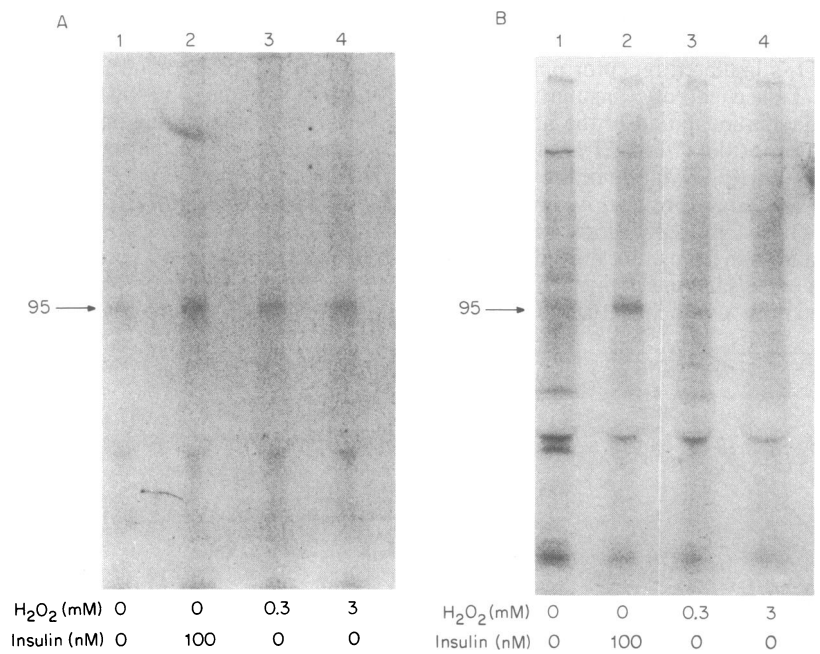

FIG. 6. Insulin receptor phosphorylation in cell homogenates and plasma membranes. Adipocyte homogenates (*A*) or purified plasma membranes (*B*) were incubated with no addition (lane 1), 1 μM insulin (lane 2), 0.3 mM H_2O_2 (lane 3), or 3 mM H_2O_2 (lane 4). Phosphorylation was initiated by addition of [^{32}P]ATP. Insulin receptors, isolated by WGA-Sepharose chromatography and immunoprecipitation, were analyzed by gel electrophoresis. The arrows indicate the β subunit of the insulin receptor (shown as $M_r \times 10^{-3}$).

thus may only reflect a measurement of the binding portion of the α subunits of the receptor. However, they do not exclude the possibility that the β subunit, or some fragment of it, may be present and mediate H_2O_2's effects. Since H_2O_2 has no effect on insulin binding (12), it is probable that an α subunit capable of binding insulin may not be required.

Although the effects of insulin and H_2O_2 were qualitatively similar in intact cells, this was not always the case in broken cell systems. H_2O_2 had no effect on either autophosphorylation or tyrosine kinase activity when added directly to partially purified insulin receptors. These findings are likely supported by the studies of Haring *et al.* (34), who found that an insulinomimetic agent, spermine, also had no effect on partially purified insulin receptors. The insulin-like properties of this polyamine are dependent on the generation of H_2O_2 by an amine oxidase present in most preparations of bovine serum albumin (35). It is unclear whether sufficient bovine serum albumin was present to generate H_2O_2 in the experiments of Haring *et al.* (34).

The observation that H_2O_2 has no effect on partially purified insulin receptors suggests that nonreceptor factors or kinases that do not copurify with the receptor may be involved in H_2O_2 stimulation of phosphorylation in intact cells. In this regard, our initial experiments to define these factors showed that, although insulin stimulates receptor phosphorylation in purified plasma membranes, H_2O_2 had no effect. However, insulin and H_2O_2 stimulate receptor phosphorylation in crude adipocyte homogenates. This may indicate that nonplasma membrane constituents are involved in H_2O_2's effect. At this time it is not known whether these factors are components of the cytosol or reside in an intracellular membrane.

Recently, Chan *et al.* (22) have reported that several oxidants stimulate phosphorylation of $Glu_{80}Tyr_{20}$ and endogenous proteins in purified rat liver plasma membranes. Additionally, the oxidant vitamin K_5 modestly stimulated phosphorylation of a protein of M_r 95,000, believed to be the β subunit of the insulin receptor, in detergent extracts of these membranes purified by chromatography on WGA. In both cases, oxidant-stimulated phosphorylation required dithiothreitol. In our study, using immunoprecipitation in addition to WGA-Sepharose chromatography to assess receptor phosphorylation, addition of dithiothreitol, in the concentration used by Chan *et al.* (22), to either partially purified receptors or isolated plasma membranes did not result in H_2O_2 stimulation of receptor phosphorylation. At this time the reason for this difference is not known.

In conclusion, H_2O_2 stimulates insulin receptor phosphorylation in intact adipocytes and crude cell homogenates but not in plasma membranes and partially purified receptors. Taken together these findings are consistent with the hypothesis that the insulin receptor could serve as a substrate for other tyrosine kinase(s) stimulated by oxidants. Further studies defining the factors involved in H_2O_2's effects by reconstitution of various cytosolic or membrane fractions from cell homogenates with either purified plasma membranes or partially purified receptors should now be possible. Identification of these factors may provide insight into the postreceptor events involved in insulin action.

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