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Hydrogen peroxide stimulates tyrosine phosphorylation of the insulin receptor and its tyrosine kinase activity in intact cells

Osamu KOSHIO*, Yasuo AKANUMA* and Masato KASUGA*†‡

*The Institute for Diabetes Care and Research, Asahi Life Foundation, 1-6-1 Marunouchi, Chiyoda-ku, Tokyo, Japan 100, and †The 3rd Department of Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan 113

H-35 rat hepatoma cells were labelled with [^{32}P]orthophosphate and their insulin receptors isolated on wheat germ agglutinin (WGA)–agarose and anti-(insulin receptor) serum. The incubation of these cells with 10 mM-H_2O_2 for 10 min increased the phosphorylation of both the serine and tyrosine residues of the β subunit of the insulin receptor. Next, insulin receptors were purified on WGA–agarose from control and H_2O_2-treated H-35 cells and the purified fractions incubated with [γ-^{32}P]ATP and Mn^{2+}. Phosphorylation of the β subunit of insulin receptors obtained from H_2O_2-treated cells was 150% of that of control cells. The kinase activity of the WGA-purified receptor preparation obtained from H_2O_2-treated cells, as measured by phosphorylation of *src*-related synthetic peptide, was increased about 4-fold over control cells. These data suggest that in intact cell systems, H_2O_2 may increase the insulin receptor kinase activity by inducing phosphorylation of the β subunit of insulin receptor.

INTRODUCTION

Structurally, insulin receptors consist of α subunits of M_r 135000 and β subunits of M_r 95000 linked together through disulphide bonding to form tetramers ($\alpha_2\beta_2$) (Massague *et al.*, 1980; Van Obberghen *et al.*, 1981). The insulin receptor is an insulin-dependent tyrosine kinase which displays autophosphorylation activity of the β subunit (Kasuga *et al.*, 1982*a*,*b*; Van Obberghen & Kowalski, 1982; Roth & Cassell, 1983). Recently, sequencing of cloned cDNA from mRNA of the human insulin receptor has revealed that the β subunit contains a single transmembrane domain and kinase domain that has similar sequences to other tyrosine kinases (Ullrich *et al.*, 1985; Ebina *et al.*, 1985).

Insulin treatment of intact cells results in autophosphorylation of the β subunit of the insulin receptor on tyrosine residues and the rapid activation of its kinase activity (Klein *et al.*, 1986; Yu & Czech, 1986). In pathological conditions such as diabetes and obesity, the kinase activity of the insulin receptor is altered and it suggested that this may lead to the insulin-resistance state accompanying these pathologies (Kadowaki *et al.*, 1984; Le Marchand-Brustel *et al.*, 1985). In addition, in insulin receptors whose tyrosine residues 1162 and 1163, which are thought to be autophosphorylated, have been replaced with phenylalanine by site-directed mutagenesis, both the insulin-dependent autophosphorylation and the insulin-stimulated uptake of 2-deoxyglucose were decreased (Ellis *et al.*, 1986). Together, these data suggest that the activity of the insulin receptor kinase may participate in the transduction of signals from insulin molecules.

There are several agents which have insulin-like effects in many types of cells and which are known as insulin mimickers. One example of these agents is H_2O_2. Although this is a simple molecule it is able to stimulate 3-*O*-methylglucose uptake (Czech *et al.*, 1974), lipogenesis (May & de Haën, 1979), and to activate pyruvate dehydrogenase (May & de Haën, 1979). Using the rat hepatoma cell line H-35, in this study we have examined the possibility that H_2O_2 elicitates its insulin-like effects by stimulating tyrosine phosphorylation of the insulin receptor and activating its receptor kinase.

MATERIALS AND METHODS

Materials

Wheat germ agglutinin (WGA)–agarose was purchased from E.Y. Laboratory (San Mateo, CA, U.S.A.). Protein A bacterial adsorbent was obtained from Miles Scientific (Naperville, IL, U.S.A.). The synthetic peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) related to the tyrosine phosphorylation site between amino acid residues 414 and 424 of the *src*-gene product (*src*-related peptide) was from Peninsula Laboratory (Belmont, CA, U.S.A.). Bovine serum albumin (fatty acid-free radioimmunoassay grade) was from Oriental Yeast Co. (Tokyo, Japan). [γ-^{32}P]ATP (7000 Ci/mmol) and [^{32}P]orthophosphate (carrier-free in water) were from New England Nuclear. H_2O_2 (35%) was from Wako Pure Chemical Ind. Co. (Osaka, Japan). All other reagents were of the best commercial grade.

Labelling of insulin receptors with [^{32}P]orthophosphate

Rat hepatoma H-35 cells were grown to 50–70% confluence in 25 cm^2 dishes (Corning) containing 4 ml of Eagle's modified essential medium (Gibco) with 6% fetal bovine serum. The culture medium was changed to serum-free Eagle's medium for 2 days and then to Krebs–Hensleit–Hepes buffer containing 1% BSA and 2 mM-sodium pyruvate.

For labelling, [^{32}P]orthophosphate was added to the

Abbreviations used: WGA, wheat germ agglutinin; PMSF, phenylmethanesulphonyl fluoride; BSA, bovine serum albumin; PAGE, polyacrylamide-gel electrophoresis.

‡ To whom correspondence and reprint requests should be addressed, at the University of Tokyo address.

medium (without phosphate) (final concentration 0.4 mCi/ml). After about 4 h, the cells were treated with 10^{-7} M-insulin for 1 min or with 10 mM-H_2O_2 for 10 min at 37 °C. After incubation, the medium was aspirated quickly, and the cell monolayers frozen with liquid N_2. The cells were thawed, solubilized and scraped out on ice with solubilizing buffer (0.4 ml/dish) containing 50 mM-Hepes (pH 7.4), 1% (v/v) Triton X-100, 50 mM-NaF, 10 mM-sodium pyrophosphate, 1 mM-sodium orthovanadate, 1 mM-PMSF and 1000 units of aprotinin/ml. The solubilized cells were centrifuged at 13000 *g* for 60 min at 4 °C. The supernatant of the whole-cell extract was incubated with WGA–agarose for 20 min on ice. The WGA–agarose was washed extensively with buffer A containing 50 mM-Hepes (pH 7.4), 50 mM-NaF, 20 mM-*p*-nitrophenylphosphate, 10 mM-sodium pyrophosphate, 1 mM-sodium orthovanadate, 0.5 mM-PMSF and 1000 units of aprotinin/ml and then with buffer B containing 50 mM-Hepes (pH 7.4), 0.1% Triton X-100 and 0.5 mM-PMSF. It was then eluted with buffer B supplemented with 0.3 M-*N*-acetylglucosamine.

The eluate was incubated on ice for 90 min with autoantibodies to the insulin receptor that had been obtained from a patient with insulin resistance and acanthosis nigricans B-5 (kindly provided by Dr C. R. Kahn at Joslin Diabetes Center, Boston, MA, U.S.A.) at a dilution of 1:150 and then with Protein A bacterial adsorbent for 45 min. The receptor-bound Protein A was washed with 50 mM-Hepes buffer (pH 7.4) containing 1% Triton X-100 and 0.1% SDS. The bound proteins were solubilized in sampling buffer for SDS/PAGE (Laemmli, 1970) containing 100 mM-dithiothreitol and boiled for 5 min, the protein A was removed by centrifugation, and the supernatant was analysed by SDS/PAGE using a 7.5% polyacrylamide resolving gel followed by autoradiography as previously described (Kasuga *et al.*, 1982*a*).

Insulin receptor preparations for kinase assay

In order to prepare the insulin receptor fraction for the assay of kinase activity, the H-35 cells were grown in 65 cm^2 dishes (Nunc) in the Krebs–Hensleit–Hepes buffer described above. They were then treated with 10 mM-H_2O_2 or 10^{-7} M-insulin for 10 min, immediately washed twice with ice-cold Dulbecco's phosphate buffered saline and incubated with buffer A (0.3 ml/dish) without Triton X-100 on ice. H-35 cells were scraped from the dish and homogenized with a Teflon/glass homogenizer at 4 °C in buffer A without Triton X-100. The nuclear fraction was removed by centrifugation at 300 *g* for 3 min. The supernatant was solubilized by addition of Triton X-100 (final concentration 1%) and shaken for 10 min. Insoluble material was removed by centrifugation at 200000 *g* for 30 min. The supernatant was applied twice to a WGA–agarose column. The agarose gel (0.4 ml) was washed with 10 ml of buffer A and 15 ml of buffer B at 4 °C. The bound glycoproteins, including the insulin receptor, were eluted with buffer B supplemented with 0.3 M-*N*-acetylglucosamine. The protein concentration of the eluate was determined using the Bio-Rad protein assay solution with BSA as a standard.

Phosphorylation assays with insulin receptor fraction

Phosphorylation of the WGA-purified fraction was carried out at room temperature in a 60 μl reaction mixture containing 2–3 μg of WGA-purified protein, 50 mM-Hepes (pH 7.4), 0.1% Triton X-100, 8.3 mM-$MnCl_2$ and 0.25 M-*N*-acetylglucosamine. After a 45 min incubation with or without 10^{-7} M-insulin the reaction was initiated by adding 5 μCi of [γ-^{32}P]ATP (final concentration of ATP, 8 μM). The incubation was stopped after 6 min by addition of 5 μl of 0.1 M-ATP and 20 μl of 5-fold concentrated sample buffer for SDS/PAGE (Laemmli, 1970) containing 100 mM-dithiothreitol and then boiling for 5 min. Phosphorylated proteins were separated by SDS/PAGE and identified by autoradiography as previously described (Kasuga *et al.*, 1983). Bands containing the phosphoproteins were identified by autoradiography, cut out from the gel and counted for radioactivity in a liquid scintillation counter.

Phosphorylation of the *src*-related peptide was performed as follows (Kasuga *et al.*, 1983). Portions (30 μl) of reaction mixture containing 1–1.5 μg of protein of receptor fraction, 50 mM-Hepes (pH 7.4), 0.1% Triton X-100, 10 mM-$MgCl_2$, 3 mM-$MnCl_2$ and 0.25 M-*N*-acetylglucosamine were incubated with or without 10^{-7} M-insulin for 45 min at room temperature followed by the addition of *src*-related peptide (final concentration 1.1 mM) and 5 μCi of [γ-^{32}P]ATP (final concentration of ATP, 11 μM). The reaction was terminated by the addition of BSA solution (final concentration 0.21%) and trichloroacetic acid solution (final concentration 2.6%). After incubating at 0 °C for 30 min, the precipitated protein was sedimented by centrifugation for 5 min in a table top centrifuge (TOMY MC-15A). The supernatant was adsorbed onto two squares (1.5 cm × 1.5 cm) of phosphocellulose paper (Whatman) and the paper washed extensively in acetic acid and then acetone. The radioactivity was counted as previously described (Kasuga *et al.*, 1983).

Phosphoamino acid analysis

The radioactive band in the dried polyacrylamide gel was cut out and washed once with 25% propan-2-ol and once with 10% ethanol. The swelling gel was redried in a vacuum oven. The dried gel was then swelled in 500 μl of 50 mM-NH_4HCO_3 (pH 8.9) and digested overnight with 0.1 mg of 'TPCK'-trypsin at 37 °C. The supernatant was lyophilized. Acid hydrolysis was carried out with 0.3 ml of 6 M-HCl at 110 °C for 70 min in either vacuum-sealed Pyrex glass tubes or screw capped polypropylene minivials. HCl was evaporated through solid KOH. The hydrolysate was lyophilized twice, solubilized in 10 μl of thin layer electrophoresis solvent (pyridine/acetic acid/water, 1:10:189, by vol. pH 3.5), and spotted onto a cellulose-coated glass plate (Merck no. 5716) with standard phosphoamino acids. Thin layer electrophoresis was performed with flat-bed electrophoresis equipment (Pharmacia FBE-3000) at 2000 V for 30 min at 4 °C. The plate was dried and the phosphoamino acids were detected with ninhydrin. Radioactive spots were identified by autoradiography,

RESULTS

Phosphorylation of the insulin receptor in H-35 hepatoma cells in the presence of insulin or H_2O_2

To examine the effect of H_2O_2 on the phosphorylation of the insulin receptor, monolayers of H-35 rat hepatoma cells were labelled with [^{32}P]orthophosphate, stimulated with either H_2O_2 or insulin and then extracted with

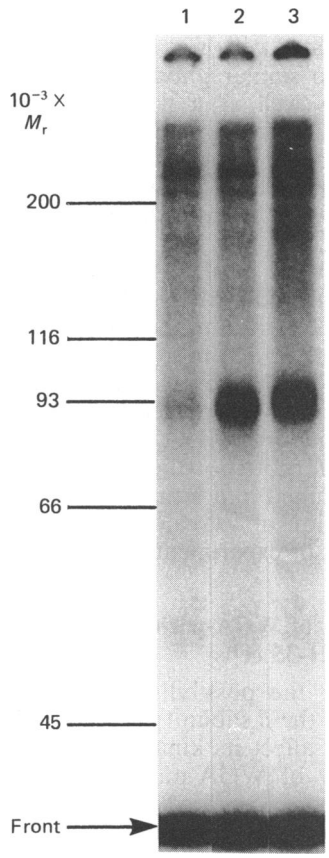

Fig. 1. Autoradiogram of insulin receptors from control, insulin-treated and H_2O_2-treated H-35 cells previously labelled with [^{32}P]orthophosphate

Rat hepatoma H-35 cells were labelled and equilibrated with [^{32}P]orthophosphate in Krebs–Hensleit–Hepes buffer, and stimulated with 10^{-7} M-insulin for 1 min or with 10 mM-H_2O_2 for 10 min at 37 °C. The receptors from control (lane 1), insulin-treated (lane 2) and H_2O_2-treated (lane 3) cells were purified using WGA–agarose and immunoprecipitation with anti-(insulin receptor) serum and Protein A. The precipitates were subjected to SDS/PAGE (7.5% acrylamide). The comigrated M_r markers are shown on the left side of the gel. The radioactivities of the M_r 93000 bands determined by Čerenkov counting were 70 c.p.m. (lane 1), 275 c.p.m. (lane 2) and 330 c.p.m. (lane 3), respectively. 'Front' means the front of protein migration.

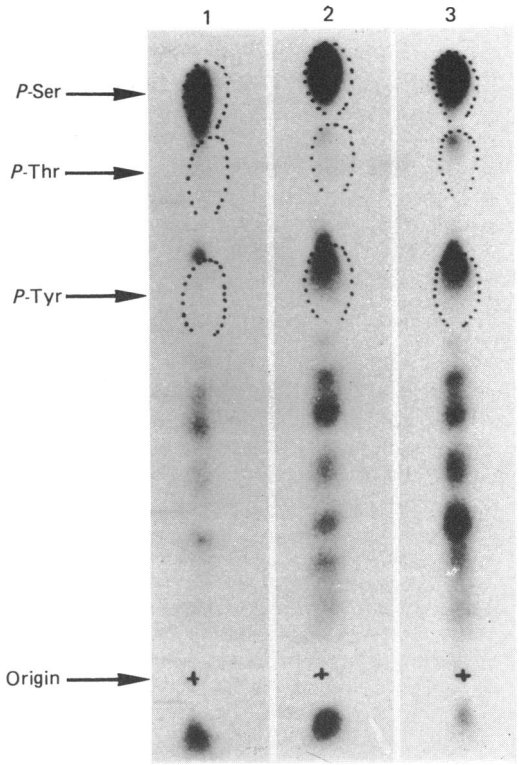

Fig. 2. Phosphoamino acids of the β-subunit of insulin receptors from control and insulin or H_2O_2-treated cells

The β-subunit of insulin receptor was purified from [^{32}P]-orthophosphate-labelled H-35 cell as described in the legend to Fig. 1. Trypsinization and hydrolysis of sample were carried out as decribed under 'Materials and methods'. The hydrolysed β-subunits from control (lane 1), insulin-treated (lane 2) or H_2O_2-treated (lane 3) cells were spotted with cold phosphoamino acids as standards and separated by thin layer electrophoresis. *P*-Ser, *P*-Thr and *P*-Tyr on the left side indicate respectively the positions of co-migrated phosphoserine, phosphothreonine and phosphotyrosine standards.

Hepes buffer containing 1% Triton X-100. Insulin receptors were then isolated by WGA affinity chromatography and immunoprecipitation using autoantibodies to the insulin receptor. On SDS/PAGE, one radioactive band of M_r 93000 was identified in the absence of stimulation (Fig. 1, lane 1). This radioactive band is presumably due to the phosphorylation of the β subunit of the insulin receptor as previously described (Kasuga *et al.*, 1982*a*). When hepatoma cells were incubated with 10^{-7} M-insulin at 37 °C for 10 min after preloading with [^{32}P]orthophosphate, the autoradiogram revealed an increase in the covalent ^{32}P labelling of the β subunit of insulin receptor (Fig. 1, lane 2). Incubation of the hepatoma cell with 10 mM-H_2O_2 for 10 min at 37 °C also stimulated the incorporation of ^{32}P into the β subunit of the insulin receptor (Fig. 1, lane 3). The extent of stimulation of β subunit phosphorylation by 10^{-7} M-insulin and 10 mM-H_2O_2 was roughly the same (about 3.5-fold stimulation) (Fig. 1, lanes 2 and 3).

Identification of phosphoamino acids

To determine the phosphoamino acids in the M_r 93000 ^{32}P-labelled phosphoprotein, this band was cut from the gel and partially hydrolysed in 6 M-HCl for 70 min at 110 °C. The hydrolysates were then separated by one-dimensional electrophoresis in the presence of unlabelled phosphoserine, phosphothreonine and phosphotyrosine and the autoradiogram of the separated phosphoamino acids was compared with ninhydrin-stained standards. Using one-dimensional electrophoresis, under basal conditions, the M_r 93000 phosphoprotein contained primarily phosphoserine (Fig. 2, lane 1). After stimulation of the intact hepatoma cells with 10^{-7} M-insulin, the phosphoprotein contained increased amounts of phosphoserine and phosphotyrosine (Fig. 2, lane 2). This finding is consistent with previous data (Kasuga *et al.*, 1983). After incubation of the intact hepatoma cells with 10 mM-H_2O_2, the phosphoprotein also contained increased amounts of phosphoserine and phosphotyrosine (Fig. 2, lane 3). The ratio of phospho-

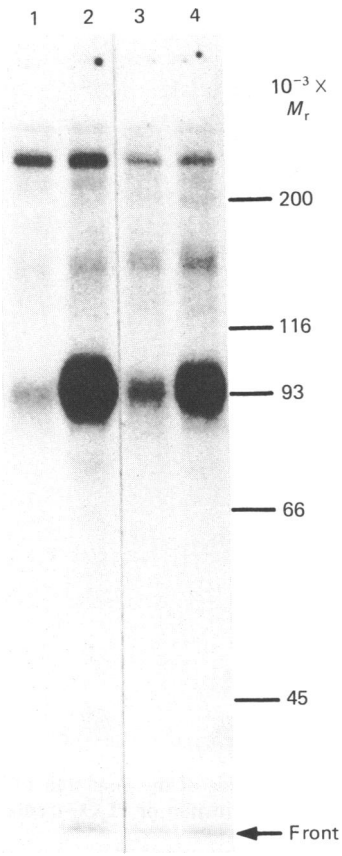

Fig. 3. Autoradiogram of the phosphorylation *in vitro* of WGA-purified fractions

The WGA–agarose-eluted fractions from control (lanes 1 and 2) and H_2O_2-treated (10 mM for 10 min) (lanes 3 and 4) H-35 cells were incubated with [γ-^{32}P]ATP and Mn^{2+} for 6 min at 23 °C and analysed by SDS/PAGE (7.5% acrylamide) after incubation without (lanes 1 and 3) or with (lanes 2 and 4) 10^{-7} M-insulin.

serine and phosphotyrosine generated by 10^{-7} M-insulin or 10 mM-H_2O_2 was roughly the same (Fig. 2, lanes 2 and 3). The ratio of phosphoserine and phosphotyrosine was different before and after stimulation with either insulin or H_2O_2. This latter may suggest that these treatments did not simply increase the specific activity of the ^{32}P-labelled ATP pool in the cell.

Phosphorylation of the WGA-purified fraction obtained from H_2O_2-treated cells

To examine whether the H_2O_2 treatment of intact cells could affect the phosphorylation of WGA-purified proteins in a cell-free system, H-35 rat hepatoma cells were treated with 10 mM-H_2O_2 for 10 min at 37 °C, solubilized by Triton X-100 and purified by WGA affinity chromatography. These WGA-purified fractions were then incubated with [γ-^{32}P]ATP and Mn^{2+} at 23 °C for 6 min, and analysed by SDS/PAGE and autoradiography. When the WGA-purified fractions from non-treated cells were incubated with [γ-^{32}P]ATP and Mn^{2+}, two phosphoprotein bands of M_r 240000 and 93000 were identified (Fig. 3, lane 1). The phosphoprotein band of M_r 93000 was the β-subunit of insulin receptor, since only this phosphoprotein was immunoprecipitated by autoantibodies to insulin receptor (results not shown). When the same amount of the WGA-purified fraction from H_2O_2-treated cells was incubated with [γ-^{32}P]ATP and Mn^{2+}, two similar phosphoproteins of M_r 240000 and 93000, as well as a new phosphoprotein of M_r 160000, were identified (Fig. 3, lane 3). The incorporation of ^{32}P into the phosphoprotein of M_r 93000 was increased in the fractions from H_2O_2-treated cells, while the incorporation of ^{32}P into the phosphoprotein of M_r 240000 was decreased.

When the WGA-purified fractions from non-treated cells were incubated with [γ-^{32}P]ATP and Mn^{2+} in the presence of 10^{-7} M-insulin, the phosphorylation of the β-subunit of insulin receptor was stimulated markedly (Fig. 3, lane 2). However, the insulin effect on the phosphorylation of this protein was attenuated in WGA fractions purified from H_2O_2-treated cells. In each case, the M_r 93000 protein bands were excised, their ^{32}P content determined in a liquid-scintillation counter and the incorporation of ^{32}P calculated. The results of four individual sets of experiments are summarized in Table 1.

Kinase activity of WGA-purified insulin receptor from H_2O_2-treated H-35 cells

To examine the possibility that the tyrosine phosphorylation of the β subunit induced by H_2O_2-treated of cells may also affect its kinase activity, we assayed the kinase activity of WGA-purified insulin receptors towards a synthetic *src*-related peptide. Since phosphorylation of the *src*-related peptide showed no saturating profile for at least 4 min at 23 °C (results not shown), the WGA-purified fractions were incubated with [γ-^{32}P]ATP, Mn^{2+}, Mg^{2+} and 1.1 mM-*src*-related peptide for 4 min at 23 °C in the absence or presence of 10^{-7} M-insulin (Table 1). During the cell-free assay in the absence of insulin, fractions obtained from H_2O_2-treated cells had a greater ability to phosphorylate the *src*-related peptide than did fractions obtained from control cells. The phosphorylation of *src*-related peptide by these fractions was stimulated by the presence of insulin. However, in fractions prepared from H_2O_2-treated cells the insulin stimulation of phosphorylation of this peptide was decreased (Table 1).

To compare the ability of insulin (10^{-7} M) and H_2O_2 (10 mM) to stimulate insulin receptor kinase activity in intact cells, H-35 rat hepatoma cells were treated with 10^{-7} M-insulin for 10 min at 37 °C, solubilized by Triton X-100 and purified by WGA affinity chromatography. These WGA-purified insulin receptors were assayed for the autophorylation and for the kinase activity towards a synthetic *src*-related peptide as described above. During the cell-free assay in the absence of insulin, fractions obtained from insulin-treated cells had a greater ability to autophosphorylate the β subunit of insulin receptor and to phosphorylate the *src*-related peptide than did fractions obtained from control cells (Table 1). Furthermore, WGA-purified fractions from insulin-treated cells always showed more ^{32}P incorporation into the β subunit of insulin receptor and *src*-related peptide than did fractions obtained from H_2O_2-treated cells (Table 1).

Time- and concentration-dependency of H_2O_2 treatment of H-35 cells on the activity of insulin receptor kinase

To characterize further the effect of H_2O_2 on the kinase activity of insulin receptors in intact cells, the

Table 1. Protein kinase activity of the WGA-purified insulin receptor from H-35 hepatoma cells treated with H_2O_2 or insulin

H-35 cells were treated with either no additions (control), H_2O_2 (10 mM) or insulin (10^{-7} M) for 10 min. Receptors were purified by Triton X-100 extraction of whole cells and WGA affinity chromatography. The protein kinase reactions were performed as described under 'Materials and methods'. Insulin (+) indicates that the kinase reactions were performed in the presence of 10^{-7} M-insulin during the cell-free assay. The results represent the average of four individual experiments; S.D. values are shown in parentheses. Significance of the difference was examined by Student's *t*-test for paired samples; *P* values < 0.05 were taken as significant. *Indicates significant difference between control and H_2O_2-treated cells; **indicates significant difference between control and insulin-treated cells.

	Kinase activity (pmol of ^{32}P incorporated/min per mg of protein)			
Substrate ...	β subunit of insulin receptor		*src*-related peptide	
Cell / Insulin (10^{-7} M) ...	−	+	−	+
Control	1.3 (0.2)	15.1 (2.9)	14 (5)	295 (84)
10 mM-H_2O_2-treated	1.9* (0.3)	6.7* (1.9)	51* (13)	220* (50)
10^{-7} M-insulin-treated	2.9** (0.6)	11.0** (2.7)	67** (15)	250** (46)

time- and concentration-dependency of H_2O_2 treatment were examined. First, H-35 cells were treated with 10 mM-H_2O_2 for different periods, WGA-purified fractions were prepared and assayed for their insulin receptor kinase activity towards *src*-related peptide. The maximal stimulation of kinase activity was observed after a 10 min incubation and incubations at 3 min or 30 min did not reach the same level (Fig. 4*a*). Next H-35 cells were treated with several concentrations of H_2O_2 for 10 min and the kinase activity of WGA-purified fraction toward *src*-related peptide was examined. Maximal stimulation was observed at a H_2O_2 concentration of 10 mM. Concentrations lower or higher than 10 mM caused a lower stimulation of kinase activity (Fig. 4*b*).

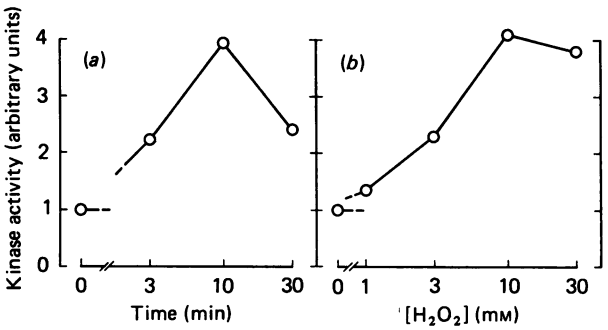

Fig. 4. The time and concentration dependency of H_2O_2 treatment of intact H-35 cells upon the phosphorylation of *src*-related peptide

The assay for time dependency (*a*) was carried out using cells treated with 10 mM-H_2O_2. The assay for concentration dependency (*b*) was carried out using cells treated with different concentration of H_2O_2 for 10 min. The *src*-related peptide was incubated with [γ-^{32}P]ATP, Mn^{2+}, Mg^{2+} and the WGA-purified fractions obtained from H_2O_2-treated cells for 4 min at 23 °C. The degree of phosphorylation is indicated in arbitrary units.

Effect of H_2O_2 treatment in a cell-free system on the insulin receptor kinase activity

It was possible that the effect of H_2O_2 on the insulin receptor kinase was due to a direct effect of H_2O_2 on the cell-free assay system. Consequently, several concentrations of H_2O_2 (10 μM–10 mM) were incubated with WGA-purified fractions at 23 °C for 10 min and insulin receptor kinase activity towards *src*-related peptide in the cell-free assay was examined. The phosphorylation of *src*-related peptide by the WGA-purified insulin receptor was slightly decreased when H_2O_2 was present during the assay (Fig. 5). A similar effect of H_2O_2 on the ^{32}P-labelling of the M_r 93000 protein in the cell-free assay system using WGA-purified insulin receptors was observed (results not shown). These results suggest that the effects of H_2O_2 on the activation of the insulin receptor were not expressed in the cell-free assay system.

DISCUSSION

We have demonstrated that the treatment of H-35 rat hepatoma cells with H_2O_2 increased the incorporation of ^{32}P into the tyrosine residues of the β subunit of insulin receptors. We have also shown that WGA-purified insulin receptors obtained from H_2O_2-treated cells similarly demonstrate an increased ^{32}P-labelling of the β subunit and an increased receptor kinase activity towards *src*-related peptide. The effect of H_2O_2 on the kinase activity was found to be time- and dose-dependent. On the other hand, the direct addition of H_2O_2 to a cell-free assay system slightly decreased the ^{32}P-labelling of the β subunit and the insulin receptor's kinase activity towards the *src*-related peptide. These results raise the possibility that in an intact cell system H_2O_2 may increase the insulin receptor kinase activity by inducing tyrosine phosphorylation of the β subunit.

Several agents are reported to have insulin-like effects on intact cells. These include anti-(insulin receptor) antibodies (Kahn *et al.*, 1977; Kasuga *et al.*, 1978), concanavalin A (Cuatrecasas & Tell, 1973), orthovanadate (Dubyak & Kleinzeller, 1980; Tamura *et al.*, 1984),

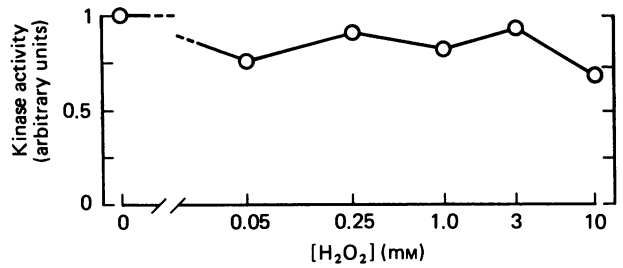

Fig. 5. Effect of H_2O_2 on the cell-free assay for phosphorylation of the *src*-related peptide

The WGA-purified insulin receptor fraction was incubated with the indicated concentrations of H_2O_2 at 23 °C for 10 min, and then with [γ-^{32}P]ATP, Mn^{2+}, Mg^{2+} and *src*-related peptide for 4 min. Phosphorylated *src* peptides were separated and counted as described under 'Materials and methods'. The activities are shown as a percentage of the basal activity.

spermine (Lockwood & East, 1974), vitamin K_5 (Livingston *et al.*, 1977), and H_2O_2 (Czech *et al.*, 1974; May & de Haën, 1979). Among these, only orthovanadate has been reported to increase the tyrosine phosphorylation of the β subunit of insulin receptors in an intact cell system (Tamura *et al.*, 1984). Considering that orthovanadate has insulin-like effects such as the stimulation of 2-deoxglucose uptake, glucose oxidation and glycogen synthase (Dubyak & Kleinzeller, 1980; Tamura *et al.*, 1984) and that the insulin-stimulated insulin receptor phosphorylation of tyrosine residues may be important for its action (Ellis *et al.*, 1986), it is possible to speculate that the insulin-like effects of orthovanadate may be mediated through the tyrosine phosphorylation of insulin receptors. From a similar point of view, it may be speculated that the insulin-like effects of H_2O_2 on intact cells are also mediated through the tyrosine phosphorylation of insulin receptors and the subsequent activation of their kinase activity.

In spite of the similarity in the action of insulin and H_2O_2 on the insulin receptor kinase activity, specific differences also exist. For example, the time course of insulin receptor kinase activation by these two agents is clearly different. Insulin (10^{-7} M) only needs 15 s for its maximal activation of insulin receptor kinase (Klein *et al.*, 1986); however, H_2O_2 (10 mM) needs 10 min. Furthermore, although H_2O_2 (10 mM) stimulated the same degree of tyrosine phosphorylation of insulin receptor compared with 10^{-7} M-insulin, H_2O_2-stimulated insulin receptor kinase activities were always lower than insulin (10^{-7} M)-stimulated kinase activities. These data suggest that the mechanisms and sites of tyrosine phosphorylation of the β subunit of insulin receptor induced by insulin and H_2O_2 are not exactly the same. Thus, further characterization of the H_2O_2-induced tyrosine phosphorylation of β subunit of insulin receptors may provide important clues to disclose the mechanisms of insulin-induced activation of insulin receptor kinase activity.

One final point of interest from the study concerns the endogenous substrates for the insulin receptor kinase. We have found that WGA-purified insulin receptors obtained from H_2O_2-treated cells demonstrate the ^{32}P-labelling of a M_r 160000 protein in the cell-free assay. Furthermore, this protein was phosphorylated in the presence of insulin during the cell-free assay. Preliminary data demonstrated that WGA-purified insulin receptors obtained from insulin-treated cells also phosphorylated a protein of M_r 160000 (O. Koshio, Y. Akanuma & M. Kasuga, unpublished work). These data suggest that a glycoprotein of M_r 160000 can serve as the substrate for the insulin receptor kinase. At the same time, these data provide additional support for the notion that H_2O_2 stimulates the tyrosine kinase activity of the insulin receptor in intact cells.

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