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Insulin Signaling Is Inhibited by Micromolar Concentrations of H_2O_2

EVIDENCE FOR A ROLE OF H_2O_2 IN TUMOR NECROSIS FACTOR α-MEDIATED INSULIN RESISTANCE*

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Both hyperglycemia and tumor necrosis factor α (TNFα) were found to induce insulin resistance at the level of the insulin receptor (IR). How this effect is mediated is, however, not understood. We investigated whether oxidative stress and production of hydrogen peroxide could be a common mediator of the inhibitory effect. We report here that micromolar concentrations of H_2O_2 dramatically inhibit insulin-induced IR tyrosine phosphorylation (pretreatment with 500 μM H_2O_2 for 5 min inhibits insulin-induced IR tyrosine phosphorylation to 8%), insulin receptor substrate 1 phosphorylation, as well as insulin downstream signaling such as activation of phosphatidylinositol 3-kinase (inhibited to 57%), glucose transport (inhibited to 36%), and mitogen-activated protein kinase activation (inhibited to 7.2%). Both sodium orthovanadate, a selective inhibitor of tyrosine-specific phosphatases, as well as the protein kinase C inhibitor Gö6976 reduced the inhibitory effect of hydrogen peroxide on IR tyrosine phosphorylation. To investigate whether H_2O_2 is involved in hyperglycemia- and/or TNFα-induced insulin resistance, we preincubated the cells with the H_2O_2 scavenger catalase prior to incubation with 25 mM glucose, 25 mM 2-deoxyglucose, 5.7 nM TNFα, or 500 μM H_2O_2, respectively, and subsequent insulin stimulation. Whereas catalase treatment completely abolished the inhibitory effect of H_2O_2 and TNFα on insulin receptor autophosphorylation, it did not reverse the inhibitory effect of hyperglycemia. In conclusion, these results demonstrate that hydrogen peroxide at low concentrations is a potent inhibitor of insulin signaling and may be involved in the development of insulin resistance in response to TNFα.

Oxidatively modified proteins accumulate during aging, oxidative stress, and in some pathological conditions (1, 2). In particular it has been shown that oxidative stress is increased *in vivo* in the diabetic state (3, 4). Noninsulin-dependent diabetes mellitus (NIDDM)[1] is associated with accelerated production of oxygen-free radicals as well as a decreased scavenging of these proteins (2). A possible role of the accumulation of reactive oxidant species in the development of the late complications in diabetes has been discussed (5–7). Recently the question of a relationship between oxidative stress and insulin action (8) was raised, and it was suggested that changes in the physicochemical state of the plasma membrane and increases in intracellular calcium concentrations might be involved.

Clinically overt NIDDM is characterized by defects in insulin secretion and insulin resistance of all major target tissues (9). Both genes and the environment contribute to the development of the disease (10). Increasing knowledge of the signaling molecules involved in insulin action has led to the proposal of several possible candidates that could contribute to insulin resistance (11). Insulin mediates its action through phosphorylation of a transmembrane-spanning tyrosine kinase receptor, the insulin receptor. Binding of insulin to the insulin receptor leads to activation of the intrinsic tyrosine kinase activity and subsequently to tyrosine phosphorylation of a number of substrates that mediate the metabolic and mitogenic effects of insulin (12).

Conflicting data on the effect of hydrogen peroxide on insulin signaling have been reported. Whereas several reports claimed that H_2O_2 had insulinomimetic effects (13–15), a recent report found reduced insulin responsiveness in response to oxidative stress (16). The latter effect, an inhibition of glucose transport, was explained through changes of the level of GLUT1 and GLUT4 (17) transcription.

Various factors including hyperinsulinemia, hypoinsulinemia, phorbol esters, adenosines, and catecholamines have been shown to regulate insulin receptor function (11). We and others have found previously that hyperglycemia induces insulin resistance at the level of the insulin receptor (IR) in various cell systems as well as in animals (18–22). Similar inhibitory effects on the IR kinase are observed when cells are treated with tumor necrosis factor α (TNFα) (23, 24). It has been suggested that analogous mechanisms may be responsible for the reduced insulin receptor kinase activity in NIDDM patients. However, how glucose or TNFα can mediate this effect is not understood. Because hyperglycemia leads to the production of hydrogen peroxide within the cell (8), we investigated whether H_2O_2 affects insulin receptor kinase activity and the activation of insulin-induced signal transduction pathways. Furthermore we addressed the possibility that the production of H_2O_2 may mediate TNFα-induced insulin resistance.

EXPERIMENTAL PROCEDURES

Materials

Recombinant human insulin was obtained from Novo-Nordisk, Bagsvaerd, Denmark. Anti-p85 and anti-insulin receptor substrate 1 (IRS-1) antisera were produced by immunization of rabbits with synthetic peptides spanning amino acids 710–723 from mouse p85 and amino acids 1220–1233 from rat IRS-1, respectively. Polyclonal rabbit anti-

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[1] The abbreviations used are: NIDDM, noninsulin-dependent diabetes; 2DG, 2-deoxyglucose; IR, insulin receptor; IRS-1, insulin receptor substrate 1; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; PI-3 kinase, phosphatidylinositol 3-kinase; GLUT, glucose transporter; GST, glutathione *S*-transferase; PY, phosphotyrosine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

serum against the insulin receptor was produced by immunization with synthetic peptides covering the last 15 C-terminal amino acids of the β-subunit. Anti-phosphotyrosine antibodies (PY20) were obtained from Transduction Laboratories. Anti-active mitogen-activated protein kinase antibodies were purchased from Promega. Protein assay reagent and horseradish peroxidase-coupled secondary antibodies were obtained from Bio-Rad, and nitrocellulose membrane (Schleicher & Schüll, BA85) was obtained from Protran. Protein A-Sepharose was from Amersham Pharmacia Biotech.

The cDNAs for the type A insulin receptor (25) and IRS-1 (26) were cloned into a cytomegalovirus promotor enhancer-driven expression vector. The expression plasmid GST-ELK1 has been described before (27) and was grown and expressed according to the GST Gene Fusion System manual, Amersham Pharmacia Biotech.

Methods

Cell Incubations—Human embryonic kidney fibroblasts (HEK293; ATCC CRL 1573) and NIH3T3 fibroblasts overexpressing the human insulin receptor (NIH-B cells, kindly provided by R. Schumacher and A. Ullrich) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Life Technologies), 10 mg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a 5% CO_2 enriched, humidified atmosphere. 3T3-L1 cells were grown in DMEM containing 4500 mg/liter glucose supplemented with 10% newborn calf serum (Life Technologies, Inc.), 1 mM sodium pyruvate (Life Technologies, Inc.), 2 mM glutamine (Life Technologies, Inc.), 10 mg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a 5% CO_2-enriched, humidified atmosphere.

Cell Transfection—HEK293 cells were transiently transfected using $CaCl_2$ as described by Chen and Okayama (28) and Graham and Van der Eb (29). 24 h later cells were starved overnight in medium containing 5 mM glucose and 0.5% fetal bovine serum.

Differentiation of 3T3L1 Cells—Cells were grown to confluence and left for 2 days. Differentiation medium I containing differentiation promoting factors (DMEM containing 4500 mg/liter glucose, 10 mM Hepes, 0.2 μM insulin, 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone) was then added. After 2 days differentiation medium II (DMEM containing 4500 mg/liter glucose, 10% fetal calf serum, 0.2 μM insulin) was added for 5–7 days with medium changes every second day. Differentiated 3T3-L1 cells used for the glucose transport assay were incubated in differentiation medium II with 0.5% fetal calf serum but without insulin overnight before the assay was done.

Cell Lysis, Western Blotting, and ECL—After stimulation the cells were washed once with phosphate-buffered saline and homogenized in lysis buffer containing 20 mM Tris-acetate, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 1% Triton X-100, 5 mM $Na_4P_2O_7$, 10 mM $C_3H_7O_6PNa_2$, 1 mM benzamidine, 1 mM dithiothreitol, and 4 μg/ml leupeptin. After the removal of cellular debris (15,000 × *g* for 10 min at 4 °C), the protein content in each sample was measured using Bio-Rad protein assay dye reagent concentrate according to the manufacturer's instructions (Bio-Rad). Equal amounts of cell lysate were dissolved in 2 × Laemmli buffer and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membranes (Schleicher & Schüll, BA85). Immunoreactive proteins were visualized using horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Immunoprecipitation—After treatment of NIH-B cells with insulin, H_2O_2, or a combination of both, the cells were lysed in HNTG buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerin, 1 mM EGTA, 2 mM orthovanadate, 20 mM NaF, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 50 mM sodium pyrophosphate. Insoluble material was removed by centrifugation. The samples were diluted 1:1 with the same buffer containing only 0.1% Triton X-100. Immunoprecipitates were collected by adding anti-p85, anti-IRS-1, anti-phosphotyrosine-specific antibodies, and Protein A-Sepharose 4B (Amersham Pharmacia Biotech) for 3 h at 4 °C followed by brief centrifugation. The precipitates were washed twice in HNTG buffer containing 0.1% Triton X-100 and twice in the same buffer without Triton X-100.

Phosphatidylinositol 3-Kinase (PI-3) Kinase Assay—NIH-B cells were lysed and immunoprecipitated as described. PI-3 kinase assays were performed essentially as described by Seedorf *et al.* (30). Briefly, the immunoprecipitates were incubated with kinase buffer containing 30 mM Hepes, pH 7.4, 30 mM $MgCl_2$, 0.2 mM adenosine, 40 μM ATP, 0.2 mg/ml sonicated phosphatidylinositol and phosphatidylserine, and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) for 10 min at 30 °C. The phospholipids were extracted with chloroform/methanol (1:1), washed twice with methanol, 1 N HCl (1:1), and finally spotted onto thin layer chromatography (TLC) plates. After one-dimensional chromatography, the plates were exposed to the PhosphorImager to quantitate radioactive lipids corresponding to authentic phosphatidylinositol monophosphate standards.

MAPK Assay—NIH-B cells were starved overnight and subsequently stimulated with insulin or H_2O_2 or pretreated with H_2O_2 prior to insulin stimulation. Cell lysis, SDS-PAGE, and Western blotting were performed as described. The data from the extracellular signal-regulated kinase 1/2 Western blot were scanned and quantitated using ImageQuant software (Molecular Dynamics).

2-Deoxyglucose (2DG) Uptake—Differentiated 3T3-L1 cells were starved overnight in DMEM containing 5 mM glucose and 0.5% fetal calf serum. The cells were washed and incubated for 3 h in preheated serum starvation medium (DMEM containing 5 mM glucose and 0.2% bovine serum albumin). The cells were then washed three times in preheated Krebs-Ringer-Hepes (KRH) buffer, pH 7.5 (136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, and 20 mM Hepes, pH 7.5) containing 0.2% bovine serum albumin. 1 ml KRH buffer/0.2% bovine serum albumin was added to the cells, and they were left untreated or stimulated with insulin, H_2O_2, a combination of the two, cytochalasin B, or cytochalasin B plus insulin at 37 °C. For the last 5 min the 2DG uptake was determined by adding 50 μl of a start solution containing 2 mM 2DG and 2 μCi/ml (0.1 μCi/ml final specific activity) 2-deoxy-D-[2,6-^{3}H]glucose. The glucose uptake was terminated by washing the cells in ice-cold phosphate-buffered saline without Ca^{2+} and Mg^{2+} and lysis in 500 μl of 0.1 M NaOH. The cell-associated radioactivity was determined by mixing 400 μl of cell lysate with 2.6 ml of scintillation fluid (Optiphase "Supermix") followed by counting in a β-counter for 10 min. The remaining 100 μl of cell lysate was used for protein determination. Non-carrier-mediated 2DG uptake was determined in parallel in the presence of 20 μM cytochalasin B and subtracted from both basal and stimulated glucose uptake measurements (31). The results are presented as cpm/mg protein.

Statistical Analysis—Data are presented as the mean ± S.D. Group comparisons were made by unpaired Student's *t* test. *p* values less than 0.05 were considered significant.

RESULTS

H_2O_2 Inhibits Insulin-induced Tyrosine Phosphorylation of the IR β-Subunit—To investigate whether hydrogen peroxide has a direct effect on IR autophosphorylation, we pretreated NIH-B cells for 5 min with increasing concentrations of H_2O_2 (0.1–5 mM) prior to insulin stimulation (10^{-7} M for 5 min). As shown by Western blot analysis using total cell lysates probed with anti-phosphotyrosine-specific antibody, an inhibitory effect of H_2O_2 on tyrosine phosphorylation of the insulin receptor β-subunit is seen with 100 μM H_2O_2 (Fig. 1*A*). The effect becomes more pronounced with higher concentrations of H_2O_2. Fig. 1*B* shows a time course of the inhibitory effect. When pretreated with 500 μM H_2O_2 prior to insulin stimulation the inhibition can be observed after 2 min of preincubation with H_2O_2 and is further enhanced after 5 and 10 min. Interestingly, after 20 min the tyrosine phosphorylation is only slightly affected, but longer incubation with H_2O_2 is once again inhibitory. This suggests that the inhibitory effect of H_2O_2 on insulin receptor activity is oscillating. As a control the *lower panels* in Fig. 1, *A* and *B,* show a Western blot of the same samples probed with an antibody against the IR β-subunit.

Insulin-induced Tyrosine Phosphorylation of IRS-1 Is Inhibited by H_2O_2—Next we investigated whether proximal events in insulin signaling were also affected by pretreatment with H_2O_2. Because the endogenous IRS-1 was difficult to detect in NIH-B cells, we used 293 cells transiently transfected with the insulin receptor and IRS-1. As a control, cells were also transfected with IR alone (*IR + Vec*) or vehicle only (*Vec*). As shown on the anti-phosphotyrosine blot in the *upper panel* of Fig. 2, 500 μM of H_2O_2 inhibited tyrosine phosphorylation of the IR β-subunit in these cells in a time-dependent manner. In parallel with the inhibitory effect on the IR β-subunit (and the IR precursor) we saw a reduced tyrosine phosphorylation of IRS-1

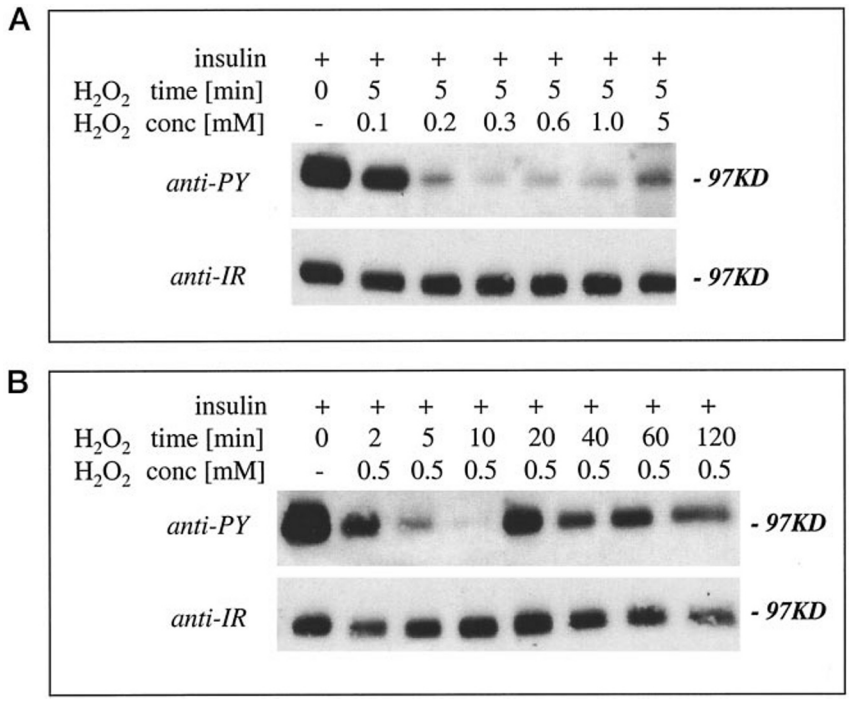

FIG. 1. **Effect of H_2O_2 on insulin-induced IR tyrosine phosphorylation in NIH-B cells.** NIH-B cells were cultured to 80% confluency and starved overnight in DMEM containing 0.5% FBS. The cells were pretreated with H_2O_2, for the times and with the concentrations (*conc*) indicated, prior to stimulation with 10^{-7} M insulin for 5 min. The cells were lysed, and protein concentration of the lysates were normalized. Proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. The blots were incubated with anti-PY (*upper panel*) or anti-IR-specific antibodies (*lower panel*). Immunoreactive proteins were visualized with horseradish peroxidase-coupled secondary antibodies and the ECL™ detection method.

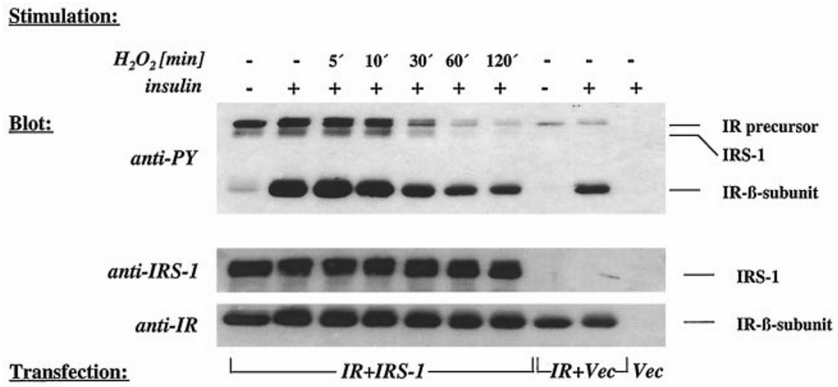

FIG. 2. **Insulin-induced tyrosine phosphorylation of IRS-1 is reduced after H_2O_2 pretreatment of HEK293 cells.** HEK293 cells were transfected with human insulin receptor and IRS-1 expression plasmid together (*IR + IRS-1*), IR alone (*IR + Vec*), or vector (*Vec*) alone, as indicated, and starved overnight in medium containing 0.5% FBS. The cells were pretreated with 500 μM H_2O_2 for the times indicated prior to stimulation with 10^{-7} M insulin for 5 min (where indicated). Cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with anti-PY (*upper panel*), anti-insulin receptor substrate 1- (*anti-IRS-1*, *middle panel*), or anti-insulin receptor-specific (*anti-IR*, *lower panel*) antibodies. Immunoreactive proteins were visualized with horseradish peroxidase-coupled secondary antibodies and the ECL™ detection method.

after 30 min of H_2O_2 pretreatment. Compared with the result in the NIH-B cells (Fig. 1*B*), the inhibitory effect was not as strong and occurred after longer times of pretreatment. Whereas we reproducibly saw oscillations of the effect in NIH-B cells, we could not detect such a phenomenon in the 293 cells under the above conditions. The two *lower panels* in Fig. 2 show the expression of IRS-1 and the IR in these samples as a control.

Insulin-induced PI-3 Kinase Activity, Glucose Transport, and MAPK Activity Are Inhibited by Hydrogen Peroxide—To further investigate whether reduced IR autophosphorylation and insulin-induced tyrosine phosphorylation of IRS-1 affects both mitogenic and metabolic signaling pathways, we studied insulin-induced MAPK activation and glucose transport, respectively. A key event in the stimulation of insulin-induced glucose transport is the activation of PI-3 kinase. We therefore initially determined the insulin-induced PI-3 kinase activity in NIH-B cells in the presence and absence of H_2O_2 pretreatment (500 μM, 5 min). A representative diagram is shown in Fig. 3. Insulin-induced PI-3 kinase activity in combined immunoprecipitates (anti-PY + anti-IRS-1 + anti-p85) was reduced to about 57% in the presence of H_2O_2. H_2O_2 alone showed a slightly insulinomimetic effect. The diagram shows mean and S.D. of two representative experiments done in triplicate. Significance was determined using the Student's *t* test.

Because NIH-B cells are not suited to study insulin-induced glucose transport (they lack the insulin-sensitive glucose transporter GLUT-4), we turned to 3T3-L1 cells to measure glucose uptake. The cells were differentiated into adipocytes as described under "Methods." Glucose transport was measured as [^{3}H]2DG uptake, corrected for the protein content in the sample, and expressed as percent of maximal insulin-stimulated transport. Insulin induced about a 5-fold increase in 2DG uptake, and both the basal and insulin-induced glucose transport were completely blocked in the presence of cytochalasin B (data not shown). When the cells were pretreated with 500 μM H_2O_2 for 20 min prior to insulin stimulation (10^{-7} M), 2DG uptake was clearly inhibited. In these experiments H_2O_2 had a slightly positive effect on basal 2DG uptake. The diagram shows mean and S.D. of a representative example of 5 experiments per-

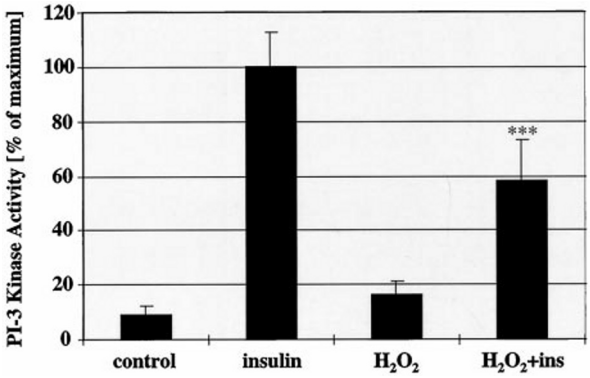

FIG. 3. **Insulin-induced PI-3 kinase activity is inhibited by hydrogen peroxide.** NIH-B cells were cultured until 80% confluency and starved overnight in DMEM containing 0.5% FBS. Cells were left untreated (*control*), stimulated with 10^{-7} M insulin for 5 min or 500 μM H_2O_2 for 5 min, or pretreated with 500 μM H_2O_2 for 5 min prior to 10^{-7} M insulin (*ins*) stimulation for 5 min. Cells were lysed and lysates were subjected to immunoprecipitation with a mixture of anti-IRS-1, anti-p85-, and anti-phoshotyrosine-specific antibodies. Immunoprecipitates were washed and subjected to PI-3 kinase assays as described under "Experimental Procedures." Radioactive lipids were separated on TLC plates, and phosphatidylinositol monophosphate was quantified using PhosphorImager technology. *Error bars* represent the standard deviation obtained from two independent experiments done in triplicate. ***, $p \leq 0.001$.

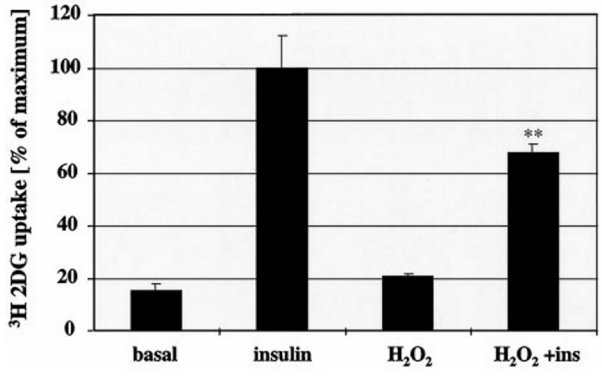

FIG. 4. **Insulin-mediated glucose transport in 3T3L1 cells is attenuated by H_2O_2.** 3T3L1 cells were differentiated into adipocytes as described and starved overnight in DMEM with 5 mM glucose and 0.5% FBS. Cells were left untreated (*control*), incubated with insulin (10^{-7} M, 15 min), H_2O_2 (500 μM, 5 min), or pretreated with H_2O_2 prior to insulin stimulation (*H_2O_2 + ins*). Total cell lysates were prepared, and protein concentration was determined from an aliquot. 2DG uptake was measured as described, and the results were corrected for protein concentration. Results are expressed as percentage of maximum. The experiment was repeated five times. A representative experiment is shown; *error bars* represent the standard deviation of one experiment performed in triplicate. **, $p \leq 0.01$.

formed in triplicate. Significance was determined using the Student's *t* test (Fig. 4).

To investigate whether H_2O_2 also regulates the mitogenic signaling of insulin, we studied MAPK activation in NIH-B cells. As shown in the *lower panel* of Fig. 5*A*, using an antibody recognizing the activated extracellular signal-regulated kinase 1/2 proteins (anti-MAPK active), insulin induced a rapid induction of MAPK activity, which peaked around 7.5 min and thereafter declined but remained elevated throughout the experiment (120 min). H_2O_2 treatment alone (500 μM) also resulted in MAPK activation; however, activated extracellular signal-regulated kinase 1/2 was observed after 20 min and peaked around 60 min. When the cells were pretreated with H_2O_2 (500 μM 10 min) prior to insulin stimulation, the initial peak observed at 7.5 min of insulin stimulation was completely inhibited. However, MAPK activity at later time points was slightly increased as compared with the maximum in the samples treated with insulin alone and seemed to follow the curve observed with H_2O_2 treatment alone. To monitor insulin receptor phosphorylation the samples were probed with an anti-phosphotyrosine-specific antibody as shown in the *upper panel* of Fig. 5*A*. Both the inhibitory effect of H_2O_2 on insulin-induced tyrosine phosphorylation of the IR as well as the oscillations of this effect can be seen, leading to delayed activation of the receptor. In the case of the insulin-treated samples there is a good correlation between tyrosine phosphorylation and MAPK activity. This is also the case in the samples that are pretreated with hydrogen peroxide prior to insulin stimulation. In contrast, the samples treated with H_2O_2 alone show MAPK activation in the absence of IR tyrosine phosphorylation, indicating that a different mechanism is involved. The *middle panel* shows the expression level of the insulin receptor as a control. A quantitation of the MAPK data using ImageQuant software (Molecular Dynamics) is shown in Fig. 5*B*. The data are representative of 4 experiments.

Catalase Prevents H_2O_2- and TNFα-induced but Not Hyperglycemia-induced Inhibition of Insulin-induced IR Tyrosine Phosphorylation—Because TNFα and hyperglycemia mediated similar inhibitory effects on IR autophosphorylation and because both agents create cellular stress, we investigated the possibility that H_2O_2 could be a common mediator for their inhibitory effect. To eliminate endogenously produced H_2O_2 in the cell we incubated 293 cells overexpressing the IR and IRS-1 in the absence or presence of 11,700 units of the H_2O_2 scavenger catalase overnight. As shown in the *upper panel* in Fig. 6*A* (anti-phosphotyrosine blot) pretreatment with 25 mM D-glucose or 25 mM 2-deoxyglucose results in a reduction of insulin-induced tyrosine phosphorylation of the IR-β-subunit, similar to the pretreatment with H_2O_2, which was used as a control (*lanes 3–5*). As expected, pretreatment with catalase prevented the inhibitory action of H_2O_2 (*lane 10*). However, it did not change the hyperglycemia-induced inhibition (*lanes 8* and *9*, the inhibitory effect of 2-deoxyglucose was even enhanced, as determined in several independent experiments). The *upper panel* of Fig. 6*B* presents an anti-phosphotyrosine blot of samples pretreated with 5.7 nM TNFα (10 min) or H_2O_2 (10 min) prior to insulin stimulation. Comparable inhibition of insulin-induced IR tyrosine phosphorylation is observed for the two preincubation conditions (*lanes 3* and *4*). Interestingly, treatment of the cells with catalase completely prevents the inhibitory effect of TNFα (*lanes 7*), suggesting that production of hydrogen peroxide is involved in the mechanism. Control blots showing the IR expression level are represented in the *lower panels* of Fig. 6, *A* and *B,* respectively.

Vanadate and PKC Inhibitors can Prevent the Inhibitory Effect of H_2O_2—Tyrosine-specific phosphatases as well as the action of serine/threonine kinases, in particular protein kinase C, have been discussed as potential mediators of insulin receptor kinase inhibition (32). To further investigate the mechanism of the H_2O_2-induced inhibition of insulin signaling, we therefore used specific inhibitors of these proteins. Pretreatment of NIH-B cells with the specific tyrosine phosphatase inhibitor sodium orthovanadate (250 μM, 30 min) abolished the inhibitory effect of hydrogen peroxide on insulin receptor tyrosine phosphorylation, as shown on an anti-phosphotyrosine blot in the *upper panel* of Fig. 7*A*. As a control the samples were also probed with an antibody against the IR β-subunit (Fig. 7*A*, *lower panel*). The specific protein kinase C inhibitor Gö6976, which mainly inhibits the Ca^{2+}-dependent classical PKC isoforms, partially reversed the inhibitory effect of H_2O_2 in a concentration-dependent manner (Fig. 7*B*, *upper panel,* H_2O_2 + insulin, 58% of insulin, pretreatment with 10 μM Gö6976

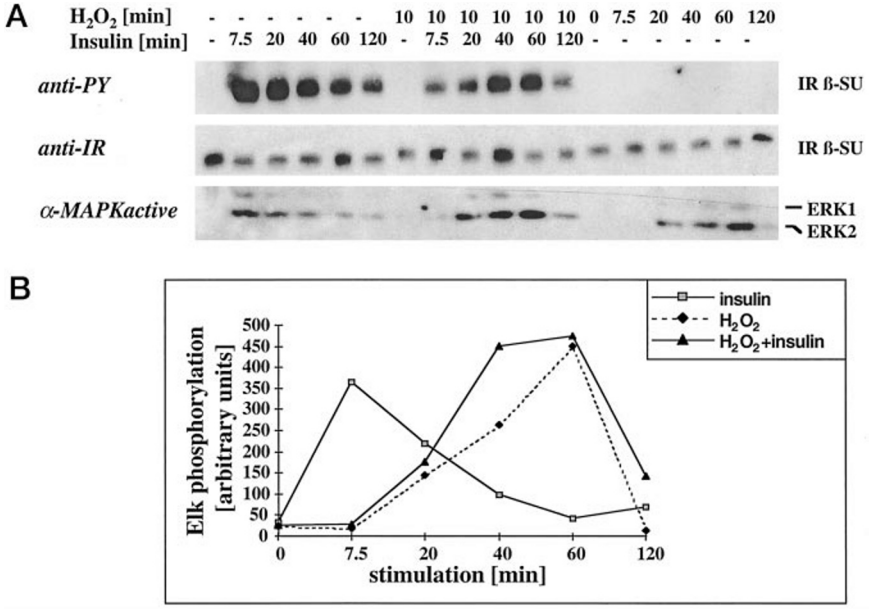

FIG. 5. **Hydrogen peroxide inhibits insulin-induced mitogen-activated protein kinase activity.** *A,* NIH-B cells were cultured until 80% confluency and starved overnight in in DMEM containing 0.5% FBS. Cells were stimulated with insulin (10^{-7} M), H_2O_2 (500 μM), or pretreated with 500 μM for 5 min prior to insulin stimulation (10^{-7} M) for the times indicated. Protein concentration was normalized and the cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with anti-PY (*upper panel*), anti-insulin receptor antibodies (*anti-IR*, *middle panel*), or antibodies directed to the activated form of extracellular signal-regulated kinase 1/2 (*anti-MAPKactive, lower panel*). Proteins were made visible using horseradish peroxidase-coupled secondary antibodies and the ECL™ detection method. *SU*, subunit. *B*, the MAPK data from the *lower panel* of Fig. 5*A* were quantitated using ImageQuant software (Molecular Dynamics). A representative example of 4 experiments is shown.

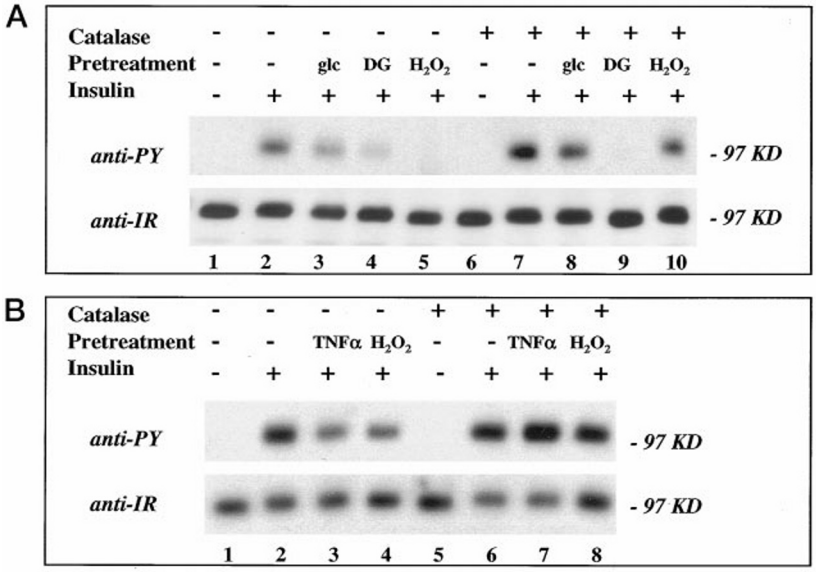

FIG. 6. **Catalase prevents TNFα and H_2O_2 but not hyperglycemia-induced inhibition of insulin-induced receptor phosphorylation.** HEK293 cells were transfected with human insulin receptor expression plasmid and starved overnight in medium containing 0.5% FBS. The cells were incubated overnight in the presence or absence of 11,700 units of catalase. The cells were washed 3 times with phosphate-buffered saline, and fresh medium containing 0.5% FBS was added. Thereafter 25 mM glucose (*glc*), 25 mM 2DG, or 500 μM H_2O_2 were added for the times indicated prior to stimulation with 10^{-7} M insulin for 5 min (where indicated). Cell lysates were subject to SDS-PAGE, transferred to nitrocellulose, and incubated with anti-PY (*upper panel*) or anti-insulin receptor-specific (*anti-IR*, *lower panel*) antibodies. Immunoreactive proteins were visualized with horseradish peroxidase-coupled secondary antibodies and the ECL™ detection method.

85%). The *lower panel* of Fig. 7*B* shows the level of insulin receptor expression as a control.

DISCUSSION

In this study, we demonstrate that micromolar concentrations of H_2O_2 have a strong inhibitory effect on insulin responsiveness in two different fibroblast cell lines and 3T3-L1 adipocytes. We present evidence for inhibition of the insulin receptor kinase as well as downstream signaling processes such as IRS-1 phosphorylation and PI-3 kinase activation. In addition, the metabolic (glucose transport) and mitogenic (MAPK) responses to insulin were reduced after pretreatment with low doses of H_2O_2. The finding of reduced insulin responsiveness is in agreement with recent reports by Rudich *et al.* (16), which described an inhibitory effect of oxidative stress on insulin-induced glucose uptake, lipogenesis, and glycogen synthase *a* activity in 3T3-L1 cells. These defects could not be attributed to early events of the insulin signaling cascade (33). The effects on glucose transport were explained by elevated GLUT1 and a decreased GLUT4 expression level as well as a defect in insulin-induced GLUT4 translocation (16, 17). This discrepancy with the present study could possibly be explained by the different mechanisms of stress induction in the two studies. Whereas we treated cells directly with H_2O_2 for short periods of time, Rudich *et al.* (16, 17) exposed them to prolonged low grade oxidant stress through exposure to glucose oxidase for 18 h.

Previous studies have shown insulinomimetic effects of H_2O_2 on insulin receptor and IRS-1 tyrosine phosphorylation and activation of PI-3 kinase, MAPK, and glucose uptake (13, 34–37). Similarly, other tyrosine kinases such as the epidermal growth factor receptor (38), Src family tyrosine kinases (39), Ha-Ras, and Raf-1 kinase (40) have been reported to be activated by H_2O_2-generated oxygen radicals, UV irradiation, and xanthine production. Although we clearly detected the insulinomimetic effects of H_2O_2 on MAPK activity, we could only detect minor effects on PI-3 kinase activity or glucose transport. Although insulin treatment resulted in the activation of PI-3 kinase, glucose transport, and MAPK, the addition of H_2O_2 resulted in impaired insulin-induced activation of these pathways (Figs. 3–5). This clearly shows that H_2O_2 in the concentrations and for the times used in this study is slightly insulinomimetic on its own; however, it is strongly inhibitory

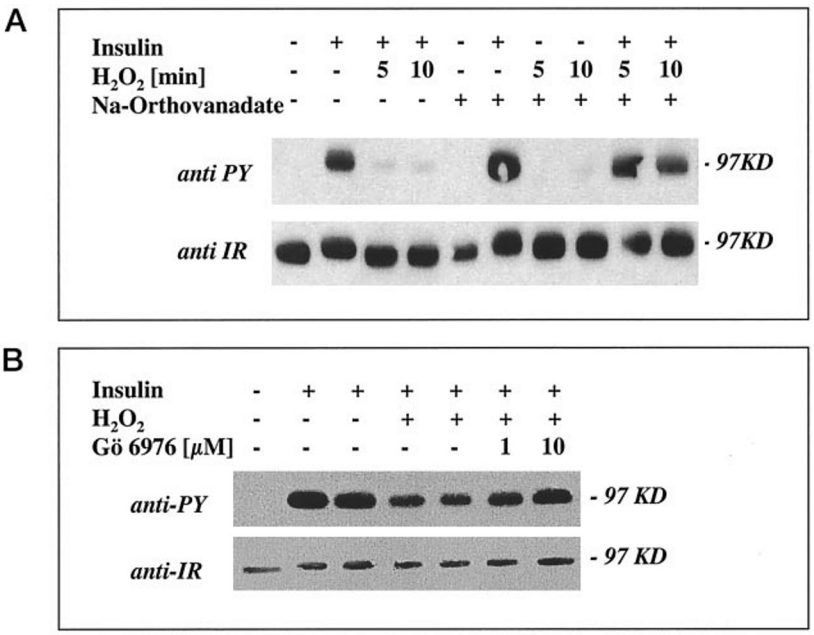

FIG. 7. **Vanadate and PKC inhibitors can prevent or reduce the inhibitory effect of H_2O_2 on IR tyrosine phosphorylation.** HEK293 cells were transfected with a human insulin receptor expression plasmid and starved overnight in medium containing 0.5% FBS. The cells were left untreated or pretreated with 250 μM sodium orthovanadate for 1 h (*A*) or 1 or 10 μM PKC inhibitor Gö6976 (*B*) for 1 h. Thereafter 500 μM H_2O_2 was added for the times indicated prior to stimulation with 10^{-7} M insulin for 5 min (where indicated). Cell lysates were subject to SDS-PAGE, transferred to nitrocellulose, and incubated with anti-PY (*upper panel*) or anti-insulin receptor-specific (*anti-IR*, *lower panel*) antibodies. Immunoreactive proteins were visualized with horseradish peroxidase-coupled secondary antibodies and the ECL™ detection method.

on different insulin-induced signaling pathways.

Several regulatory mechanisms seem to be involved in the effect of H_2O_2 on insulin signaling. Pre-incubation of the cells with orthovanadate prevents the inhibitory effect of H_2O_2 on insulin signaling suggesting an involvement of tyrosine phosphatases. However, H_2O_2 has in general an inhibitory effect on phosphatases, as seen when we measure the overall activity of phosphatases in our cell extracts after stimulation with H_2O_2 (data not shown). This finding is in agreement with other recent reports (41–43). We would therefore argue that H_2O_2 specifically activates one particular IR-specific phosphatase. Such specific protein tyrosine phosphatase activity would not have to be detectable in the overall protein tyrosine phosphatase activity measured in the lysates.

We detected partial prevention of the inhibitory effect of H_2O_2 by the protein kinase C inhibitor Gö6976, indicating that activation of PKC is also involved. PKC has long been known to be able to modulate cross-talk between several signal transduction pathways (44). It is also known to play an important role in insulin signaling (45, 46). PKC is a large family of proteins with multiple subspecies (47, 48), and so far it is not clear which of these isoforms play a role. Activation of PKC by H_2O_2 has been described previously for several isoforms from the three subclasses of PKC (49, 50). The activation was independent of receptor-coupled hydrolysis of inositol phospholipids and occurred through tyrosine phosphorylation. Another study reported a dual effect of H_2O_2 on PKC with an initial activation by mild oxidative modification and a subsequent inactivation by further oxidation (51). Such dual activation/inactivation could possibly be responsible for the oscillations that we observed in this study. Depending on the cell line there seem to be defined "time windows" that allow observation of the inhibitory effect at a given concentration (Fig. 1*B*).

Decreases in insulin receptor tyrosine kinase activity have been observed in various insulin resistant states (52). The data that we obtained when we studied the effect of H_2O_2 on insulin signaling bore strong resemblance to the effects of hyperglycemia and TNFα on insulin signaling in these cells (18, 53). Both, hyperglycemia and TNFα-mediated modulation of the insulin receptor have been suggested to be important for acquired insulin resistance in skeletal muscle (32). The importance of hyperglycemia for the development of the metabolic insulin resistance syndrome in NIDDM patients is well documented. On the other hand, TNFα has been proposed to play a role in obesity-linked insulin resistance (23, 54, 55). Because both hyperglycemia and TNFα create cellular stress, we evaluated the possibility that the modulation of IR kinase activity might occur through the generation of oxidative stress and H_2O_2 production. Both $MnCl_2$ (data not shown), which mimics a function of the intracellular scavenger, superoxide dismutase (56), and catalase, an enzyme that specifically catalyzes the dismutation of H_2O_2 to O_2 and H_2O, prevented the H_2O_2-induced attenuation of insulin signaling. Interestingly, catalase prevented the inhibitory effect of TNFα completely, whereas it had no effect on hyperglycemia-induced inhibition of insulin receptor tyrosine phosphorylation. This suggests that production of H_2O_2 in response to TNFα could be an important step in the molecular mechanism involved in causing insulin resistance. Different pathways for hyperglycemia- and TNFα-mediated insulin resistance at the level of the insulin receptor have been proposed before (53).

Elevated levels of hydrogen peroxide have been linked to noninsulin-dependent as well as insulin-dependent diabetes and seem to increase with the duration of the disease (2–4, 57). Oxidative stress is believed to play a major role in the development of diabetic complications (5–7). Because we have studied only short term effects of hyperglycemia, we cannot rule out that high blood glucose over sustained periods of time leads to the generation of H_2O_2 within the organism and thereby induces insulin resistance. However, other metabolic changes of the diabetic milieu such as hyperinsulinemia could also be responsible for the increase in hydrogen peroxide. H_2O_2 is produced in response to insulin (58–60), and we could therefore speculate that this effect of insulin could be part of a feedback mechanism involved in signal termination. This would suggest that hyperinsulinemia, through production of H_2O_2, could cause premature termination of insulin signaling.

The present study suggests that cellular events leading to increased H_2O_2 production might not only be connected to late complications of diabetes but possibly play a role in the induction of insulin resistance in early phases of the disease. Additional work is required to understand the detailed mechanism of this decreased insulin responsiveness and to study its physiological relevance in models of insulin resistance and patients with NIDDM.

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