Insulin-induced Activation of NADPH-dependent H₂O₂ Generation in Human Adipocyte Plasma Membranes Is Mediated by Go₁₂*  

(Received for publication, August 5, 1996, and in revised form, January 15, 1997)

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Human fat cells possess a multireceptor-linked H₂O₂-generating system that is activated by insulin. Previous studies revealed that manganese was the sole cofactor required for a hormonal regulation of NADPH-dependent H₂O₂ generation in vitro. In this report it is shown that the synergistic activation of NADPH-dependent H₂O₂ generation by Mn²⁺ and insulin was blocked by GDPβS (guanosine 5’-O-(2-thiotriphosphate)), pertussis toxin and COOH-terminal anti-Go₁₂ or the corresponding peptide.

Consistently, manganese could be replaced by micromolar concentrations of GTPγS (guanosine 5’-O-(3-thiotriphosphate)), which increased NADPH-dependent H₂O₂ generation by 20–40%. Insulin shifted the dose response curve for GTPγS to the left (10-fold) and increased the maximal response. In the presence of 10 μM GTPγS, the hormone was active at picomolar concentrations, indicating that insulin acted via its cognate receptor.

The insulin receptor and Go were co-adsorbed on anti-Ga₅ and anti-insulin receptor β-subunit (anti-IRβ) affinity columns. Partially purified insulin receptor preparations contained Ga₅, Ga₂βγ, and Gaβ (but no Gaα or Gaβγ). The functional nature of the insulin receptor-Go₁₂ complex was made evident by insulin’s ability to modulate labeling of Ga by bacterial toxins. Insulin action was mimicked by activated Ga, but not by Gaα or Gaγ, indicating that insulin’s signal was transduced via Go₁₂. Thus, NADPH oxidase is the first example of an effector system that is coupled to the insulin receptor via a heterotrimERIC G protein.

The insulin receptor is a heterotetrameric transmembrane protein consisting of two α- and β-subunits (1). Insulin binding produces a conformational change leading to activation of its intrinsic tyrosine kinase activity. This tyrosine kinase activity is one of the earliest steps in insulin action and may be essential for many of insulin’s biological effects. Recently, we demonstrated that insulin activates a H₂O₂-generating system in human fat cell plasma membranes via a mechanism bypassing the receptor kinase (2–4). Together with the results of others (5–9), these findings indicated that tyrosine phosphorylation may not be essential in all cases for insulin receptor signaling.

An alternative pathway of insulin receptor signaling for which tyrosine phosphorylation may not be essential could be via G proteins (6–11). In intact cells, pertussis toxin, which ADP-ribosylates the α-subunits of members of the Gα/Gβ, family and uncouples them from receptors, inhibited a number of insulin-stimulated cellular events, such as glucose transport and its metabolism (9–11), whereas the effects of the bacterial toxin on insulin’s antilipolytic action have remained controversial (11–13).

Plasma membranes from BC3 H-1 myocytes, adipocytes, and hepatocytes have been used to show that insulin promotes GTP binding or GDP release (7, 14, 15). It is also reported that insulin attenuates the pertussis toxin sensitivity of a 40-kDa Gγ-like protein (6, 8). Finally, GTPγS, a nonhydrolyzable GTP analog, inhibited insulin binding to its receptor and modulated autophosphorylation of the receptor and its phosphotransferase activity (16, 17). Consistently, the insulin receptor has at least two G protein binding sites that coincide with autophosphorylation sites and appears to be associated with several distinct GTP-binding proteins, including a 40-kDa pertussis toxin substrate and a 60–67-kDa protein (18–22).

Several lines of evidence indicated that the insulin receptor kinase has no role in the communication between the insulin receptor and G proteins: (i) no phosphotyrosine was detectable in anti-Gα immunoprecipitates from lysates of 32P-labeled insulin-stimulated hepatocytes (6); (ii) ATP and ATP analogs had no effect on the insulin-induced acceleration of binding of GTPγS to BC3 H-1 membranes (7); and (iii) a monoclonal antibody to the insulin receptor (Ma-20) inhibited pertussis toxin (PTX)-catalyzed ADP-ribosylation of Gα, without activating insulin receptor kinase (8).

Thus, there is evidence to suggest that a non-kinase-dependent pathway of insulin receptor signaling involving G proteins exists. However, an effector system that couples to a G protein associated with the insulin receptor has not yet been identified. Thus, despite many efforts, a role of G proteins in insulin receptor signaling remains to be established.

EXPERIMENTAL PROCEDURES

Materials

Synthetic peptides corresponding to residues 345–354 of Ga₁₂, 345–354 of Gao₃, and 386–394 of Gaα, and the corresponding COOH-terminal antibodies, recombinant α-subunits of Gαᵢ₁, Gαᵢ₂, Gαᵢ₃, and Gαₑ, as well as the A-protomers of cholera and pertussis toxin were from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Purified βγ-subunits and α₁₀₃-subunits from brain were kindly provided by Dr. G. Schultz (Institut für Pharmakologie, Freie Universität Berlin, Germany).

 Peroxidase-conjugated anti-rabbit IgG was from Dianova (Hamburg, Germany), and rabbit polyclonal anti-Gβ and protein A-agarose were from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit polyclonal guanosine 5’-O-(2-thiotriphosphate); MOPS, 3-(N-morpholino)propane sulfonic acid; PTX, pertussis toxin; CTX, cholera toxin; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MES, 4-morpholineethanesulfonic acid; WGA, wheat germ agglutinin; IGF, insulin receptor β-subunit; IGF, insulin-like growth factor.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, FRG. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: G protein, regulatory guanine nucleotide binding protein; GTPγS, guanosine 5’-O-(3-thiotriphosphate); GDPβS,

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anti-Gαi2 (1-20) was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit insulin receptor antibody (β-subunit) was from Transduction Laboratories (Lexington, KY). Antiserum raised against internal decapeptide sequences of Gβi1 (MIKKI), Gβi2 (IINES), or against the COOH terminus of Gαi2 (VLEUM) were kindly donated by Dr. Y. J. Oh (Department of Medical Chemistry, University of Helsinki, Finland). [32P]P[NAD (800 Ci/mmol) was purchased from NEN, Du Pont, Bad Homburg, Germany, Hybond PVDF membranes were from Amer sham (Braunschweig, Germany). Insulin, GTPγS, GDPβS, and GDP were from Boehringer Mannheim (Mannheim, Germany). Human serum albumin and luminol were from Behring Werke (Marburg, Germany) and Fluka AG (Basel, Switzerland), respectively.

Methods

Subjects, Preparation of Fat Cells, and Fat Cell Ghosts—Experiments were described in detail elsewhere (2–4). Adipose tissue was from nonobese subjects undergoing elective abdominal or cosmetic breast surgery. The tissue specimens were cut into small pieces, and fat cells were isolated by the method of Rodbell (23) in a HEPES-buffered Krebs-Henseleit solution, pH 7.4, containing 20 mM HEPES, 10 mM NaHCO3, 5 mM glucose, 20 g/liter albumin, and 1 mg/ml collagenase CLS (Worthington). After 30 min, fat cells were washed and resuspended in 10 volumes of an ice-cold lysing medium containing 20 mM MES, pH 6.0, 0.2 mM MgCl2, 1 mM CaCl2, 5 mM KCl, and 100 mM/liter soybean trypsin inhibitor. Cell lysis was completed by mechanical shaking, and fat cell ghosts were collected by low speed centrifugation (1000 × g, 4 °C, 20 min).

Receptor-mediated Modulation of NADPH-dependent H2O2 Generation in Fat Cell Ghosts—A two-step procedure was used as reported elsewhere (2–4). Plasma membranes were first exposed to insulin and various cofactors (activation step) and were then assayed for NADPH oxidase activity. The activation step was carried out in 30 mM MOPS, pH 7.5, containing 120 mM NaCl, 1.4 mM CaCl2, 2.5 mM MgCl2, 10 mM NaHCO3, and 0.1% human albumin. Membranes were first incubated with various concentrations of insulin for 5 min to allow receptor occupation. Thereafter, guanine nucleotides or Mn2+ were added as indicated in the legends to figures and tables. After 20 min, ghosts were collected by centrifugation, washed, and then resuspended in 30 mM MES, pH 5.8, containing 120 mM NaCl, 4 mM MgCl2, 1.2 mM KH2PO4, 1 mM NaN3, 250 µM NADPH, and 10 µM FAD for determination of NADPH-dependent H2O2 generation (2–4).

Treatment of Membranes with Bacterial Toxins—For toxin labeling, membranes (100 µg) were incubated for 45 min at 37 °C in 1 ml of 30 mM MOPS, pH 7.5, containing 2.5 mM MgCl2, 14.4 mM CaCl2, 30 mM NaCl, 10 mM thymidine, 10 mM arginine, 100 µM ATP, and 10 µM [32P]P-NAD (10 µCi/assay), 0.01% bovine serum albumin, and 0.2 µg of PTX A protomer, or 5 µg of cholera toxin (CTX) subunit A. In the absence of insulin, 100 µM GDP (pertussis toxin) or 10 µM GTPγS (cholera toxin) were routinely included in the reaction media.

For assessing the effects of insulin on the action of both toxins as well as modulation of PTX labeling by M22 in plasma membranes, GTPγS or GDP was omitted (6, 24). The ligand-induced ADP-ribosylation of Gαi2 by cholera toxin was initiated by the simultaneous addition of 100 nM insulin and CTX (5 µg/sample). By contrast, for determining the inhibition of PTX labeling, membranes were first exposed to 10 nM insulin or 3 mM MnCl2, respectively. After 15 min, PTX A-protomer (0.2 µg/ml) was added, and incubations were continued for another 20 min. Reactions were terminated by centrifugation; the pellets were solubi lized with 150 µl of Laemmli buffer (25). Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 11% gel), electrophoretically transferred to Hybond PVDF, and visualized by autoradiography. Autoradiographic data were quantified by laser densitometry.

The conditions used for assessing the effects of bacterial toxins on NADPH-dependent H2O2 generation were identical with those described above, except that [32P]P-NAD was omitted and albumin concentration was increased to 0.1% (w/v). Control values containing all reagents except bacterial toxins were run in parallel for each condition. After 45 min, membranes were pelleted, resuspended in 30 mM MOPS, pH 7.5, containing 120 mM NaCl, 1.4 mM CaCl2, 2.5 mM MgCl2, 10 mM NaHCO3, and 0.1% human serum albumin, and were then subjected to the two-step procedure for determining NADPH-dependent H2O2 generation described above.

Partial Purification of Insulin Receptors—The purification of insulin receptors was performed as described by Katota (26). Plasma membranes were solubilized in 25 mM HEPES, pH 7.6, containing 4 mM EDTA, 4 mM EGTA, 1% Triton X-100, 0.2 mM phenylmethylylsulfonyl fluoride, 1 unit/ml aprotinin, and 2 µM leupeptin. After centrifugation, the supernatant was applied to a column of wheat germ agglutinin (WGA) coupled to agarose. Bound material was eluted with 0.3 M acetyl-D-glucosamine in 25 mM HEPES pH 7.6, containing 0.1% Triton X-100 and 0.2% 2-ME. Bound insulin receptors were identified by immunoblotting.

Western Blot Analyses—Proteins resolved by 11% SDS-PAGE were transferred to Hybond PVDF membranes. The membranes were blocked for 2 h with 5% polycrylamide-gelatin and then were exposed for 12–14 h at 4 °C to polyclonal antibodies raised against decapeptide sequences of Gαi2 and Gβi2, or against the subunit of insulin receptor. Western blotting was done at room temperature for 1 h with 0.1% Tween 20 (TBS-T buffer) and then incubated for 1 h at room temperature with peroxidase-conjugated anti-rabbit IgG (Dianova; 1:20,000) in TBS-T buffer with 1% Tween 20. Visualization was accomplished by enhanced chemiluminescence (Amersham). Immunoaffinity Chromatography—Immunoadfinity columns were prepared using CNBr-activated Sepharose (Pharmacia Biotech Inc.) and polyclonal antibodies (1-20) raised against a peptide corresponding to amino acids 93–112 of Gαi2 or directed against the β-subunit of insulin receptor, respectively. Antibodies were dialyzed against coupling buffer, pH 8.9 (0.1 M NaHCO3 containing 0.5 M NaCl), added to the gel, and incubated for 4 h at room temperature. After excess antibodies were removed with coupling buffer, the remaining active groups were blocked with 0.75 mM ethanamine, pH 8.0, for 3 h; and the gel was washed with 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl. Solubilized membrane proteins were applied to 1.6 ml of the immunoaffinity matrix in 20 mM Tris, pH 7.4, containing 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.4 mM phenylmethylsulfonyl fluoride, 10 µg/ml Aprotinin, 1% Nonidet-P-40 and 2% Triton X-100. After 12–14 h, columns were washed with 10 column volumes of the same buffer. Gαi2 or IRb was associated with proteins eluted with 0.1 M glycin buffer, pH 2.75, containing 0.1 M NaCl.

Reconstitution with Gαi2 Subunits—Purified or recombinant Gαi2 was activated by incubation with 30 mM MOPS, pH 7.5, containing 120 mM NaCl, 1.4 mM CaCl2, 2.5 mM MgCl2, 0.1% human serum albumin, and 500 µM GTPγS at room temperature for 60 min (27). GDP-ligated Gαi2 was prepared under identical conditions except that GTPγS was replaced by 500 µM GDP.

To determine the effects of activated Gαi2 subunits on NADPH-dependent H2O2 generation, 60–70 µg of membrane protein was incubated with 0.5 µM nucleotide-ligated G protein α-subunits for 25 min at 25 °C in a total volume of 400 µl without prior removal of free guanine nucleotides. Controls contained 25 µM GTPγS or GDP, respectively, corresponding to the concentrations of free nucleotides carried over by the addition of Gαi2-GTPγS or Gαi2-GDP. Incubations were terminated by washing followed by determination of NADPH-dependent H2O2 generation as described above.

**RESULTS**

Effects of Stable Guanine Nucleotide Analogues—As reported previously and shown in Table I (2, 4), the stimulatory effect of insulin on NADPH-dependent H2O2 generation was critically
dependent on supraphysiological concentrations of manganese. In the absence of Mn\(^{2+}\), insulin increased NADPH-dependent H\(_2\)O\(_2\) generation by approximately 10%, whereas a 2-fold increase was seen in the presence of the divalent cation. As pointed out previously and discussed below, divalent cations, such as manganese, have a variety of biological effects involving changes in G protein function (28–30). To explore the possibility that a G protein regulated NADPH-dependent H\(_2\)O\(_2\) generation, we evaluated whether the stimulatory effect produced by insulin in the presence of 3 mM Mn\(^{2+}\) could be inhibited by GDP\(\beta\)S. At a concentration of 100 \(\mu\)M, the GDP-analogue suppressed the basal rate of H\(_2\)O\(_2\) generation by approximately one-third. Concomitantly, insulin’s stimulatory action was almost completely reversed, suggesting that receptor-mediated activation of NADPH-dependent H\(_2\)O\(_2\) generation was in fact mediated by a G protein (Table I). Consistently, manganese, which is active at millimolar concentrations only, could be replaced by micromolar concentrations of GTP\(\gamma\)S (Table I, Fig. 1).

Fig. 1 shows dose-response curves for GTP\(\gamma\)S in the absence and presence of insulin (5 nM). GTP\(\gamma\)S caused a concentration-dependent enhancement of NADPH-dependent H\(_2\)O\(_2\) generation, which at maximum concentrations amounted to an increase of about 40% above basal levels. Half-maximal effects were observed at approximately 10 \(\mu\)M GTP\(\gamma\)S. Insulin stimulated NADPH-dependent H\(_2\)O\(_2\) generation by increasing the sensitivity and maximal responsiveness to activation by the nonhydrolyzable GTP analogue. This resulted in a more than 10-fold shift in the apparent affinity; the maximal response to the GTP analogue was markedly increased.

Fig. 2 shows activity profiles produced by increasing concentrations of insulin in the simultaneous presence of 3 mM Mn\(^{2+}\) or 10 \(\mu\)M GTP\(\gamma\)S, respectively. The dose-response curves were superimposable over the whole range of insulin concentrations tested, suggesting that identical mechanisms of signal transmission were involved under both conditions. As noted in a previous publication (3), insulin was extremely potent in stimulating NADPH-dependent H\(_2\)O\(_2\) generation. Significant increases in NADPH-dependent H\(_2\)O\(_2\) generation were observed at 1 \(\mu\)M, and the maximal effect occurred with about 100 \(\mu\)M insulin under both conditions, indicating that insulin acted via its cognate receptor.

**Effects of Bacterial Toxins**—To characterize further the G proteins that transduce insulin’s stimulatory effect on NADPH-dependent H\(_2\)O\(_2\) generation, the effects of pretreating membranes with the A protomers of PTX and CTX were tested. A pretreatment of membranes with 1 \(\mu\)g/ml of the A protomer of PTX for 30 min had little effect on nonstimulated rates of NADPH-dependent H\(_2\)O\(_2\) generation. However, the stimulatory effect of insulin was impaired, regardless of whether GTP\(\gamma\)S or Mn\(^{2+}\) was used as a cofactor (Fig. 3).

Cholera toxin catalyzes the ADP-ribosylation of G\(_{\alpha}\) leading to its constitutive activation (31). The members of the G/G\(_{\alpha}\) family can also, under some conditions, be ADP-ribosylated by CTX (32–34). Regarding the requirements for CTX-catalyzed ADP-ribosylation of G proteins, two situations have emerged. G\(_{\alpha}\) can be ADP-ribosylated in the absence of ligands, whereas proteins of the G\(_{\alpha}\) class are only sensitive to CTX in the presence of their corresponding agonist-activated receptors. Indeed, when cholera toxin-catalyzed ADP-ribosylation was performed in the presence of 0.1 mM GTP without insulin, radioactivity was incorporated into two proteins of \(\approx 43\) and 45 kDa, which could be shown by immunoblotting to represent the short and long form of G\(_{\alpha}\). In the absence of exogenously added guanine nucleotides, radiolabel was also incorporated into a polypeptide of 40 kDa, resembling G\(_{\alpha}\) on Western blots (Fig. 6). The addition of insulin enhanced the cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide in a concentration-dependent manner but did not affect the incorporation of radioactivity into either the 43- or 45-kDa bands (Fig. 6).

Concomitantly, a pretreatment of membranes with CTX alone caused a small decrease in basal activity but had no influence on insulin-stimulated rates of NADPH-dependent...
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**Fig. 3.** Effects of pertussis toxin on insulin-stimulated rates of NADPH-dependent \( \text{H}_2\text{O}_2 \) generation. Membranes were preincubated in the absence or presence of 2 \( \mu \text{g/ml} \) pertussis toxin A-protomer for 45 min. After washing, membranes were incubated for 5 min with 3 mM MnCl\(_2\), 10 mM GTP-S, or both either alone or in combination with 5 nM insulin, as indicated. Insulin-induced activation of NADPH-dependent \( \text{H}_2\text{O}_2 \) generation was determined after washing as described under “Methods.” Values are means ± S.D. of 6–10 paired experiments carried out with different membrane preparations. The stimulatory effect of insulin is abolished by PTX regardless of whether GTP-S or Mn\(^{2+}\) is used as a cofactor. * and **, significantly different from corresponding controls (*, \( p \leq 0.001; \) **, \( p \leq 0.01; \) Student’s \( t \) test for paired observations).

**Fig. 4.** Pertussis toxin-catalyzed ADP-ribosylation of \( \text{G}_i \) and the resulting suppression of insulin action are prevented by MnCl\(_2\). Membranes were incubated in the presence and absence of 3 mM MnCl\(_2\), as indicated. After 15 min, pertussis toxin A-protomer (2 \( \mu \text{g/ml} \)) was added, and membranes were incubated for another 30 min at 37°C. Panel A shows the effect of MnCl\(_2\) on ADP-ribosylation of human fat cell membrane proteins by pertussis toxin. The samples were subjected to SDS-PAGE, and autoradiography was performed as described under “Methods.” Panel B shows that the PTX-induced suppression of insulin’s stimulatory action was prevented by MnCl\(_2\). Membranes were activated by 5 nM insulin in the presence of 10 mM GTP-S, followed by washing and determination of NADPH-dependent \( \text{H}_2\text{O}_2 \) generation as described under “Methods” and in the legend to Fig. 1. Values are means ± S.D. of three separate experiments. *, significantly different from values obtained in membranes treated with PTX alone \( (p \leq 0.05; \) Student’s \( t \) test for paired observations).

**Fig. 5.** Inhibition of pertussis toxin-induced ADP-ribosylation by insulin. Fat cell plasma membranes were exposed to pertussis toxin in the absence or presence of 100 nM insulin and subjected to SDS-PAGE and autoradiography as described under “Methods.” A, representative autoradiograph of pertussis toxin-catalyzed ADP-ribosylation in the presence and absence of insulin. B, densitometric analyses of PTX labeling of \( \text{G}_i \) in the absence (control) or presence of insulin \( (10^{-7} \text{M}) \). Values are means ± S.D. of three separate experiments. *, significantly different from values obtained in membranes treated with PTX alone \( (p \leq 0.05; \) Student’s \( t \) test for paired observations).

**Fig. 6.** Enhancement of cholera toxin labeling of \( \text{G}_i \) by insulin. Membranes were incubated with 50 \( \mu \text{g/ml} \) of cholera toxin A-subunit and \( ^3\text{H}-\text{NAD} \) (37°C, 45 min) either alone (control) or in combination with various concentrations of insulin in the absence of exogenously supplied guanine nucleotides. Subsequently, membranes were solubilized and subjected to SDS-PAGE and autoradiography as described under “Methods.” A, insulin 100 nM caused a selective increase in cholera toxin labeling of \( \text{G}_i \) without affecting ADP-ribosylation of \( \text{G}_i \) in the absence of GTP. B, densitometric analysis of cholera toxin labeling of \( \text{G}_i \) in the absence and presence of two different concentrations of insulin. Values are means ± S.D. of three separate experiments. *, significantly different from values obtained in membranes treated with CTX alone \( (p \leq 0.05; \) Student’s \( t \) test for paired observations).

The stimulatory effect of insulin on NADPH-dependent \( \text{H}_2\text{O}_2 \) generation was not affected when membranes were pretreated for 45 min with 10 nM insulin alone. However, when insulin was applied together with CTX in the absence of exogenous GTP, the subsequent response to a maximal concentration of insulin was reduced from 100% above basal levels to approximately 15% (Fig. 7). Effects of COOH-terminal Antibodies and the Corresponding Peptides—The identity of the G protein(s) involved in insulin receptor-NADPH oxidase coupling was further probed with antibodies against the carboxyl-terminal region of G protein \( \alpha \)-subunits and synthetic peptides corresponding to specific regions of the carboxyl termini of \( \text{G}_a \). These antibodies and peptides have been shown to prevent G protein interaction with various receptors (35, 36). Membranes were preincubated on ice for 1 h with various dilutions of anti-\( \text{G}_{\alpha_1} \), anti-\( \text{G}_{\alpha_2-3} \), or anti-\( \text{G}_{\alpha_6} \), respectively. At the concentrations used, none of the antisera or peptides had a significant effect on basal rates of NADPH-dependent \( \text{H}_2\text{O}_2 \) generation. The stimulatory effect of insulin was selectively blocked by antibodies to \( \text{G}_{\alpha_1-2} \), regardless of whether insulin action was assessed in the presence of 10 \( \mu \text{M} \) GTP-S or 3 mM Mn\(^{2+}\). Antibodies directed toward \( \text{G}_{\alpha_3} \) or \( \text{G}_{\alpha_6} \) were without effect (Table II). Consistently, the COOH-
FIG. 7. Insulin action is impaired upon exposure of membranes to 100 nM insulin and 50 μg/ml cholera toxin but remains unaffected by a pretreatment with either agent alone. Membranes were first exposed to 50 μg/ml of cholera toxin A-subunit (37 °C, 45 min) in the absence or presence of 100 nM insulin, washed, and activated by 5 nM insulin and 10 μM GTP-S for 25 min. Activated membranes were washed again and assayed for NADPH-dependent H2O2 generation as described under “Methods.” Values are means ± S.D. of six experiments. *, significantly different from controls (p ≤ 0.01; Student’s t test for paired observations).

TABLE II

Selective inhibition of insulin-stimulated H2O2 generation by COOH-terminal anti-Gα1-2

Membranes were preincubated with antibodies (dilution 1:1000) directed against the carboxyl termini of G protein α-subunits for 1 h on ice and subsequently activated by 5 nM insulin in the simultaneous presence of 10 μM GTP-S or 3 mM MnCl2, respectively. Incubations were terminated by washing, and membranes were assayed for NADPH-dependent H2O2 generation, as described under “Methods.” Values are means ± S.D. of three separate experiments.

| Antibody | Basal | Insulin | Insulin, GTP-S | Insulin, MnCl2 |
|----------|-------|---------|----------------|----------------|
| None     | 27 ± 5| 57 ± 10 | 54 ± 3         |                |
| Anti-Gα1-2 | 28 ± 4| 30 ± 5* | 35 ± 5*        |                |
| Anti-Gα3 | 33 ± 6| 58 ± 7  |                |                |
| Anti-Gα2 | 30 ± 4| 65 ± 11 |                |                |

* Significantly different from corresponding controls (p ≤ 0.01; Student’s t test).

terminal peptide from Gaα3, at concentrations up to 20 μM, failed to affect the stimulatory effect of insulin on NADPH-dependent H2O2 generation. In contrast, the Gaα2 peptide, at a concentration of 20 μM, inhibited insulin’s stimulatory effect by approximately 80% (Table III).

Collectively, the experiments using bacterial toxins as well as antibodies against the COOH-terminal sequences of Gaα or the corresponding peptides demonstrated that insulin’s stimulatory action on NADPH-dependent H2O2 generation was transduced via Gα1-2 and strongly supported the view that the insulin receptor interacted with Gα1, Gα2, or both of these proteins directly.

Physical Association of Gaα2 and the Insulin Receptor—Since the insulin receptor and the G protein transducing the insulin signal to NADPH oxidase seemed to interact directly, the association of G proteins with the insulin receptor was investigated. Fig. 8 shows the relative distribution of Gaα and G proteins in fat cell plasma membranes and partially purified insulin receptor preparations using antibodies directed toward internal sequences of Gaα1 and Gaα2, as well as COOH-terminal anti-Gaα3 and anti-Gα. Human fat cell membranes contained Gα and the three isoforms of Ga, a finding that is in general agreement with a previous report showing that human adipocytes contain Gα1, Gα2, and low amounts of Gα3 (37). Interestingly, Gα2 was selectively enriched in partially purified insulin receptor preparations. Only Gaα1, Gaα2, and Gβγ were recovered after WGA chromatography, whereas neither Gaα3 nor Gα3 could be detected. Essentially the same results were obtained by immunoaffinity chromatography on Sepharose coupled to antibodies directed against the β subunit of the insulin receptor (Fig. 9). Together these findings suggested that the insulin receptor may be associated with heterotrimeric Gα2 (and Gα3). Consistently, the Gα2 present in insulin receptor fractions could be labeled by pertussis toxin, which ribosylates heterotrimeric Gα only (Fig. 10). A treatment of lectin-purified insulin receptors with 100 nM insulin for 15 min resulted in a dramatic decrease in PTX labeling (>90%), indicating that virtually all Gα2 recovered after WGA chromatography was functionally associated with the insulin receptor (Fig. 10).
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**FIG. 10.** Inhibition of pertussis toxin-induced ADP-ribosylation by insulin in partially purified insulin receptor preparations.**Partially purified insulin receptor complexes were solubilized in acetyl-d-glucosamine eluates after adsorption to WGA were exposed to pertussis toxin and 10 \( \mu \)M GDP in the absence or presence of 100 nM insulin for 30 min and subjected to SDS-PAGE and autoradiography as described under "Methods."** The experiment was reproduced three times using different membrane preparations.

**FIG. 11.** Co-immunoadsorption of IR\( \beta \) and \( \text{Go}_{\alpha} \) by anti-IR\( \beta \)-Sepharose.**Plasma membranes were solubilized and the lysates were subjected to immunoadsorption chromatography on Sepharose coupled to anti-IR\( \beta \) as described under "Methods."** The experiment was reproduced three times using different membrane preparations.

**DISCUSSION**

The Stimulatory Effect of Insulin on NADPH-dependent \( \text{H}_2\text{O}_2 \) generation was critically dependent on millimolar concentrations of manganese but occurred in the absence of exog-
Mn$^{2+}$, can convert heterotrimeric G proteins to forms that cannot be ADP-ribosylated by bacterial toxin, indicating that the receptors for insulin and IGF-1 utilize G proteins to mediate their stimulatory effects on NADPH-dependent H$_2$O$_2$ generation. Values are means ± S.E. of 3–6 experiments, as indicated in the right-hand column.

### TABLE IV

**Stimulation of NADPH-dependent H$_2$O$_2$ generation by recombinant and purified G protein subunits**

Membranes (60–80 μg) were incubated with nucleotide-ligated purified (0.3 μM) or recombinant (0.5 μM) G protein subunits (rGαi) as well as Gβγ (0.2 μM) in the absence or presence of 5 nm insulin (Ins), as indicated. Controls contained 25 μM free GTPγS or GDP corresponding to the concentrations of free nucleotides carried over by the addition of Gα2v-GTPγS or Gα2v-GDP. Incubations were terminated by washing. Therefore, neither free guanine nucleotides, buffer components, nor detergent was carried over into the assay for determination of NADPH-dependent H$_2$O$_2$ generation. Values are means ± S.E. of 3–6 experiments, as indicated in the right-hand column.

| Additions | H$_2$O$_2$ generation (nmol min$^{-1}$ mg$^{-1}$) |
|-----------|-----------------------------------------------|
| GDP       | 30 ± 7                                        |
| GTPγS     | 36 ± 6                                        |
| Ins, GTPγS| 65 ± 8                                        |
| Brain Gαv-GTPγS | 73 ± 8                                    |
| Brain Gαv-GDP | 33 ± 5                                    |
| rGα1-GTPγS | 80 ± 5                                       |
| rGα2v-GTPγS | 77 ± 9                                       |
| rGα2v-GTPγS | 53 ± 5                                       |
| rGβγ-GTPγS | 32 ± 7                                       |
| rGα1-GTPγS | 33 ± 7                                       |
| Ins, GTPγS, Gβγ | 29 ± 5                                    |
| Ins, GTPγS, rGα2v-GDP | 60 ± 7                                     |

* Significantly different from controls (p ≤ 0.01; Student’s t test).

Concentrations of Mn$^{2+}$ are also essential for a ligand-induced activation of receptor protein-tyrosine kinases in cell-free preparations (29). Thus, the Mn$^{2+}$ requirement seemed to provide further evidence in support of the widely held view that the receptor kinase activity is essential for most, if not all, of insulin’s biological effects (1). However, the mechanism(s) by which insulin activated NADPH-dependent H$_2$O$_2$ generation were independent of ATP, indicating that an alternative pathway bypassing the receptor kinase had been activated (2). As pointed out in the introduction, one potential pathway could be via G proteins. Indeed, divalent cations, such as Mn$^{2+}$, are known to potentiate the effects of GTP in some systems (28–30), and this could explain why no exogenous GTP was required in the presence of Mn$^{2+}$. Accordingly, several independent lines of evidence indicated that the stimulatory effects of insulin on NADPH-dependent H$_2$O$_2$ generation were in fact mediated via G proteins. First, the increase in NADPH-dependent H$_2$O$_2$ generation induced by insulin in the presence of Mn$^{2+}$ was inhibited by GDPβS, a specific antagonist of GTP at G proteins. Second, the stimulatory effect of insulin seen in the presence of Mn$^{2+}$ was prevented by pretreating the membranes with pertussis toxin, consistent with several reports suggesting that a pertussis toxin substrate may contribute to insulin receptor signaling (6–8). Conversely, a prior treatment with 3 mM Mn$^{2+}$ prevented the PTX-induced ADP-ribosylation of G$i$. Concomitantly, the bacterial toxin lost its ability to block insulin’s stimulatory effect on NADPH-dependent H$_2$O$_2$ generation (3). The latter observations confirm early reports, demonstrating that divalent cations, such as Mn$^{2+}$, can convert heterotrimeric G proteins to forms that cannot be ADP-ribosylated by bacterial toxins (28) and provide additional evidence in support of the view that Mn$^{2+}$ action involved a change in G protein function. Third, studies using antibodies directed against the carboxyl terminus of the α-subunits of different heterotrimeric G proteins and synthetic peptides corresponding to specific regions of the carboxyl termini of Gα showed that the insulin-induced increase of NADPH-dependent H$_2$O$_2$ generation was mediated via Gα$_{1-2}$ or a related protein. Finally, manganese, which is active at millimolar concentrations, could be replaced by micromolar concentrations of GTPγS. The synergistic activation of NADPH-dependent H$_2$O$_2$ generation by insulin and GTPγS was also blocked by GDPβS, pertussis toxin, and anti-Gα$_{1-2}$ or the corresponding peptide, indicating that insulin activated the same G protein, regardless of whether Mn$^{2+}$ or GTPγS was used as a cofactor.

Overall, it thus appeared that the involvement of Gα$_{1-2}$ in insulin receptor signaling had been masked by Mn$^{2+}$ in previous studies (2–4), possibly because the divalent cation potentiated the effect of endogenous GTP. This observation has important practical implications. As pointed out above, investigations into the functions of receptor protein-tyrosine kinases are routinely carried out in the presence of millimolar concentrations of manganese, because this cation is thought to be essential for a ligand-induced activation of their catalytic activity in cell-free preparations. Manganese is not the sole compound routinely present in receptor kinase assays that tends to obscure an involvement of G proteins in insulin receptor signaling. Another example of this type is the phosphatase inhibitor, vanadate, which dissociates heterotrimeric G proteins and prevents pertussis toxin labeling of G$i_0$ (6). Thus, one of the main obstacles in elucidating the role of G proteins in signaling by receptor kinases may reside in the fact that the conditions thought to be optimal for assessing phosphorylation-dependent signaling events are inappropriate for investigating G protein-dependent pathways of signal transduction in cell-free preparations.

**Specificity of Insulin Action—**Insulin, insulin-like growth factor (IGF)-1, and IGF-2 have distinct cell surface receptors, each of which can bind insulin, IGF-1, and IGF-2 with varying affinity (39). The receptors for insulin and IGF-1 are both αββ$_2$-heterotetramers and have approximately 60% sequence identity; the receptor for IGF-2 consists of a single membrane-spanning polypeptide that lacks intrinsic tyrosine kinase activity. Type I IGF receptors are not detectable in rat adipocytes (40). However, there is indirect evidence that they may be present in human adipocytes (41). Indeed, using antibodies against the β-subunit of the type I IGF receptor, we were able to show that this protein is in fact present in human fat cell membranes. However, it is unlikely that IGF-1 receptors mediate insulin’s stimulatory effect on NADPH-dependent H$_2$O$_2$ generation for two reasons. First, the stimulatory effect of insulin was observed at picomolar concentrations, which would cause negligible cross-reactivity with IGF-1 receptors. Second, although IGF-1 is another stimulator of NADPH-dependent H$_2$O$_2$ generation (3), its effect was not inhibited by pertussis toxin, indicating that the receptors for insulin and IGF-1 utilized different pathways of receptor signaling.

**The Insulin Receptor Interacts with G$_i$ Directly—**Heterotrimeric G proteins are typically coupled to seven-helix receptors. However, it is becoming increasingly clear that G proteins may be responsible for transducing signals of other types of receptors as well, including receptor protein-tyrosine kinases (42, 44–46). The present findings confirm and extend previous observations suggesting that the insulin receptor is an example of a non-seven-transmembrane receptor that appears to be associated with multiple G proteins, including G$i$ (20).

The association with G$i$ was detectable after immunoprecipitation with antibodies directed against Gαi or IRβ and survived wheat germ agglutinin chromatography as well as immunoaffinity chromatography on Sepharose coupled to anti-Gαi or anti-IRβ, respectively. Partially purified insulin receptor preparations contained only Gα$_{12}$ and Gβγ (but not Gα$_{16}$ or Gα$_{63}$), indicating that the insulin receptor was associated with the Gα$_{12}$ heterotrimer.

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2. H. I. Krieger-Brauer and H. Kather, unpublished results.
The functional nature of the insulin receptor-G\textsubscript{i2} complex is made evident by the GTP-dependence of insulin's stimulatory effect on NADPH-dependent \textsubscript{H}_2\textsubscript{O}_2 generation and by its ability to modulate the ADP-ribosylation of Ga\textsubscript{a}/Go by bacterial toxins. Insulin reduced the PTX-induced ADP-ribosylation of total G\textsubscript{i}/Go present in crude plasma membranes by approximately one-third, indicating that the insulin receptor caused a conformational change of a major fraction of plasma membrane-bound G\textsubscript{i}/Go upon ligand binding (38). Accordingly, pertussis toxin labeling of G\textsubscript{i} was virtually abolished in WGA extracts of insulin-treated membranes. The latter finding demonstrated that nearly all G\textsubscript{i} recovered in WGA extracts is functionally associated with the insulin receptor. It has been reported that G\textsubscript{i} comprises approximately half of total G\textsubscript{i}/Go present in human adipocyte plasma membranes (37). Given that insulin inhibited PTX labeling of total plasma membrane-bound G\textsubscript{i}/Go by one-third and considering that the insulin receptor selectively interacts with G\textsubscript{i2}, it thus appears that the ligand-occupied insulin receptor communicates with at least two-thirds of the total G\textsubscript{i2} present in human fat cell membranes, suggesting that this alternative pathway may be as important for insulin receptor signaling as its intrinsic tyrosine kinase activity.

Another strategy for confirming the functional nature of the insulin receptor-G\textsubscript{i2} complex was based on the ability of cholera toxin to ADP-ribosylate G\textsubscript{a} activated by a ligand-occupied receptor in the absence of GTP (32–34). As yet, a ligand-induced ADP-ribosylation of G\textsubscript{a} has only been demonstrated for ligands of members of the superfamily of heptahelical receptors. In this paper we show that the addition of insulin, a ligand of a tyrosine kinase receptor, to membranes that are maintained in the absence of GTP stimulated the CTX-dependent ADP-ribosylation of G\textsubscript{a}. The observation that the ability of mediating a ligand-induced ADP-ribosylation of G\textsubscript{a} by cholera toxin is not restricted to heptahelical receptors but is shared by at least one receptor protein-tyrosine kinase strongly suggested that both types of receptors activated G\textsubscript{a} via similar mechanisms. Indeed, as pointed out previously (2–4) and shown herein, the stimulatory effect of insulin occurred in the absence of ATP, indicating that the interaction between G\textsubscript{a} and the insulin receptor took place via a noncovalent mechanism that may be similar to that used by heptahelical receptors.

**NADPH Oxidase Is an Effector for Ga**—Current knowledge suggests that activation of heterotrimeric G proteins by ligand-receptor complexes is achieved by exchange of GDP for GTP on the \(\alpha\)-subunit, and this is thought to facilitate dissociation into \(\alpha\)-and \(\beta\gamma\)-subunits (47). G protein-sensitive effectors are then directly regulated by GTP-ligated \(\alpha\)-subunits, \(\beta\gamma\)-subunits, or both (48). Accordingly, the mechanism(s) by which insulin stimulated NADPH-dependent \textsubscript{H}_2\textsubscript{O}_2 generation was membrane-delimited and independent of soluble second messengers, making it likely that activated G\textsubscript{a} acted upon NADPH oxidase directly, although indirect mechanisms of action involving intermediate membrane-associated effectors cannot entirely be ruled out.

Recently, evidence has been presented to suggest that stimulation of the MAP kinase pathway by IGF-1 receptor requires the participation of \(\beta\gamma\)-subunits derived from PTX-sensitive G proteins (49). Because the receptors for insulin and IGF-1 are closely related, it appeared possible that the insulin-induced increase in NADPH-dependent \textsubscript{H}_2\textsubscript{O}_2 generation was mediated by \(\beta\gamma\)-subunits derived from G\textsubscript{a}. However, insulin's stimulatory effect was mimicked by activated Ga subunits, whereas \(\beta\gamma\)-subunits suppressed insulin-activated rates of NADPH-dependent \textsubscript{H}_2\textsubscript{O}_2 generation. G\textsubscript{a2} appeared to cause a greater activation than G\textsubscript{a11} or G\textsubscript{a13}, and G\textsubscript{a} was without effect, suggesting specificity in the interaction with the effector NADPH oxidase, but detailed titration studies will be required to determine the relative potency and efficacy of various PTX-sensitive Ga subunits. Identification of unambiguous effectors for Ga subunits has been difficult (50). The present findings implicate NADPH oxidase as an effector system for activated PTX-sensitive Ga proteins, fulfilling most of the criteria utilized to establish adenylate cyclase as an effector for Ga.

There is precedent for an involvement of G proteins in regulating plasma membrane-bound redox activities. The activation of NADPH oxidases of endothelia and professional phagocytes can be triggered by peptides binding to G protein-coupled receptors (51). In parietal cells, the opening-closing behavior of a housekeeping Cl\textsuperscript{−} channel is controlled by superoxide production mediated by a PTX-sensitive GTP-binding protein (52). Finally, intestinal smooth muscle cells appear to possess a hormone-sensitive NO synthetase that is localized to the plasma membrane and is coupled to G\textsubscript{1–2} (53). Thus, a scheme emerges in which G protein-coupled redox systems activated by hormones or cytokines and sensitive to pertussis toxin are responsible for the generation of reactive oxygen species at the plasma membrane.

**NADPH Oxidase Is the First Example of an Effector System Coupled to the Insulin Receptor via a Heterotrimeric G Protein**—As pointed out in the introduction, an apparent association of insulin receptors with G proteins has been supported by several indirect studies, and most recent evidence indicated that Ga\textsubscript{a} is a critical regulator of insulin action in vivo (54). However, despite intensive efforts, an effector system that is regulated by the ligand-occupied insulin receptor via the intermediacy of a G protein has not yet been identified. It has been proposed that adenylate cyclase or glycosyl-phosphatidylinositol-specific phospholipase C may be coupled to the insulin receptor via Ga or a related protein (55, 56). However, recent work has indicated that insulin's regulatory effects on these latter systems are a consequence of its stimulatory action on protein phosphatase activity and glucose metabolism, respectively (57, 58). Thus, the stimulus-sensitive NADPH oxidase of human fat cells is the first example of an effector system that is coupled to the insulin receptor via a heterotrimeric G protein.

Previous work demonstrated that the stimulus-sensitive \textsubscript{H}_2\textsubscript{O}_2-generating system that is present in human fat cell plasma membranes meets important criteria of a universal effector system for hormones and cytokines (2–4). By demonstrating that the stimulatory effect of insulin on NADPH-dependent \textsubscript{H}_2\textsubscript{O}_2 generation is transmitted via Ga\textsubscript{a2}, the present findings provide further evidence in support of the idea that \textsubscript{H}_2\textsubscript{O}_2 has a second messenger function. Most recent work using transgenic mice harboring RNA antisense to the gene for Ga\textsubscript{a2} has suggested that Ga\textsubscript{a2} may be critical for insulin action (54), and evidence has been presented to suggest that the levels and functions of G\textsubscript{a} proteins may be altered in diabetes (8, 43). Together with these latter observations, the current findings argue for a central role of NADPH-dependent \textsubscript{H}_2\textsubscript{O}_2 generation in insulin receptor signaling.

In conclusion, previous studies showed that the mechanism(s) by which insulin stimulated NADPH-dependent \textsubscript{H}_2\textsubscript{O}_2 generation were membrane-delimited and independent of the receptor kinase. The present findings confirmed that this alternative pathway of insulin receptor signaling exists and demonstrated that the transduction of insulin's signal to NADPH oxidase takes place via Ga\textsubscript{a2}, whose participation had been obscured in former studies because NADPH oxidase was assayed in the presence of manganese, which is generally believed to be essential for a ligand-induced activation of receptor protein-tyrosine kinases in cell-free preparations. Thus, NADPH oxidase joins an expanding list of effector molecules.
that are influenced, possibly directly, by heterotrimeric G proteins and is the first example of an effector system that is coupled to the insulin receptor via a heterotrimeric G protein.

Acknowledgments—We are indebted to Brigitte Sattel for expert technical assistance, and we thank Dr. Y. J. Ohisalo (University of Helsinki, Finland) for supplying antibodies against Go-subunits and Dr. G. Schultz (Freie Universität Berlin) for supplying purified G protein subunits.

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Insulin-induced Activation of NADPH-dependent H$_2$O$_2$ Generation in Human Adipocyte Plasma Membranes Is Mediated by G $\alpha_\text{i2}$

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J. Biol. Chem. 1997, 272:10135-10143.
doi: 10.1074/jbc.272.15.10135

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