Inhibitory Effect of Isoproterenol on NADPH-dependent H$_2$O$_2$ Generation in Human Adipocyte Plasma Membranes Is Mediated by $\beta\gamma$-Subunits Derived from G$_s^*$

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Previous studies revealed that human fat cell plasma membranes contain a multireceptor-linked H$_2$O$_2$-generating system that is under antagonistic control by hormones and cytokines and is stimulated by insulin via G$_{\alpha_\text{ag}}$. In this report, it is shown that the inhibitory action of the $\beta$-adrenergic agonist isoproterenol is mediated by G protein $\beta\gamma$-subunits, based on observations that its action was specifically reversed by anti-$\alpha$-antagonists or by a C-terminal $\beta$-adrenergic receptor kinase-1 fragment containing the G$_{\beta\gamma}$-binding site of the enzyme, and was mimicked by exogenously supplied G protein $\beta\gamma$-subunits. Isoproterenol signals through a prototypical G$_s$-coupled receptor. Consistent with these results, direct activation of G$_s$, by cholera toxin or by an anti-G$_{\alpha}$ antibody exhibiting $\beta\gamma$-adrenergic receptor-mimetic proper-

There is growing evidence that redox-active biomolecules play important roles in signaling by hormones and cytokines (1–5). Ligand-induced changes in cellular redox status modulate tyrosine phosphorylation-dependent pathways of signal transduction; alter DNA binding and transactivation activities of many transcriptional activators; and may influence key steps in the synthesis, degradation, and action of cAMP and cGMP as well (2, 3). Previous work showed that human fat cells possess a plasma membrane-bound H$_2$O$_2$-generating system that is under antagonistic control by a large and diverse group of hormones and cytokines, including insulin, the $\beta$-adrenergic agonist isoproterenol, and different isoforms of fibroblast and platelet-derived growth factors (4–7). The mechanisms by which hormones and cytokines regulated NADPH-dependent H$_2$O$_2$ generation were confined to the plasma membrane, operating in the absence of ATP, and were independent of soluble second messengers, indicating that established pathways of signal transduction were not involved. These findings placed human fat cell oxidase in a position comparable with adenyl cyclase and suggested a physical interaction between receptors and NADPH oxidase or receptor-effector coupling via signal-transducing protein(s). Indeed, recent work revealed that the stimulatory effect of insulin on NADPH-dependent H$_2$O$_2$ generation is transduced via G$_{\alpha_m}$ (7).

In this study, we have investigated whether the effects of the $\beta$-adrenergic agonist isoproterenol are also mediated by heterotrimeric G protein(s). Using specific antibodies against the $\alpha$- and $\beta\gamma$-subunits of heterotrimeric G proteins and a peptide that specifically binds G$_{\beta\gamma}$, it is shown that the inhibitory effects of isoproterenol on NADPH-dependent H$_2$O$_2$ generation are mediated by $\beta\gamma$-subunits. Taking advantage of the unique properties of a commercially available antibody directed against residues 100–119 within the $\alpha$-helical domain of G$_s$ (K-20) and of the peptide corresponding to its target sequence, which are summarized in a recent publication (8), it is shown that the $\beta\gamma$-subunits mediating the inhibitory action of isoproterenol were derived from G$_s$.

**EXPERIMENTAL PROCEDURES**

**Materials**

The characteristics and sources of the antibodies and G$_s$-derived peptides used in these experiments are listed in Table I. Cholera toxin subunit $\alpha$, recombinant G$_{\alpha_m}$, and protein $\alpha$-agarose were from Calbiochem. Hybond polyvinylidene difluoride membranes were obtained from Amersham Pharmacia Biotech (Braunschweig, Germany). Insulin, GTP$\gamma$S, GDP$\beta$S, and GDP were from Roche Molecular Biochemicals (Mannheim, Germany). Isoproterenol was from Sigma (München, Germany). Human albumin and luminol were obtained from Behring Werke (Marburg, Germany).

Purified $\beta\gamma$-subunits and $\alpha/\alpha'_{\text{subunits}}$ from brain were kindly donated by Dr. G. Schultz (Institut für Pharmakologie, Freie Universität, Berlin, Germany) (9). The GST fusion protein containing a carboxyl-terminal fragment (residues 546–670) of $\beta$-adrenergic receptor kinase-1 (GARK1-CT/GST) and the corresponding GST protein were a gift of Dr. R. J. Lefkowitz (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC) (10).

**Methods**

**Subjects and Preparation of Fat Cells and Fat Cell Ghosts**—Experimental details have been described in detail elsewhere (4–7). Briefly, adipose tissue was from nondiabetic subjects undergoing elective abdominoal or cosmetic breast surgery. The specimens were cut into small pieces, and fat cells were isolated by the method of Rodbell (11) in a HEPES-buffered Krebs-Henseleit solution, pH 7.4, containing 20 mM HEPES, 10 mM NaHCO$_3$, 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, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 5 mM KCl, and 100 mg/liter soybean trypsin inhibitor. Cell lysis was completed by mechan-

* This work was supported by a grant from the Deutsche Forschungs-gemeinschaft, Bonn-Bad Godesberg, Federal Republic of Germany. 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.

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1 The abbreviations used are: GTP$\gamma$S, guanosine 5’-O-(3-thiotriphospho-
phate); GDP$\beta$S, guanosine 5’-O-(2-thiodiphosphate); GST, glutathione S-transferase; GARK1-CT, $\beta$-adrenergic receptor kinase-1 carboxyl-terminal fragment 546–670; MES, 2(N-morpholino)ethanesulfonic acid; MOPS, 3(N-morpholino)propanesulfonic acid.
ical, shaking, and fat cell ghosts were collected by low speed centrifugation (1000 × g, 4 °C, 20 min) and washed with lysing medium. These crude fat cell plasma membranes (fat cell ghosts) contained <10% of total cellular NADPH:cytochrome P430 reductase and <2% of NADPH-dependent glutamate dehydrogenase (4).

Receptor-mediated Modulation of NADPH-dependent H 2O2 Generation—A two-step procedure was used, as reported elsewhere (4–7). Plasma membranes from untreated or insulin-treated cells were first exposed to hormones and growth factors 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 hormones and growth factors for 5 min to allow receptor occupation. Thereafter, guanine nucleotides were added. 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 NaNO3, 250 μM NADPH, and 10 μM FAD for determination of NADPH-dependent H 2O2 generation. Reactions were stopped with 0.1 N HCl. H 2O2 was assayed using oxidation of luminol in the presence of peroxidase as the indicator reaction (5).

To assess the effects of anti-G protein antibodies and peptides corresponding to their target sequences on NADPH-dependent H 2O2 generation, membranes were exposed to both types of agents at 0 °C for 45 min, as indicated in the figure and table legends, and were then subjected to the two-step procedure described above. For cholera toxin A subunit antibody or a peptide corresponding to its antigenic site on adenylyl cyclase activity, membranes were exposed to the two-step procedure described above. For cholera toxin A subunit antibody or peptide, membranes were washed and assayed for NADPH-dependent H 2O2 generation as described under "Methods." Values are means ± S.D. of three separate experiments. Plasma membranes from insulin-stimulated adipocytes produced H 2O2 at a rate of 48.7 ± 3 nmol × (mg × min)−1. Isoproterenol caused a marginal decrease in the absence of exogenously supplied GTP.S at a rate of 3 nmol × (mg × min)−1, which respectively reflected the presence of trace amounts of endogenous GTP. B, dose-response curve for isoproterenol in the presence of 50 μM GTP.S. Plasma membranes were incubated with 5 mM insulin and increasing concentrations of isoproterenol (Iso) in the presence of 50 μM GTP.S for 25 min and assayed for NADPH-dependent H 2O2 generation as described under "Methods." Values are means ± S.D. of three experiments carried out with different membrane preparations.

RESULTS AND DISCUSSION

Isoproterenol Acts as an Inhibitor of NADPH-dependent H 2O2 Generation in the Presence of GTP.S—Previous work has shown that isoproterenol causes an inhibition of NADPH-dependent H 2O2 generation in human fat cell plasma membranes that can be overcome by the β-adrenergic receptor antagonist propranolol, indicating that it is mediated by a β-adrenergic receptor (5). Initial studies suggested that the regulatory effects of hormones and cytokines on NADPH-dependent H 2O2 generation may be critically dependent on supraphysiological concentrations of manganese (4–6). However, as pointed out previously (7), divalent cations such as Mn2+ may obscure an involvement of G proteins in receptor signaling. Indeed, manganese, which is active at millimolar concentrations, can be replaced by micromolar concentrations of GTP.S.

Fig. 1 illustrates the mutual interrelationship of the effects of isoproterenol and GTP.S. In the absence of exogenously supplied GTP.S, isoproterenol caused a small decrease in NADPH-dependent H 2O2 production of ~3 nmol × (mg protein × min)−1, which probably reflected the presence of trace amounts of endogenous GTP (data not shown). The synergistic effect of GTP.S became apparent at ~0.3 μM and was half-maximal at 3 μM (Fig. 1A). At a maximal concentration of GTP.S (50 μM), insulin-stimulated rates of NADPH-dependent H 2O2 generation were suppressed by two-thirds in the presence of 1 μM isoproterenol. Fig. 1B shows a concentration-response curve for isoproterenol in the presence of a maximal concentration of GTP.S (50 μM). The isoproterenol-induced inhibition of insulin-stimulated H 2O2 generation was half-maximal at ~0.3 μM and maximal at ~2 μM. Thus, it appeared that the inhibitory effects of isopro-

| Antibody | Peptide sequence | Specificity | Amino acids | Ref. or source |
|----------|------------------|------------|-------------|---------------|
| Goa (RM/1) | (C) RMRILQCELL | Goa | 385–394 | 45 |
| Goa (K-20) | KEAIITIYAMSLVPPVLEL | Goa | 100–119 | a |
| Goa (K-20) | KYSGDGQRAQDDLRMMADT | Goa | 90–109 | a |

a Santa Cruz Biotechnology.

TABLE I Antibodies and peptide sequences
Values are means assayed for NADPH oxidase activity as described under "Methods." Incubated with 5 nM insulin, 50 μM GTPγS, and 3 nM isoproterenol for 25 min at 37 °C. Incubations were terminated by washing, and membranes were assayed for NADPH-dependent H2O2 generation as described under "Methods." B, membranes were incubated with 5 nM insulin, 50 μM GTPγS, and 5 μM isoproterenol for 25 min at 37 °C in the presence of increasing concentrations of βARK1-CT (10), a β-adrenergic receptor kinase-1 fragment that includes the Gβγ-binding region of this kinase (Fig. 2B). At a concentration of 10 μM, the peptide reversed the inhibition seen in the presence of 0.5 μM isoproterenol completely, whereas the GST protein alone had no effect.

Consistent with the results summarized above, addition of Gβγ purified from bovine brain caused a concentration-dependent inhibition of insulin-stimulated NADPH-dependent H2O2 generation that was maximal at 200 nM Gβγ (Fig. 3A). Addition of 300 nM GDP-liganded α-subunits from bovine brain or recombinant Gαs resulted in complete reversal of the inhibition induced by Gβγ (Fig. 3B). Boiled Gβγ (200 μM) had no effect on NADPH-dependent H2O2 generation. Thus, three independent lines of evidence indicated that the inhibitory effects of isoproterenol on NADPH-dependent H2O2 generation were transmitted by βγ-subunits.

G Protein βγ-Subunits Mediating the β-adrenergic Inhibition of H2O2 Generation Are Derived from Gαs—Mammalian β-adrenergic receptors are prototypical examples of G protein-coupled receptors that are highly selective for Gαs, the G protein stimulatory with respect to adenyl cyclase (13), suggesting that the βγ-subunits mediating the inhibitory effects of isoproterenol on NADPH-dependent H2O2 generation might have been derived from Gαs. Antibodies directed against the C termini of Gα-subunits and peptides corresponding to their target sequences are thought to block receptor-G protein recognition and activation and are therefore widely used in functional studies for assessing receptor-G protein coupling (14–17). Anti-Gαs C terminus antibody and the peptide corresponding to its antigenic site were highly efficient in suppressing the stimulatory effect of isoproterenol on adenyl cyclase, which is transduced through Gαs (8). However, we previously showed that both compounds failed to reverse the suppression of NADPH-dependent H2O2 generation seen in the presence of isoproterenol over the whole range of concentrations tested (8). One possibility to explain this unexpected finding would be that activation of Gαs may not yield enough, or inappropriately composed, βγ-subunits to inhibit NADPH-dependent H2O2 generation, as has been proposed for the regulation of type II adenyl cyclase (18, 19).
latter contention implies that isoproterenol may be able to activate multiple G proteins, which seems in fact to be the case under certain conditions (20).

However, as pointed out elsewhere (8), the effects of anti-GαC terminus antibodies on Gβγ-mediated responses are variable and may depend on receptors, G proteins, and cell types studied (14–17). Therefore, the lack of effects of anti-GαC antibody (and of the peptide corresponding to its target sequence) does not necessarily indicate that the βγ-subunits transmitting the inhibitory effect of isoproterenol were derived from G proteins other than Gs.

Considering the variability in antibody action, the effects of cholera toxin, which directly activates Gs (21), were explored next. Indeed, a direct activation of Gs by cholera toxin resulted in a concentration-dependent inhibition of NADPH-dependent H2O2 generation. At the concentration employed in previous studies (50 μg/ml), the bacterial toxin caused a rightward shift of the dose-response curve for insulin by 2 orders of magnitude, but did not suppress the maximal response seen at 10 nM insulin (data not shown). At higher concentrations, the toxin also inhibited the response seen in the presence of 10 nM insulin (Fig. 4A). The latter effect was dose-dependent and was paralleled by a corresponding increase in Gs labeling (Fig. 4B). At the highest toxin concentration used (200 μg/ml), insulin-stimulated H2O2 production was suppressed by approximately two-thirds, suggesting that trimeric Gs contained appropriately composed G protein βγ-subunits to transduce a pronounced inhibition of human fat cell oxidase.

To provide definitive proof that the isoproterenol-induced inhibition of NADPH-dependent H2O2 generation was mediated by functionally specific Gβγ derived from Gs, we used a novel approach circumventing the shortcomings of anti-GαC terminus antibodies. As shown recently (8), a commercially available antibody recognizing residues 100–119 within the α-helical domain of Gαs (K-20) stimulates Gs in a manner similar to activated β-adrenergic receptors. This receptor-mimetic antibody was as efficacious as isoproterenol in suppressing NADPH-dependent H2O2 generation, indicating that Gs can in fact provide sufficient amounts of appropriately composed βγ-subunits to account for the inhibition of NADPH oxidase activity seen in the presence of isoproterenol (8). More important, a peptide corresponding to the target sequence of K-20 effectively antagonized the β-adrenergic inhibition of NADPH-dependent H2O2 generation, apparently through competition between the peptide and Gs for binding to activated β-adrenergic receptors (8). At a concentration of 2.5 μM, the peptide reduced the inhibitory action of a maximally effective concentration of isoproterenol by ~80%, whereas the homologous sequence of Gαa had no effect at all (data not shown). Considering the specificity of K-20 and its target sequence, the latter finding provided definitive proof that the βγ-subunits mediating the β-adrenergic inhibition of NADPH-dependent H2O2 generation were in fact mainly, if not completely, derived from Gs. Consistent with these results, activation of Gαs-coupled receptors has been shown to produce sufficient amounts of βγ-subunits to provide membrane anchorage of G protein-coupled receptor kinase (22), to activate mitogen-activated protein kinases (23, 24), or to inhibit type I adenyl cyclase (25).

Conclusions—The mechanisms by which H2O2 and other reactive oxygen species such as superoxide are generated has been extensively studied in neutrophils and macrophages (26–28). The phagocyte system, which is critical to the inflammatory response, is a multicomponent enzyme that is activated by assembling four cytosolic components (p40phox, p47phox, p67phox, and the small GTPase Rac) with the transmembrane electron carrier flavocytochrome b558 (26–28). Human fat cell oxidase differs from neutrophil oxidase in that all components required for H2O2 generation are membrane-bound. In addition, the system produces nontoxic amounts of H2O2, consistent with its potential role in signaling by hormones, growth factors, and cytokines. Despite these differences, human fat cell plasma membranes contain a spectrally detectable cytochrome b558 (4), suggesting that some of the components of human fat cell oxidase may be similar or identical to those of phagocyte oxidase, as has also been proposed for stimulus-sensitive redox activities of other non-phagocytic cells such as endothelia (29), fibroblasts (30, 31), mesangial cells (32), chondrocytes (33), vascular smooth muscle cells (34–36), and adventitial cells (37).

As pointed out previously, there is precedent for an involvement of heterotrimeric G proteins in the regulation of membrane-bound redox activities (7). The NADPH oxidases of endothelia and professional phagocytes can be triggered by peptides binding to G protein-coupled receptors (26–29). In parietal cells, the opening-closing behavior of a housekeeping Cl− channel is controlled by superoxide production mediated by a pertussis toxin-sensitive GTP-binding protein (38). Finally,
intestinal smooth muscle cells appear to possess a hormone-sensitive NO synthetase that is localized to the plasma membrane and that is coupled to GIα (39). However, human fat cell oxidase is the first example of a ligand-regulated redox system that is under antagonistic control by α- and βγ-subunits derived from different G proteins, as has also been reported for other important effector systems, including certain types of adenylyl cyclase (18, 19, 25), phospholipase C (40), and inwardly rectifying K+ channels (41).

The mechanisms by which Gαq and Gβγα modulate the activity of human fat cell oxidase remain to be defined. Current knowledge suggests that activation of heterotrimeric G proteins by ligand-receptor complexes is achieved by exchange of GDP for GTP on the α-subunit, and this is thought to facilitate dissociation into α- and βγ-subunits (42, 43). G protein-sensitive effectors are then directly regulated by GTP-ligated α-subunits, βγ-subunits, or both. Consistent with these results, the mechanisms by which insulin and isoproterenol modulate NADPH-dependent H2O2 generation were confined to the plasma membrane and are independent of soluble second messengers, making it likely that activated Gαq and Gβγα act upon NADPH oxidase directly, although indirect mechanisms of action involving intermediate membrane-associated effectors such as Rac cannot be ruled out (26, 31).

In conclusion, the present findings show that the β-adrenergic agonist isoproterenol utilizes βγ-subunits to inhibit human fat cell oxidase and illustrate that antibodies directed against the C termini of G protein α-subunits, which are often used in functional studies, are of limited value for determining their origin. The latter observation has also been made by others, but is rarely mentioned (44). Taking advantage of the serendipitous discovery that an antibody directed against residues 100–119 within the ω-helical domain of Gαs (K-20) has β-adrenergic receptor-mimetic properties, whereas the peptide corresponding to its target sequence acts as a GTP-guanine nucleotide exchange factor for GTP on the β-subunit, and Gβγγ, antagonistic control by both types of component subunits of heterotrimeric G proteins by ligand-receptor complexes is achieved by exchange of GDP for GTP on the α-subunit, and this is thought to facilitate dissociation into α- and βγ-subunits (42, 43). G protein-sensitive effectors are then directly regulated by GTP-ligated α-subunits, βγ-subunits, or both. Consistent with these results, the mechanisms by which insulin and isoproterenol modulate NADPH-dependent H2O2 generation were confined to the plasma membrane and are independent of soluble second messengers, making it likely that activated Gαq and Gβγα act upon NADPH oxidase directly, although indirect mechanisms of action involving intermediate membrane-associated effectors such as Rac cannot be ruled out (26, 31).

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