Human Fat Cells Possess a Plasma Membrane-bound H_2O_2-generating System That is Activated by Insulin via a Mechanism Bypassing the Receptor Kinase

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Abstract

Insulin caused a transient increase in H_2O_2 accumulation in human fat cell suspensions that was observed only in the presence of an inhibitor of catalase and heme-containing peroxidases, such as azide, and reached peak levels of 30 μM within 5 min. The cells contained a plasma membrane-bound NADPH oxidase, producing 1 mol H_2O_2/mol of NADPH oxidation, that was activated on exposure of intact cells to insulin at contrations that are physiologically relevant (0.1–10 nM). The hormone effect was rapid and was due to a selective increase in substrate affinity. The enzyme was magnesium dependent, required a flavine nucleotide for optimal activity, and was most active at pH 5.0–6.5. In contrast to all other hormone- or cytokine-sensitive NADPH oxidases that have been characterized in sufficient detail, the human fat cell oxidase retained its hormone responsiveness after cell disruption, and only Mn^{2+}, but no ATP, was required for a ligand-induced activation in crude plasma membranes. The results demonstrate that insulin utilizes tyrosine kinase–independent pathways for receptor signaling and strongly support the view that H_2O_2 contributes to the intracellular propagation of the insulin signal. (*J. Clin. Invest.* 1992. 89:1006–1013.) Key words: adipocytes • insulin • reactive oxygen species • signal transduction

Introduction

Insulin increases the production of H_2O_2 in rat adipocytes, and exogenously supplied H_2O_2 brings about a range of metabolic effects that are rather similar to those seen with insulin (1–3). It has therefore been proposed that H_2O_2 may act as a second messenger for insulin (1). Consistently, H_2O_2 can influence the activity of metabolic enzymes (4–6); modulates key steps in the synthesis, degradation, and action of second messengers, such as low-k_m cAMP phosphodiesterase (7), soluble guanylate cyclase (8), and protein kinase c (9), in vitro; and increases insulin receptor autophosphorylation and its phosphotransferase activity in intact adipocytes (10, 11). The latter effect may, at least in part, be due to the newly discovered inhibitory effect of H_2O_2 on phosphoprotein phosphatases (12).

In addition, most recent evidence indicates that regulation by oxidation–reduction may be a general mechanism of control of transcriptional and translational activators. For example, the bacterial transcriptional regulatory protein Oxy R and a *Drosophila* heat shock factor, which regulate the transcription of stress-inducible genes (13, 14) and nuclear factor (NF)-κB (a pleiotropic transcription factor that is present in many cells [15, 16]), are active only when oxidized, whereas the products of the protooncogenes c-*jun* and c-*fos* (17) and two translational regulators, the iron-responsive element-binding protein (18) and the adenosine-uridine binding factor, require reduced sulfhydryls for high affinity binding (19). Thus, H_2O_2 qualifies for a second-messenger function and could be involved in mediating both the short-term metabolic actions of insulin and its long-term effects on cell growth and differentiation.

Furthermore, there is precedent for an involvement of reactive oxygen species in hormone action. For NO, a role as a second messenger is now becoming widely accepted (20). Reactive oxygen species are key intermediates in thyroid hormone synthesis (21, 22), host defense by professional phagocytes (23, 24), and oocyte maturation (25, 26), processes that are controlled by thyrotropin, chemotactic peptides, or unknown stimuli, respectively. Each of these systems consists of a plasma membrane–bound NADPH oxidase that is activated by specific stimuli and generates H_2O_2 (either directly or via prior formation of superoxide anion [O_2^-]) and peroxidases catalyzing a variety of tissue-specific reactions, such as iodination of thyroglobin tyrosine residues and their coupling to thyroid hormones (21, 22), generation of hypochloric acid (23, 24), or cross-linking of shell proteins (25, 26). In addition, it is becoming increasingly clear that increases in the production of reactive oxygen species can be elicited by many hormones and cytokines; occur in a variety of other cells, such as B-lymphocytes (27), vascular endothelia (28), fibroblasts (29), osteoclasts (30), or neural cells (31, 32); and may be involved in the regulation of cell growth and differentiation (29, 33).

Intriguingly, evidence has been presented to suggest that adipocytes also possess a plasma membrane–bound NADPH oxidase that produces H_2O_2 and that is stimulated on exposure of intact cells to insulin (2). However, subsequent studies raised doubts about the validity of these findings. Based on the observation that H_2O_2 production is inversely related to free fatty acid concentration, one group concluded that H_2O_2 generation is a metabolic consequence of insulin action, making it unlikely that the effects of exogenously supplied H_2O_2 are linked to a second-messenger function for this compound (3). Despite the mounting evidence implicating H_2O_2 and oxygen radicals in signal transduction, this latter view is still prevailing, because the existence of a stimulus-sensitive NADPH oxidase in fat cell plasma membranes has never been confirmed.

Methods

Subjects. Adipose tissue was from nondiabetic subjects undergoing elective abdominal or cosmetic breast surgery. The subjects were oper-

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Received for publication 17 January 1991 and in revised form 22 August 1991.

J. Clin. Invest.

0021-9738/92/03/1006/08 $2.00
Volume 89, March 1992, 1006–1013

ated on after an overnight fast. Anesthesia was initiated with a short-acting barbiturate and maintained with halothane, nitrous oxide, and oxygen.

Preparation of fat cells. Tissue specimens were cut into small pieces and fat cells were isolated by the method of Rodbell (34) in a Hepes-buffered Krebs-Henseleit solution, pH 7.4, containing 20 mM Hepes, 5 mM glucose, 20 g/liter human serum albumin, and 1 mg/ml crude collagenase. After 30 min, fat cells were washed three times and resuspended in the same buffer (except that collagenase was omitted).

Glucose uptake and lipolysis. Glucose uptake was determined by measuring the release of $[^3H]H_2O$ from added [5-^{3}H]glucose using the method of Pollard et al. (35), whereby the whole reaction and assay are performed in a single scintillation vial. 0.15-mm aliquots of suspensions were incubated in plastic scintillation vials containing 10 mM pyruvate, 0.1 mM glucose, and 0.2 μCi [5-^{3}H]glucose in the absence and presence of insulin. After 60 min, 5 ml of extraction-scintillation cocktail was added. The vials were vigorously agitated, centrifuged, and then counted for radioactivity.

For determination of lipolysis incubations, were stopped by heating (95°C, 5 min). Glycerol was determined by a luminescence assay as described (35a).

H_2O_2 accumulation in fat cell suspensions. Immediately after washing, 1 mM NaN_3 was added, and suspensions (10^5 cells/ml) were incubated at 37°C in the absence or presence of 10 nM insulin. At the times indicated, 1-ml aliquots of suspensions were removed. Cells and media were separated by the silicone oil technique (36), and the media were assayed for H_2O_2 by the chromogenic method of Ngo and Lenhoff (37). Cell breakage and the release of H_2O_2-degrading enzymes were monitored by determining lactate dehydrogenase release (36) and by following the disappearance of exogenously supplied H_2O_2 (80 μM), respectively.

Activation of NADPH-oxidase in intact cells, cell fractionation, and preparation of fat cell ghosts. Isolated adipocytes were exposed to various concentrations of insulin for 10 min. For isolation of subcellar fractions, packed adipocytes were homogenized in 20 mM MOPS [3-(*N*-morpholino)propanesulfonic acid][1] buffer, pH 7.0, containing 2 mM sucrose, 1 mM EDTA, 1 mM PMSF, 5 mM NaF, and 100 mg/liter soybean trypsin inhibitor. Homogenates were fractionated on discontinuous sucrose gradients by the method of McKeel and Jarett (38).

For ghost preparation, packed adipocytes were resuspended in 10 vol of an ice-cold lysing medium containing 20 mM MES [2-(*N*-morpholino)ethanesulfonic acid], pH 5.8, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM KCl, and 100 mg/liter soybean trypsin inhibitor. Cell lysis was completed by mechanical shaking. Fat cell ghosts were prepared by low speed centrifugation (1,000 *g*, 4°C) for 20 min, washed, and checked for microsomal and mitochondrial contaminations using the microsomal NADH/cytochrome *c* reductase and NADPH-dependent L-glutamic acid dehydrogenase as marker enzymes (39, 40).

Receptor-mediated activation in cell-free preparations and assay of NADPH-oxidase activity. For technical reasons, e.g., different pH optima for insulin binding and NADPH-oxidase activity, interference of divalent cations, such as Mn^{2+}, with H_2O_2 determinations, essentially the same two-step procedure was used that had been employed with intact fat cells. Ghosts were first exposed to insulin, thoroughly washed, and 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 $CaCl_2$, 5 mM $MgCl_2$, 3 mM $MnCl_2$, and 10 mM $NaHCO_3$ in the absence or presence of insulin as indicated. After 25 min, the ghosts were washed; resuspended in 30 mM [2-(*N*-morpholino)ethanesulfonic acid], pH 5.8, containing 120 mM NaCl, 4 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1 mM NaN_3, and 10 μM FAD; and assayed for NADPH-oxidase activity.

Reactions were started by addition of 0.25 mM NADPH and were terminated after 30 min at 37°C by adding HCl (H_2O_2 determinations) or KOH (NADPH measurements) to yield final concentrations of 0.1 M. Proportional increases in NADPH-dependent H_2O_2 production were limited to a narrow range of protein concentrations. Therefore, assays were routinely performed at four different protein concentrations ranging from 2 to 20 μg/ml of protein.

Determination of H_2O_2. After stopping, the samples were extensively diluted (30–1,000 times) and assayed for H_2O_2, using the oxidation of luminol in the presence of peroxidase, which results in emission of light (41), as the indicator reaction. The assays contained 0.1 M Na_2HPO_4/KH_2PO_4, pH 8.2, 0.72 g/liter EDTA, 12.5 μM luminol, and 0.1 U/liter horseradish peroxidase. Reactions were carried out in the absence or presence of 110 U/liter catalase (Sigma Chemical Co., St. Louis, MO; thymol free) to account for nonspecific reactions. External standards (10–100 nM) contained all components that were present in the samples, except that membranes were omitted. Reaction kinetics were followed on a screen using the kinetic program supplied by the manufacturer of the luminescence analyzer (LB 950T; Berthold, Wildbad, FRG). Light output was stable for 20 s, and the integral of counts between 5 and 10 s was taken as a measure of H_2O_2 concentration. The detection limit for H_2O_2 was in the range of 5 to 10 nM, and light output was linearly related to H_2O_2 concentration up to 100 nM.

Contaminations (probably traces of metals) that are often present in test tubes may lead to unacceptable levels of background luminescence. Therefore, vials (Lumac, Landgraaf, The Netherlands) were first treated with 0.2 g/liter Triton X-100 for 30 min, washed, and then exposed to horseradish peroxidase (10 kU/l) for 12 h. The peroxidase treatment was terminated by addition of 0.2 g/liter Triton X-100, followed by extensive washing.

NADPH concentrations were determined by the bacterial NAD(P)H-linked luciferase (31). Protein concentrations were measured by the method of Lowry et al. (43).

Superoxide generation and ferricytochrome c reductase activity. Superoxide generation and ferricytochrome *c* reductase activity were monitored at 25°C by the rates of ferricytochrome *c* reduction in the absence or presence of 10 μg/ml SOD (44). Superoxide formation corresponds to the difference in the rates of cytochrome *c* reduction in the absence and presence of SOD under these conditions, whereas the residual (SOD-insensitive) fraction of ferricytochrome *c* reduction reflects cytochrome *c* reductase activity.

The assay contained 50 μM ferricytochrome *c*, 20 mM MOPS, pH 7.0, 4 mM $MgCl_2$, 120 mM NaCl, 1.2 mM KH_2PO_4, and 10–30 μg membrane protein in a total volume of 1 ml. The reaction was started by addition of 0.25 mM NADPH, and the reduction of ferricytochrome *c* was followed at 550 nm. The reduction of cytochrome *c* was calculated using an extinction coefficient of $21.1 \cdot mmol^{-1} \cdot cm^{-1}$.

Chemicals. Diphenylene iodonium was a gift of Dr. O.T.G. Jones (Department of Biochemistry, University of Bristol, Bristol, UK). Human serum albumin, luminol, catalase (thymol-free), and [5-^{3}H]-glucose were from Behring Werke (Marburg, FRG), Fluka AG (Basel, Switzerland), Sigma GmbH (München, FRG), and Amersham (Bucks, UK), respectively. Porcine insulin and the majority of enzymes and coenzymes were from Boehringer Mannheim (Mannheim, FRG).

Results

H_2O_2 accumulation in human fat cell suspensions. Similar to rat adipocytes, exogenously supplied H_2O_2 mimicked the effects of insulin on glucose transport and lipolysis in human fat cells. Maximal effects were comparable with those elicited by insulin and occurred at 10 mM (data not shown). The insulinomimetic effects of H_2O_2 were abolished by catalase, whereas insulin action was not influenced at all, consistent with similar observations in rat adipocytes (2).

Exogenously supplied H_2O_2 (80 μM) disappeared from the media in the absence of cells, and its half-life varied between 20 and 50 min with different batches of albumin. Human adipo-

1. *Abbreviation used in this paper:* MOPS, 3-(*N*-morpholino)propanesulfonic acid.

cytes are fragile. On the average, 10% of total cellular lactate dehydrogenase was released into the media per hour of incubation (36). Accordingly, used media contained cell-derived peroxidase(s) and catalase, and the $t_{1/2}$ of exogenously supplied H_2O_2 was reduced to 5–10 min. In suspensions (10^5 cells/ml), > 90% of added H_2O_2 was removed within 5 min, indicating that a considerable proportion of exogenously supplied H_2O_2 was consumed inside intact cells.

The disappearance of exogenously supplied H_2O_2 was considerably delayed by 1 mM azide ($t_{1/2} \sim 20$ min at a density of 10^5 cells/ml). Accordingly, it was only in the presence of 1 mM azide that a spontaneous accumulation of H_2O_2 could be detected in cell suspensions (Fig. 1). The appearance of measurable amounts of H_2O_2 was critically dependent on cell density, and virtually no H_2O_2 could be detected at cell concentrations of $\geq 5 \times 10^5$ cells/ml. At a density of 10^5 cells/ml, H_2O_2 accumulation was transient (probably because of a continuous extrusion of H_2O_2-degrading enzymes that were resistant to the inhibitory action of 1 mM azide) and was markedly augmented by insulin. In the absence of insulin, H_2O_2 reached peak concentrations (15 μM) within 5 min and fell below detectable levels within 20 min. 1 nM insulin accelerated the initial increase in H_2O_2 concentrations, and maximal levels were two times higher than in controls.

Subcellular localization of the insulin-sensitive H_2O_2-generating system. Among the subcellular fractions obtained by sucrose density centrifugation of untreated cells, light microsomes displayed the highest rates of NADPH-dependent H_2O_2 generation, e.g., 16 nmol · (mg protein)$^{-1}$ · min^{-1}. The activities detected in dense microsomal, mitochondrial, and plasma membrane fractions were comparable to and corresponded to approximately one sixth of that seen in light microsomes. Exposure of intact adipocytes to 10 nM insulin for 10 min resulted in a selective increase in NADPH-dependent H_2O_2 production by plasma membranes that was not seen in the mitochondrial or microsomal fractions.

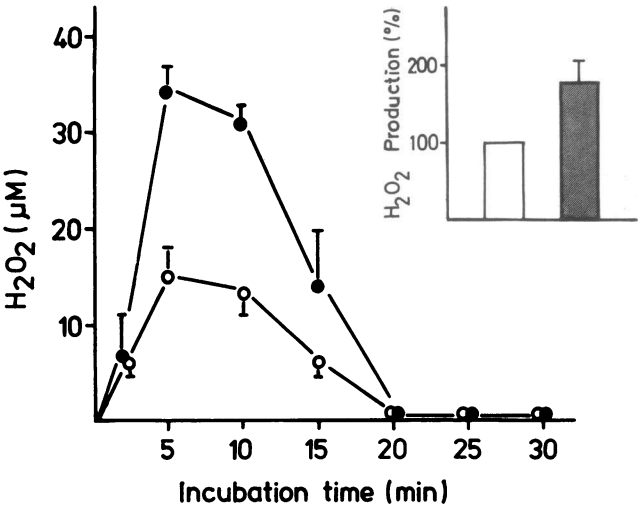

Figure 1. Effect of insulin on H_2O_2 accumulation in human fat cell suspensions. Isolated adipocytes (10^5 cells/ml) were incubated in the absence (○) or presence (●) of 10 nM insulin. The media contained 1 mM NaN_3. The kinetics of H_2O_2 accumulation varied between individual preparations, with peak levels occurring at between 2 and 10 min of incubation. Therefore, a representative time course carried out in triplicate with the same batch of cells is shown. Values given in the inset refer to the relation between peak concentrations of H_2O_2 observed in the absence (*white bar*) and presence (*shaded bar*) of 10 nM insulin and are means±SE of 10 separate experiments carried out with fat cells from different donors.

Crude fat cell plasma membranes ("fat cell ghosts") contained < 10% of total cellular NADPH/cytochrome P_{450}-reductase and < 2% of NADPH-dependent glutamate dehydrogenase, indicating that they were only moderately contaminated by microsomes and reasonably free of mitochondria. As the activity of NADPH/cytochrome P_{450}-reductase is extremely low at pH 6.0, the pH-optimum of the insulin-sensitive NADPH-oxidase, and is further suppressed by azide, the results obtained with fat cell ghosts and purified plasma membranes were virtually identical. Therefore, most of the experiments were carried out with fat cell ghosts without further purification.

Crude plasma membranes from untreated adipocytes oxidized NADPH (0.25 mM) at a rate of 2.2 nmol · min^{-1} · mg protein^{-1}. Membranes from cells that had been exposed to 10 nM insulin for 10 min displayed a twofold increase in NADPH oxidation (Table I, Fig. 2). The reaction resulted in accumulation of H_2O_2 with a ratio of ~ 1 for NADP/H_2O_2 under both conditions and was not seen with boiled membranes (Table I). The insulin-induced increase in NADPH-dependent H_2O_2 generation was rapid and depended on the insulin concentration applied to the cells. At 10 nM insulin, the increase in NADPH-oxidase activity was half-maximal at 2 min. By 5 min, H_2O_2 production was maximal and remained stable for ≥ 20 min (not shown). Exposure of intact fat cells to increasing concentrations of insulin led to a dose-dependent increase in NADPH-dependent H_2O_2 generation by fat cell ghosts (Fig. 2). Half-maximal effects were observed at ~ 0.3 nM insulin, a concentration that is physiologically relevant. The stimulatory effect of insulin was due to a selective increase in substrate affinity. The apparent K_m value for NADPH was reduced from 0.27±0.03 to 0.11±0.03 mM, whereas V_{max} values of insulin-treated preparations were identical to those seen in controls (Fig. 3).

Properties. Similar to all stimulus-sensitive NADPH-oxidases that have been identified, NADPH oxidation and H_2O_2 production were resistant to various inhibitors (1 mM KCN, 1 mM NaN_3, 10 μg/ml rotenone) and activators (1 mM ATP, 10 μg/ml antimycin A) of mitochondrial respiration and were not influenced by 2 mM NADP, an inhibitor of the microsomal NADPH/cytochrome P_{450}-reductase (data not shown). NADPH oxidation and H_2O_2 production were optimal at pH 5–6.5, consistent with early observations in the rat (2). The enzyme resembled the stimulus-dependent systems in thyrocytes and oocytes in that no "free" O_2^- could be detected (as determined in terms of the SOD-inhibitable reduction of ferricytochrome *c*).

Other properties of the human fat cell oxidase were strikingly similar to those of the respiratory burst oxidase of professional phagocytes. Like the phagocyte enzyme, the insulin-stimulated fat cell oxidase displayed more than 10-fold preference for NADPH over NADH, and the K_m values determined for both nicotinamide adenine nucleotides were similar to those measured in particulate fractions of activated phagocytes (Fig. 3). Second, NADPH oxidation and H_2O_2 production were inhibited by 0.5 mM EDTA. The inhibitory effect could be overcome by 2 mM Mg^{2+}, whereas Ca^{2+} had no effect (Table I). Third, the human fat cell enzyme required a flavine nucleotide

Table I. Effects of Different Compounds on Basal and Insulin-stimulated NADPH-oxidase Activity of Human Fat Cell Ghosts

Addition*	Control‡		Insulin (10 nmol/l)		
	H_2O_2 production	NADPH oxidation	H_2O_2 production	NADPH oxidation	*n*
None	2.5±1.0	2.2±0.5	4.5±1.5	5.1±1.0	7
FAD (10 μmol/liter)	24.2±5.0	23.7±6.0	45.1±9.0	47.7±9.0	7
FMN (10 μmol/liter)	28.0±6.0	ND	46.0±4.0	ND	3
FAD (10 μmol/liter) + EDTA (0.5 mmol/liter)	<5	ND	13.0±3.0	ND	3
FAD (10 μmol/liter) + EDTA (0.5 mmol/liter) + Ca^{2+} (2.0 mmol/liter)	<5	ND	10.0±3.0	ND	3
FAD (10 μmol/liter) + EDTA (0.5 mmol/liter) + Mg^{2+} (2.0 mmol/liter)	13.0±3.0	ND	32.0±4.0	ND	3
FAD (10 μmol/liter) + DPI (100 μmol/liter)	28.0±3.0	25.0±5.0	59.0±6.0	55.0±7.0	3

* FAD and FMN caused high levels of background luminescence that could be eliminated only by excessive dilution. Therefore, the detection limit for H_2O_2 production rose from ~0.1 to ~5 nmol · min^{-1} · mg $protein^{-1}$.
‡ Crude plasma membranes from controls and insulin-treated adipocytes were assayed for NADPH-oxidase activity as described under Methods. H_2O_2 production and NADPH oxidation are given in terms of nmol · min^{-1} · mg $protein^{-1}$. Values are means±SE of three to seven separate experiments, as indicated in the column to the right.
DPI, diphenylene iodonium.

for optimal activity. FAD (apparent K_m value ~ 4 μM) caused a 10-fold increase in V_{max} without changing the affinity for NADPH, the stoichiometry between NADPH oxidation and H_2O_2 production, or the relative extent of insulin stimulation (Table I). In contrast to the phagocyte system, FMN was as potent as FAD in increasing basal and insulin-stimulated rates of NADPH-dependent H_2O_2 production in human fat cell cell ghosts (Table I).

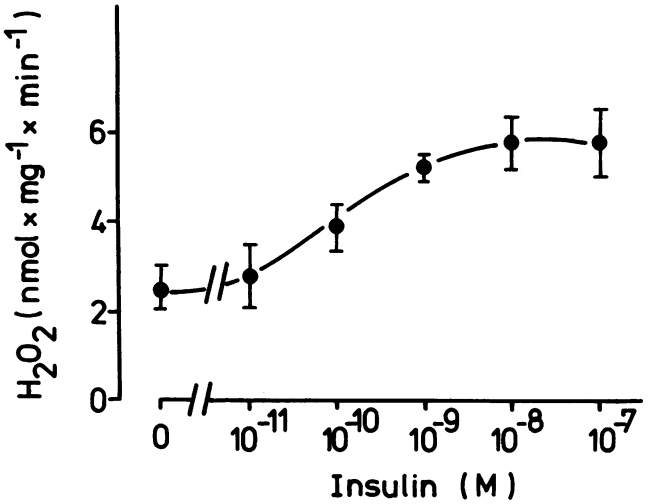

Figure 2. Effects of exposure of intact adipocytes to increasing concentrations of insulin on NADPH-dependent H_2O_2 production in crude plasma membranes. Intact adipocytes were first exposed to various concentrations of insulin for 10 min and then lysed. NADPH-dependent H_2O_2 production by fat cell ghosts was determined in the presence of 1 mM NaN_3. Values are means±SE of three separate experiments carried out in triplicate with fat cells from different donors.

The respiratory burst oxidase of professional phagocytes may consist of at least two redox active components, a low potential cytochrome b_{558}, and a flavoprotein (23, 24, 45–48). The observation that FAD (or FMN) was required for optimal activity of both the fat cell oxidase and the respiratory burst enzyme therefore invited the speculation that the NADPH oxidases of human fat cells and of professional phagocytes might also be related in structural terms (45–48). Indeed, a preliminary spectroscopic analysis revealed that human fat cell ghosts contain a low potential cytochrome *b* with spectral properties similar to, or identical with, those of the terminal oxidase of the O_2^--generating phagocyte system (45). However, diphenylene iodonium, a potent inhibitor of the respiratory burst oxidase thought to act via a covalent modification of its flavoprotein component (48), had no effect on basal or insulin-stimulated

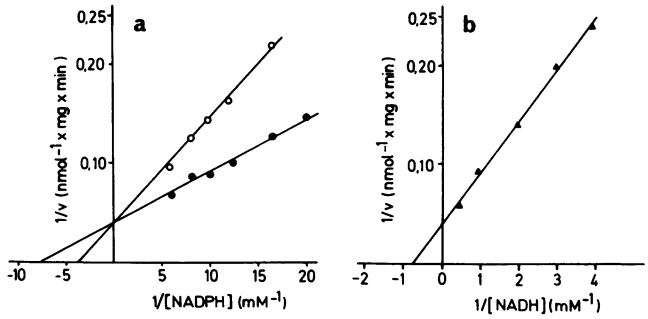

Figure 3. Double reciprocal plots of H_2O_2 production as a function of NAD concentration. Crude plasma membranes from untreated cells (○) and of adipocytes that were exposed to 10 nM insulin for 10 min before cell lysis (●, ▲) were assayed for H_2O_2 production in the presence of 1 mM NaN_3 and 10 μM FAD, as described under Methods, except that the NADH was used where indicated. Values are means of triplicate determinations obtained in one out of four experiments for each condition. The mean K_m values±SE of insulin-activated preparations are listed in Table III.

rates of NADPH-dependent H_2O_2 generation when tested over a wide range of concentrations (1–100 μM; Table I). The compound failed to suppress enzyme activity regardless of whether H_2O_2 production was assessed in the presence of the inhibitor or after a pretreatment of membranes with various concentrations of diphenylene iodonium for 30 min at 25°C, indicating that the flavoprotein component of the fat cell oxidase is either distinct from the corresponding phagocyte protein, becomes uncoupled from cytochrome b_{558} during cell disruption (49), or is lacking.

Activation of NADPH-dependent H_2O_2 generation in cell-free preparations. In contrast to all hormone- or cytokine-sensitive NADPH oxidases that have been characterized in sufficient detail, the human fat cell oxidase retained its insulin responsiveness after cell disruption. In vitro (where in vitro refers to crude plasma membranes), the stimulatory effect of insulin was critically dependent on millimolar concentrations of manganese ions, which are also essential for a ligand-induced activation of receptor protein–tyrosine kinases in cell-free preparations and have insulin-like metabolic effects in intact adipocytes (7, 50). This is illustrated in Fig. 4. A pretreatment of membranes with Mn^{2+} or insulin alone had no effect on NADPH-dependent H_2O_2 generation (Fig. 4 *a*). The stimulatory effect of insulin was half-maximal at 2 mM Mn^{2+} and attained maximal levels at 3 mM. Manganese could not be replaced by cofactors that are essential for optimal activity (FAD, FMN) or by other insulinomimetic cations, such as Mg^{2+}, Zn^{2+}, or SeO_4^{3-}, when added at concentrations of 3 mM (Zn^{2+}, SeO_4^{3-}) or up to 10 mM (Mg^{2+}), respectively (data not shown).

The stimulatory effect of insulin was rapid and occurred in the absence of NADPH-dependent catalytic turnover. At 0.5 μM insulin, the activation was completed within 1 min (Fig. 4 *b*). As in intact cells, the hormone-induced activation was quasiirreversible and could be detected after removal of insulin and Mn^{2+} (which interferes with H_2O_2 determinations) by extensive washing. Accordingly, the maximal increase in NADPH-dependent H_2O_2 generation caused by insulin in broken cell preparations was virtually identical with that observed after exposure of intact cells to the hormone, and no additive effects occurred when the system was first activated in intact cells and then exposed to insulin and Mn^{2+} in vitro. However, compared with its effects in intact cells, insulin was markedly less potent in activating NADPH-dependent H_2O_2 generation in cell-free preparations; half-maximal effects were observed at 0.1 μM insulin (not shown). It is a common experience, however, to find receptor-mediated processes much less responsive to ligands in broken cell preparations than in intact cells, and similar concentrations of insulin are required to stimulate receptor autophosphorylation in cell-free preparations (51).

Surprisingly, the stimulatory effects of insulin occurred in the absence of exogenously supplied ATP and remained unchanged on removal of residual membrane-bound ATP by hexokinase (Table II). At concentrations of ≥ 0.4 mM, ATP even suppressed the insulin-induced activation, whereas ADP, AMP, and cAMP had no effects (Table II). At 1 mM ATP, the stimulatory effect of insulin was reduced by one half. ATP had no effect when added to preparations that were already activated and did not influence catalytic activity (data not shown). The inhibitory action of the nucleotide did not involve a covalent modification, because its non-hydrolyzable analogue, AMP(PNP), had similar effects (Table II). It is also unlikely that this latter effect reflected a decrease in the concentration of "free" Mn^{2+} due to its complexation by ATP, because the inhibitory action of the nucleotide was not reversed when Mn^{2+} concentrations were increased to 4 mM. Further studies are required to establish whether the ATP-induced suppression of insulin's stimulatory effects is receptor-mediated (via P_1-purinergic receptors) or reflects a stable interaction of ATP with a component of the receptor-oxidase couple.

Guanine nucleotides were also tested. However, neither GTP and GDP, nor their stable analogues GTPγS and GDPβS, had a detectable influence on the process of hormonal activation when added over a wide range of concentrations (0.01–1 mM).

Discussion

We have confirmed that human fat cells possess a plasma membrane–bound H_2O_2 generating system that is activated on

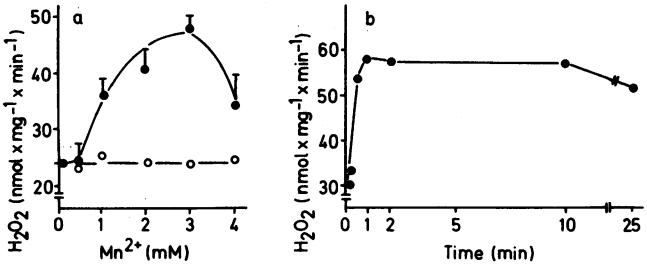

Figure 4. Manganese dependence (*a*) and kinetics of the insulin-induced activation of NADPH-dependent H_2O_2 generation in crude plasma membranes (*b*). (*a*) Ghosts from untreated adipocytes were incubated (25°C, pH 7.5) at various concentrations of $MnCl_2$ in the absence (○) or presence (●) of 0.5 μM insulin as described under Methods. After 25 min, the ghosts were washed and assayed for NADPH-dependent H_2O_2 generation in the presence of 1 mM NaN_3 and 10 μM FAD. Values are means±SE of three separate experiments. (*b*) Ghosts were exposed to 0.5 μM insulin at pH 7.5 in the presence of 3 mM $MnCl_2$. At the times indicated, aliquots were removed and assayed for NADPH-dependent H_2O_2 generation. Values are means of triplicate determinations.

Table II. Inhibition of the Insulin-induced Activation of NADPH-dependent H_2O_2 Generation in Crude Plasma Membranes by ATP and AMP(PNP)

Condition*	H_2O_2 generation
	nmol · mg^{-1} · min^{-1}
Control	32±5
ATP (1 mM)	32±3
Insulin (0.5 μM)	72±10
ATP (1 mM) + insulin (0.5 μM)	49±9
AMP(PNP) (1 mM) + insulin (0.5 μM)	43±8
GTP (1 mM) + insulin (0.5 μM)	70±7

* Crude plasma membranes from untreated adipocytes were incubated at 3 mM $MnCl_2$ in the absence or presence of insulin, either alone or together with various nucleotides, as indicated. The activation was terminated by extensive washing, and washed membranes were assayed for NADPH-dependent H_2O_2 generation as described under Methods. Values are means±SE of four separate experiments.

exposure of intact adipocytes to insulin at concentrations that are physiologically relevant, indicating that widely held views considering H_2O_2 as a metabolic byproduct require careful reconsideration. In contrast to all stimulus-sensitive NADPH oxidases that have been characterized in sufficient detail, the human fat cell enzyme remains responsive to a receptor-mediated activation in cell-free preparations (Table III). This unique property made it possible to demonstrate that the insulin-induced activation takes place via a novel type of receptor, effector coupling bypassing the receptor kinase. These aspects are discussed separately.

Properties of the insulin-sensitive H_2O_2-generating system. Table III summarizes the properties of the human fat cell oxidase and of other stimulus-sensitive NADPH oxidases that have been characterized in sufficient detail. All systems appear to be membrane-bound, to be resistant to inhibitors of mitochondrial respiration, and to have K_m values for NADPH in the micromolar range. As far as comparisons can be made, the properties of the human and rat fat cell oxidases (e.g., pH-optimum, substrate affinity, stoichiometry between NADPH oxidation and H_2O_2 production, insulin sensitivity) appear to be nearly identical. In addition, the increase in activity elicited by insulin in intact cells is preserved during cell disruption and can be measured in membrane preparations of adipocytes from both species. The latter property has also been noted with other effects of insulin, e.g., activation of glucose transport (51), and is shared by the respiratory burst oxidase of professional phagocytes (23, 24). Unlike the fat cell oxidase, the latter system mainly generates $O_2^{\cdot-}$. However, other relevant properties are strikingly similar. In contrast to the NADPH oxidases of thyrocytes and oocytes (which are Ca^{2+} and ATP dependent), the phagocyte oxidase and the human fat cell enzyme require Mg^{2+} and a flavine nucleotide for optimal activity.

The respiratory burst oxidase is a multicomponent electron transport system containing a characteristic b-type cytochrome (cytochrome b_{558}) and possibly a flavoprotein (23, 24, 45–49). The observation that the characteristic b-type cytochrome is also present in human fat cell plasma membranes, therefore, strongly supports the view that both systems are also related in structural terms, and further studies are required to delineate whether other parallels exist between the phagocyte system and the human fat cell oxidase.

Stimulatory effects of insulin in cell-free preparations. All stimulus-sensitive NADPH oxidases that have been characterized in sufficient detail become unresponsive to receptor-mediated stimuli after cell disruption, because their activation is mediated by second messengers and/or involves a translocation of cytosolic proteins to the plasma membrane (20–26). By contrast, the mechanism(s) by which insulin stimulated the rates of NADPH-dependent H_2O_2 generation in human fat cell plasma membranes were independent of second messengers and occurred in the absence of guanine nucleotides. The dependence on GTP is not a reliable criterion, however, to establish the participation of guanine nucleotide regulatory proteins (G proteins), since some of these proteins exhibit quite slow rates of GDP dissociation and GTP hydrolysis in the absence of guanine nucleotide release proteins or GTPase-activating proteins (52). However, neither GTPγS nor GDPβS had a detectable effect on the insulin-induced activation of NADPH-dependent H_2O_2-production, suggesting that G proteins, which may control certain aspects of insulin action, are probably not involved in receptor-NADPH oxidase coupling (53).

The insulin receptor is a member of the receptor protein–tyrosine kinase superfamily (51). A universal feature of these receptor kinases is that ligand binding results in a change in receptor conformation leading to immediate stimulation of the receptor kinase, and this is thought to be essential for all subsequent events (51). Indeed, the insulin-induced increase in NADPH-dependent H_2O_2 generation was preserved after extensive washing, consistent with a covalent modification. It was therefore surprising that ATP was not only not necessary for, but even suppressed, the stimulatory effects of insulin. This

Table III. Properties of the Human Fat Cell NADPH Oxidase and of Other Stimulus-sensitive Oxidases

Cell type	Thyrocytes (21, 22)	Oocytes (25, 26)	Phagocytes (23, 24, 45–49)	Human fat cells	Rat fat cells (2)
Substrate affinity (K_m)*					
NADH (*mmol/l*)	0.4		0.4–1.0	1.6±0.2	
NADPH (*μmol/l*)	12–50	40	30–80	110±30	58
Initial product	H_2O_2 ($O_2^{\cdot-}$)	H_2O_2	$O_2^{\cdot-}$ (H_2O_2)	H_2O_2	H_2O_2
pH-optimum	6.5–7.4	6.8–7.6	7.0–7.6	5.0–6.5	6.0
Cofactor requirements	Ca^{2+}, ATP	Ca^{2+}, ATP	FAD (FMN) Mg^{2+}	FAD (FMN) Mg^{2+}	?
Sensitivity to inhibitors of mitochondrial respiration	–	–	–	–	–
Stimuli	TSH	?	Formyl peptides Immunoglobulins Bioactive lipids Cytokines	Insulin	Insulin
Localization of activated enzyme	Plasma membrane	Membranes (unspecified)	Plasma membrane	Plasma membrane	Plasma membrane
Stability of activation	–	–	+	+	+

* K_m values refer to the substrate affinity observed in the activated state. K_m values for the human fat cell oxidase are means±SE of four separate experiments.

finding is in marked contrast with the hypothesis that receptor kinase activity is an absolute requirement for all biochemical actions of insulin. Indeed, it is becoming increasingly clear that insulin's effects are mediated by a diversity of pathways originating at the occupied receptor, and several authors have interpreted recent evidence as suggesting that kinase activity may not be necessary for all actions of insulin (53–55). For example, insulin promoted GTP hydrolysis in BC_3H_1 cells in a pertussis toxin–sensitive manner, and this effect was not prevented by concentrations of AMP(PNP) that block the receptor kinase, suggesting a noncovalent interaction of the receptor with a G protein (53). In addition, most recent evidence indicated that insulin receptor mutants with inactive tyrosine kinase domains are still capable of transmitting the stimulatory effect of the hormone on pyruvate dehydrogenase, indicating that this latter effect is mediated by a pathway that bypasses the receptor kinase (54). Intriguingly, pyruvate dehydrogenase has long been known to be activated by exogenously supplied H_2O_2 (5, 6).

The stimulatory effect of insulin was critically dependent on millimolar concentrations of Mn^{2+}. The concentrations of Mn^{2+} required to achieve a ligand-induced increase in NADPH-dependent H_2O_2 generation exceed its intracellular concentrations by orders of magnitude, making it unlikely that free Mn^{2+} is involved in the regulation of NADPH-dependent H_2O_2 generation in intact cells. Interestingly, supraphysiological concentrations of Mn^{2+} are also required for a ligand-induced activation of receptor protein–tyrosine kinases in cell-free preparations (50, 51). It has, therefore, been proposed that Mn^{2+} may either serve to maintain receptors in an "activatable" conformation or mimic an unknown effector that is present in intact cells (50). At this stage, it is uncertain whether the mechanism(s) by which Mn^{2+} renders receptor kinases responsive to their ligands is identical with those involved in receptor-NADPH oxidase coupling. As both effects occur at similar concentrations, this interesting possibility deserves further investigation.

Physiological considerations. As pointed out above, it is becoming increasingly clear that the biochemical actions of insulin are mediated by a diversity of independent pathways, including phosphorylation of distinct substrates and generation of second messengers. Some of the mediators of insulin action may originate from glycosyl-phosphatidylinositol anchors of membrane proteins (56). Freshly isolated adipocytes have repeatedly been shown to be devoid of functional receptors for insulin-like growth factor-1, which often complicate the analysis of insulin receptor–mediated events (57–59). By confirming that a H_2O_2-generating system exists in adipocytes that is activated on exposure of the cells to insulin at concentrations that are physiologically relevant, the present findings therefore reinforce the original idea that H_2O_2 contributes to insulin receptor signaling (1, 2).

However, the role assumed by H_2O_2 in the propagation of the insulin signal is presently unknown. A solution to this problem is difficult for several reasons. First, it is unknown whether any of insulin's biochemical actions may be triggered independently by one of the diverse pathways involved in insulin action. Second, even though H_2O_2 qualifies for a second-messenger function, this may not necessarily be its role. Recent studies demonstrated that the stimulatory effects of exogenously supplied H_2O_2 on the tyrosine kinase activity of the insulin receptor may, at least in part, be due to its inhibitory action on phosphoprotein phosphatases (12), implying that it might serve solely to facilitate and/or maintain insulin-induced protein phosphorylations. Third, it is also possible that the H_2O_2 produced in response to insulin is mainly used for a cross-talk between signal transduction pathways regulated by insulin and other hormones acting through receptors that are coupled to adenylate cyclase, guanylate cyclase, or protein kinase C (60). Thus, despite the insulin-like metabolic effects of exogenously supplied H_2O_2, the peroxide produced in response to insulin in intact cells may, of itself, be incapable of generating any of insulin's actions. Further insights into the role assumed by H_2O_2 in insulin receptor signaling will depend on the development of specific inhibitors of NADPH-dependent H_2O_2 generation and/or construction of receptor mutants that have no tyrosine kinase activity but are still capable of coupling to NADPH oxidase, or vice versa.

Acknowledgments

The authors are indebted to B. Löser, B. Sattel, and B. Nessel for expert technical assistance and wish to thank Dr. O. T. G. Jones, who kindly performed a spectral analysis of the cytochromes present in human fat cell membranes and provided diphenylene iodonium.

This work was supported by a grant of the Bundesministerium für Forschung und Technologie (Förderungskennzeichen 0704721-AZ), Bonn, FRG.

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