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SIMILAR ACTIVITIES OF NERVE GROWTH FACTOR AND ITS HOMOLOGUE PROINSULIN IN INTRACELLULAR HYDROGEN PEROXIDE PRODUCTION AND METABOLISM IN ADIPOCYTES

TRANSMEMBRANE SIGNALLING RELATIVE TO INSULIN-MIMICKING CELLULAR EFFECTS

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Abstract—Generation of hydrogen peroxide in adipocyte plasma membrane and its intracellular metabolism and regulatory role have been shown by Mukherjee and co-workers to be a major effector system for insulin [*Fedn Proc.* **35**, 1694 (1976); *Archs Biochem. Biophys.* **184**, 69 (1977); *Biochem. Pharmac.* **27**, 2589 (1978); *Fedn Proc.* **37**, 1689 (1978); and *Biochem. Pharmac.* **29**, 1239 (1980)]. The possible involvement of this mechanism in the action of structurally similar polypeptides having some insulin-like metabolic effects was investigated. The β-subunit of nerve growth factor (2.5 S NGF, mol. wt 13,500) which has a striking structural homology with proinsulin and has been reported to exert certain insulin-like metabolic effects in its own target tissues (e.g. growing neurites and sympathetic ganglia), and the insulin-derived polypeptides, desalanine-insulin and desoctapeptide-insulin, as well as proinsulin, were examined for their effects on rat adipocytes, employing the technique of formate oxidation. Both NGF and proinsulin caused increased [^{14}C]formate oxidation, showing similar intrinsic activities, up to a maximum of 140–160% of the basal rate; insulin increased the rate to 190–210% of the basal rate. The relative potencies of the hormones toward H_2O_2 formation and stimulation of the pentose phosphate pathway activity were: insulin (EC_{50}: 2.5×10^{-11} M), desalanine-insulin (EC_{50}: 2.5×10^{-10} M), proinsulin (EC_{50}: 8×10^{-9} M), and NGF (EC_{50}: 10^{-9} M). The biologically inactive derivative, desoctapeptide-insulin, did not stimulate glucose oxidation, although it caused a small increase in formate oxidation, with an EC_{50} of 5×10^{-7} M, indicating a suboptimal level of H_2O_2 formation in the elevation of the hexose monophosphate shunt activity. 3-Amino-1,2,4-triazole (50 mM), which irreversibly decomposes the peroxidatic compound II of the catalase:H_2O_2 complex, inhibited formate oxidation to a greater extent in the hormone-treated cells than in the control cells, whereas sodium azide, an inhibitor of the hemoprotein, catalase, completely inhibited it. The abilities of the polypeptides to stimulate H_2O_2 formation correlated with their abilities to promote lipogenesis from [U-^{14}C]-D-glucose, as expected of insulin. The cellular GSH/GSSG ratio increased concomitantly with the stimulation of glucose oxidation via the shunt, indicating a tight coupling between these processes. The results confirm that the hydrogen peroxide production is a common basis of the metabolic actions of growth-promoting polypeptide hormones or mitogens beyond their respective receptors.

Polypeptide hormones such as insulin are known to exert their biological effects on target cells by their interactions with plasma membrane receptors. Many authors have speculated that such interactions may cause the generation of an intracellular mediator molecule. While the production of cyclic 3′, 5′-AMP is generally recognized as the 'second messenger' for the catecholamines and a host of other hormones or drugs [1], studies have indicated that the intracellular effects of insulin may involve a mediator distinct from cAMP. However, all attempts to identify such a mediator have failed so far, and studies on the mechanism of action of insulin, as summarized in recent reviews, have not resolved the problem. The discovery of pyridine nucleotide oxidase activity, in plasma membrane of adipocytes, that is stimulated by insulin and several insulin-mimicking chemical agents [2, 3] introduces an entirely new concept of hormonal action, which involves an electron transport system in the plasma membrane of peripheral cells. The product of this reaction, H_2O_2, was found to accumulate within these cells [2–5] with profound metabolic consequences to the peroxidatic pathways [4–6]. We also found that lectins such as concanavalin A, which mimic several metabolic actions of insulin [7], also activate the NADPH oxidase in fat cell plasma membrane [4, 8, 9] as well as in thymocyte plasma membrane [10], leading to intracellular H_2O_2 accumulation and stimulation of metabolic activities. If H_2O_2 generation inside the plasma membrane [2–4] is a transmembrane signal, or messenger of insulin, this mechanism may underlie the observed insulin-like effects of some other hormones. Oxytocin, a nonapeptide hormone of pituitary origin which is structurally heterologous to insulin, activates the plasma membrane NADPH oxidase in

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adipocytes, with H_2O_2 production, with a potency which is comparable to its stimulatory effect on glucose metabolism via the pentose phosphate pathway and lipogenesis [11–13], although the receptor sites are distinct from those of insulin. The similarities between insulin and another anabolic polypeptide hormone, nerve growth factor (NGF)*, a neurotropic factor isolated from mouse submaxillary glands were investigated, in order to evaluate the potential for endogenous H_2O_2 to be a common transmembrane signal for growth-promoting factors. NGF is known to stimulate glucose metabolism [14], uridine uptake [15], and macromolecule synthesis [16], in its principal target tissues, viz. sensory ganglia of growing neural tissues *in vitro*. This study was undertaken because analysis of the primary structure of the β-subunit of this polypeptide complex (NGF) revealed a homology with proinsulin [17].

Here we present evidence that NGF increases H_2O_2 production in rat adipocytes. The metabolic effects of NGF and the transmembrane metabolic system of insulin were examined by measuring formate oxidation in fat cells [2–5]. Further, like insulin or proinsulin, NGF also stimulates glucose oxidation with a preferential increase in the pentose phosphate pathway, leading to enhanced contribution of glucose carbon units to *de novo* synthesis of glycerolipids. To evaluate, with respect to H_2O_2 production, the structure–activity relationships or polypeptides having insulin-like properties, we also examined the effects of two insulin derivatives, viz. desalanine-insulin and desoctapeptide-insulin.

MATERIALS AND METHODS

Adipocytes were prepared from the parametrial adipose tissue of femal Sprague–Dawley albino rats (125–150 g) by digestion with bacterial collagenase, according to the method of Rodbell [18], as previously described [3–6]. Briefly, pooled fragments of fat pads were distributed in 1 ounce polypropylene bottles containing 3–8 ml of 3% bovine serum albumin in Krebs–Ringer phosphate buffer (pH 7.4) containing 0.05 mM D-glucose and crystalline collagenase (*Clostridium histolyticum*, Worthington Biochemical Corp., Freehold, NJ). The glucose concentration was kept low in order to retain a redox optimum for the expression of the peroxidative pathway activities. After incubation at 37° for 50 min on a moderately shaking Dubnoff metabolic shaker, the cells were sieved through a double layer of cheesecloth and washed three times with approximately 4 vol. of Krebs–Ringer phosphate buffer (pH 7.4) containing 1% albumin. Aliquots of 0.2-ml cell suspensions were added to polypropylene tubes (17 × 100 mm) which contained 1 ml of 3% albumin in the above buffer plus 0.2 mM ^{14}C-labeled glucose (sp. act. 0.12 μCi/μmole) for the assay of the hexose monophosphate shunt and the glycolytic pathway activities by measuring $^{14}CO_2$ production from [1-^{14}C]-D-glucose and [6-^{14}C]-D-glucose respectively. The tubes were stoppered immediately with rubber serum stoppers fitted with a center well containing a strip of filter paper which that was saturated with 0.2 ml phenethylamine (New England Nuclear Corp., Boston, MA) to absorb released $^{14}CO_2$. The reaction was stopped by injecting 0.1 ml of 1 N H_2SO_4 into the medium.

Oxidation of [^{14}C]formate by adipocytes was measured in the presence or absence of the hormones, in the same manner as glucose oxidation, exploying 0.2 mM sodium formate (sp. act. 0.12 μCi/μmole), in the absence or the presence of 0.05 mM D-glucose. The reaction was stopped by injecting 0.5 ml of 1 N acetic acid into the medium; alternatively, addition of 0.2 ml sulfuric acid [3] yielded similar results. A blank correction was made by incubating tubes with albumin medium containing labeled formate (including carrier formate) [5, 6].

The effect of the hormones on lipogenesis from glucose was estimated from the incorporation of ^{14}C of the uniformly labeled D-glucose into glyceride glycerol and fatty acids as described before [6, 8, 19, 20]. Briefly, after glucose oxidation the media were filtered on GF/C filters by vacuum suction, and the lipids were extracted from the insoluble materials according to Folch *et al.* [21]. The samples were homogenized in a mixture of chloroform–methanol (2:1, v/v), in a ground glass homogenizer, and 10 ml chloroform and 18 ml water were added for extraction. Suitable aliquots of the chloroform extracts were dried *in vacuo*, redissolved in 8 ml of 5% ethanolic KOH, and heated at 60° for 3 hr. To the cooled fractions, 3.0 ml water was added; they were extracted two times with 10 ml of light petroleum. The combined light petroleum fractions were evaporated to a small volume, and aliquots were counted for radioactivity incorporated into the fatty acid fractions, using Aquasol-2 (New England Nuclear Corp.) as the scintillation fluor. The lipid fractions were quantitated as before [20, 22].

Cellular glutathione (GSH) was estimated by titration with Ellman's reagent [23] as described before [5]. Oxidized glutathione (GSSG) was determined enzymatically, using the principle of Tietz [24]; yeast glutathione reductase (1– ~ 2 μg/ml final concentration) (Boehringer-Mannheim, West Germany) was employed after blocking all GSH with *N*-ethylmaleimide. The GSH estimation was done after adding sodium metaphosphate (10^{-4} M).

Mouse nerve growth factor (2.5 S, mol. wt 13,500 daltons) was purchased from Collaborative Research, Inc., Waltham, MA. Crystalline porcine insulin (essentially free of glucagon), proinsulin, desoctapeptide-insulin and desalanine-insulin were donated by Dr. Ronald Chance of Eli Lilly & Co., Indianapolis, IN. Crystalline bovine serum albumin (fraction V) was purchased from the Sigma Chemical Co., St. Louis, MO. The radioactive chemicals, viz. [1-^{14}C]-D-glucose, [6-^{14}C]-D-glucose, [U-^{14}C]-D-glucose and Na[^{14}C]formate were purchased from the New England Nuclear Corp. 3-Amino-1,2,4-triazole was purchased from the Aldrich Chemical Co., Mil-

* Abbreviations: NGF, nerve growth factor; NADP$^+$ and NADPH, nicotinamide adenine dinucleotide phosphate, oxidized and reduced forms repectively; desalanine-insulin, insulin having the carboxyterminal alanine removed from the B-chain; desoctapeptide-insulin, the insulin derivative having the eight carboxyterminal amino acid residues of the B-chain removed by tryptic digestion; HMP shunt, hexose monophosphate shunt; GSH, glutathione; and GSSG, oxidized glutathione.

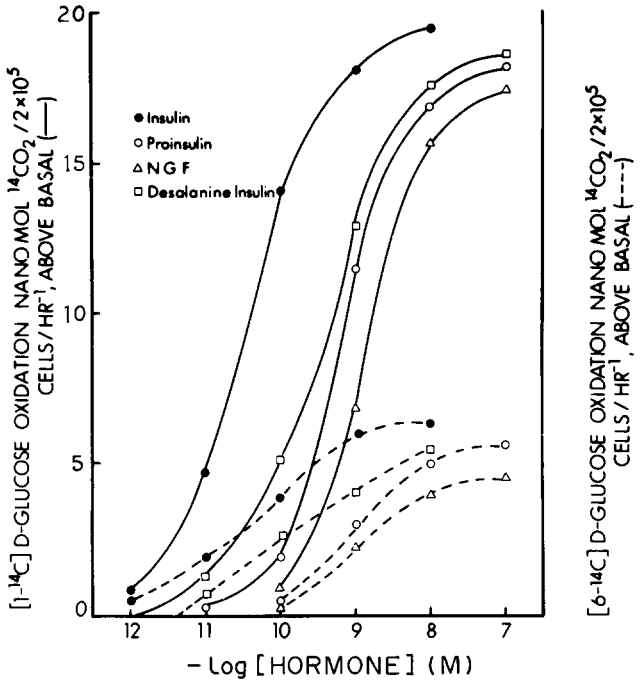

Fig. 1. Dose–response curves of the effects of insulin, proinsulin, NGF and desalanine-insulin on the rates of oxidation of [1-^{14}C]-D-glucose (solid lines) and [6-^{14}C]-D-glucose (broken lines). The basal value of [1-^{14}C]-D-glucose oxidation was 4.42 ± 0.6 and of [6-^{14}C]-D-glucose 2.6 ± 0.7 in four experiments in duplicate. The values are the averages.

waukee, WI. The organic chemicals were purchased from the Sigma Chemical Co., and the inorganic chemicals (analytical grade) were supplied by the Fisher Scientific Co., Raleigh, NC.

RESULTS

Stimulation of glucose metabolism. Glucose oxidation in adipocytes was stimulated by insulin, as expected, and a similar but less pronounced effect was observed with nerve growth factor (NGF). The dose–response curve of NGF was similar to that of proinsulin (Fig. 1 and Table 1). Larger amounts of $^{14}CO_2$ evolved from [1-^{14}C]-D-glucose than from [6-^{14}C]-D-glucose, indicating a primary effect on the hexose monophosphate shunt pathway activity. The predominance of the shunt was also evident from the relatively greater proportion of glucose carbon incorporation into glyceride fatty acid moieties than into glyceride glycerol. Net lipogenesis, on the basis of these data (Table 1), also increased in response to insulin and NGF or proinsulin, corresponding to their relative potencies in stimulating glucose oxidation. The dose–response curves for these hormonal peptides indicated that their relative potencies were: insulin > desalanine-insulin > NGF ≥ proinsulin (Fig. 1). Desoctapeptide-insulin, the insulin derivative which is devoid of eight terminal amino

Table 1. Effects of insulin, proinsulin, nerve growth factor and insulin derivatives on [U-^{14}C]-D-glucose oxidation and lipid synthesis in adipocytes*

Additions	$^{14}CO_2$ Production	^{14}C-Incorporation into total lipids	Glyceride glycerol	Glyceride fatty acids
None	5.6 ± 0.61	10.7 ± 2.4	2.6 ± 0.66	6.8 ± 1.1
Insulin	24.5 ± 5.2†	21.0 ± 2.0‡	4.4 ± 0.92	16.0 ± 2.7†
Desalanine-insulin	18.3 ± 4.0‡	16.4 ± 3.2‡	3.6 ± 0.71	12.2 ± 1.4†
Desoctapeptide-insulin	6.2 ± 1.0	9.8 ± 2.1	2.4 ± 0.61	7.0 ± 0.6
Proinsulin	14.6 ± 3.6†	15.2 ± 2.8‡	3.4 ± 0.56	10.5 ± 2.0‡
Nerve growth factor	12.8 ± 4.2‡	14.8 ± 2.6‡	3.1 ± 0.68	10.8 ± 2.0‡

* The final concentration of [U-^{14}C]-D-glucose was 0.2 mM. The assays of $^{14}CO_2$ evolution and of incorporation of the labeled glucose carbons into the lipid fractions are described in Materials and Methods. Additions: insulin, 10^{-9} M; desalanine-insulin, 10^{-8} M; desoctapeptide-insulin, 10^{-8} M; proinsulin and NGF, 10^{-8} M. The values are expressed as nmoles·(2 × 10^5 cells)·2 hr^{-1}, and the values are the averages ± S.E. of four experiments in duplicate.

† $P < 0.001$ versus control.

‡ $P < 0.02$ versus control.

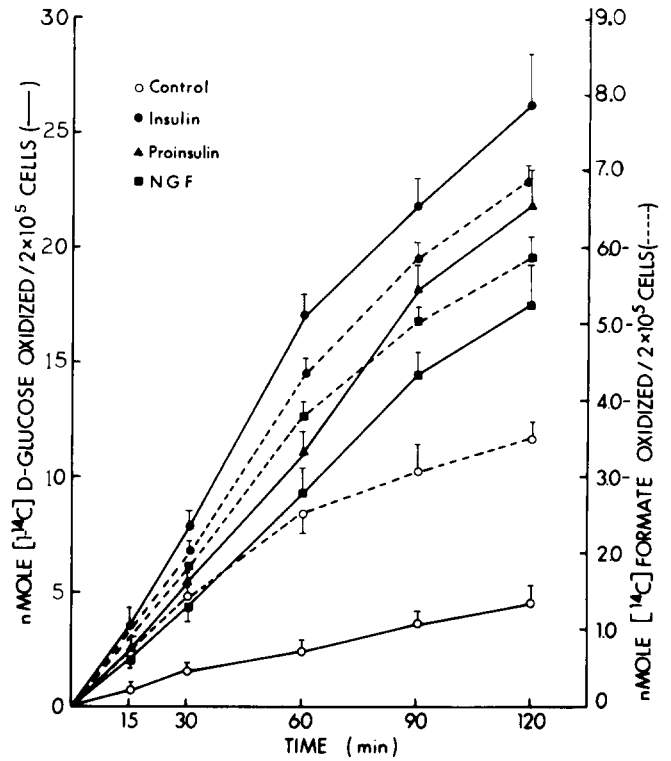

Fig. 2. Time courses of glucose and formate oxidation in adipocytes in the presence or absence of insulin (5×10^{-9} M), proinsulin (10^{-8} M) or NGF (10^{-8} M), at 37°. Values are the averages ± S.D. of three experiments in duplicate.

acid residues of the B-chain and has been reported to be biologically inactive [3, 25], had no detectable effect on glucose oxidation. The basal, was well as the hormone-stimulated, rate of glucose oxidation continued in a linear fashion for at least 90 min and was measured for 2 hr (Fig. 2).

Formate oxidation. The peroxidatic reaction of the catalase:H_2O_2 complex was assayed for intracellular H_2O_2 metabolism, employing H[^{14}C]OONa as a substrate. As with insulin, stimulation of this rate was also found in the presence of NGF, proinsulin or desalanine-insulin, and their dose–response curves (Fig. 3) indicate potencies similar to those when stimulating glucose oxidation (Fig. 1).

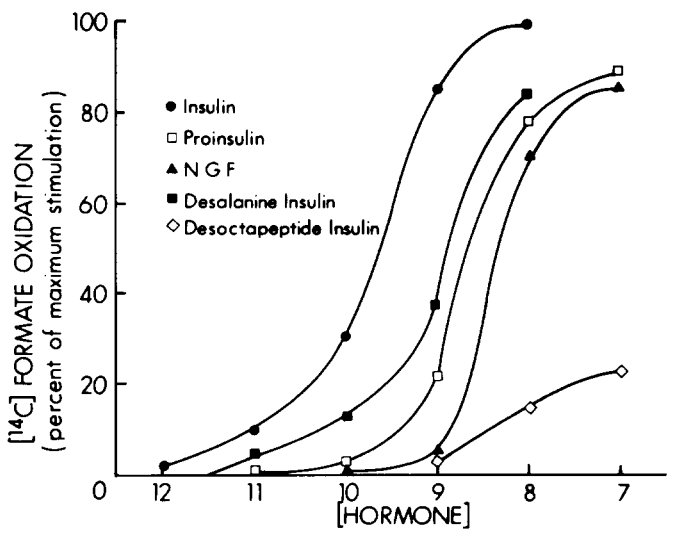

Fig. 3. Dose–response curves of the effects of insulin, insulin derivatives, and NGF on [^{14}C]formate oxidation. Values are the averages of a typical experiment in triplicate. Units on the abscissa are the negative logarithms of the molar concentrations.

Table 2. Relative effects of insulin, insulin derivatives, and nerve growth factor on H[^{14}C]OONa oxidation by adipocytes*

Additions	$^{14}CO_2$ Production from [^{14}C]formate [nmoles·(10^6 cells)$^{-1}$ hr^{-1}]			
	Only hormone (A)	Percent change	+ Aminotriazole (B)	B as percentage of A
None	2.6 ± 0.32	100	2.2 ± 0.14	84
Insulin	4.72 ± 0.5	181	3.6 ± 0.14	76
Proinsulin	3.24 ± 0.41	125	2.6 ± 0.08	80
NGF	3.3 ± 0.4	126	2.5 ± 0.12	76
Desalanine-insulin	3.7 ± 0.22	142	2.9 ± 0.14	78
Desoctapeptide-insulin	2.9 ± 0.24	112	2.25 ± 0.11	78

* The final concentration of sodium formate was 0.2 mM in the presence of 0.1% bovine albumin. Insulin was added to a final concentration of 5×10^{-9} M, and proinsulin, NGF, desalanine-insulin or desoctapeptide-insulin were added to the maximal concentration of 10^{-7} M each. 3-Amino-1,2,4-triazole was 50 mM where indicated. The values are the averages ± S.E. of five experiments in duplicate.

Desoctapeptide-insulin had a marginal, but significant effect ($P < 0.05$), having increased the rate by about 15–20% of the maximum level (Fig. 3); it failed, however, to elicit any glucose oxidation. The small increase in formate oxidation due to this agent was attributed to very low NADPH oxidase activity [3] and H_2O_2 production.

It was shown previously [4–6] that in insulin-stimulated cells the catalase:H_2O_2 compound I is partly converted to compound II, which has no effect on catalatic activity, retaining only the peroxidatic mode of action. Compound II is irreversibly inhibited by 3-amino-1,2,4-triazole. In the present study, aminotriazole caused greater inhibition of formate oxidation in cells stimulated by insulin, as well as by proinsulin, NGF or desalanine-insulin (20–25%), than in control cells (10–15%) (Table 2). This provides an estimate of the increased accumulation of H_2O_2 in this state (compound II) [26, 27]; the larger part of formate oxidation continued, however, catalyzed by catalase:H_2O_2 compound I. Under this condition, the major part of the H_2O_2 generated by the NADPH oxidase activity can be decomposed by the glutathione peroxidase activity [4–6]. Subsequent reduction of the oxidized glutathione (GSSG), catalyzed by the NADPH-dependent glutathione reductase, causes a replenishment of the cytosolic $NADP^+$, raising the $NADP^+$/NADPH ratio, which is a critical requirement for the elevation of the hexose monophosphate shunt activity. Apparently, sufficient H_2O_2 did not accumulate in response to desoctapeptide-insulin.

Sodium nitrite, a substrate that competes for the peroxidatic action of the catalase:H_2O_2 complex, maximally reduced the net oxidation of formate in both basal and hormone-stimulated cells, at a concentration of 10^{-2} M (Fig. 4A). Formate oxidation was not inhibited completely, however, in the presence of this competitive inhibitor, which indicates that the former was the preferred substrate. But sodium azide could completely inhibit formate oxidation at a concentration of 5 10^{-3} M (Fig. 4B). This illustrates that formate oxidation was dependent entirely on the catalase:H_2O_2 peroxidatic reaction, since azide is a potent inhibitor of the hemoprotein catalase. It was shown previously that nitrite or azide does not inhibit [1-^{14}C]-D-glucose oxidation [5] or the NADPH oxidase activity in the isolated adipocyte plasma membrane [3].

Relationship with glucose transport. The generation of H_2O_2 in response to insulin, proinsulin or NGF occurred even in the complete absence of glucose in the medium, although an optimal increase was noted in the presence of a low range (0.04 to 0.3 mM) of D-glucose concentrations (Fig. 5). At higher concentrations of D-glucose, the rate of formate oxidation declined, apparently due to the regeneration of additional reducing equivalents, especially glutathione [5] which may serve as an alternative source of electrons [6]. This agrees with our earlier evidence that the stimulatory effect of insulin on NADPH oxidase-linked H_2O_2 production and on hexose monophosphate shunt activity is essentially independent of the effect of the hormone on the saturable D-glucose transport component [2–6, 11]. The present data also illustrate that the sensitivity of the formate oxidation method for the detection of intracellular H_2O_2 accumulation depends on the intracellular redox optimum for the plasma membrane NADPH oxidase activity. Apparently, at higher concentrations of glucose, it is the glutathione peroxidase reaction, coupled with the hexose monophosphate shunt activity, that is the predominant pathway of H_2O_2 utilization. We observed that hormonal stimulation of plasma membrane NADPH oxidase was not inhibited and, in fact, was better sustained if the cells were preincubated with D-glucose (0.2 to 5.5 mM), an effect which we attribute to regeneration of intracellular NADPH, the substrate of the oxidase (S. P. Mukherjee, unpublished results). Moreover, formate did not compete for the D-glucose transport system in assays employing 3-*O*-[^{3}H]methyl D-glucose (not shown), which suggests that formate oxidation as an index of intracellular H_2O_2 accumulation is distinct from the known stimulatory effect of insulin on the glucose carrier system. In light of this evidence, it does not appear that the stimulation of H_2O_2 formation by insulin or NGF is secondary to increased D-glucose transport; rather, the hexose transport component

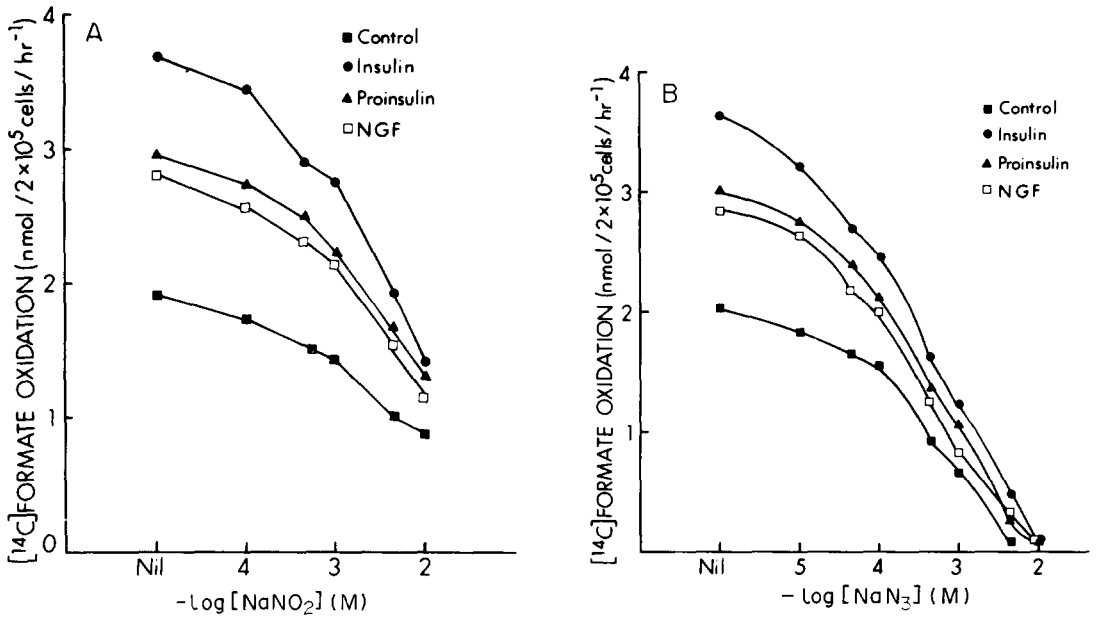

Fig. 4. (A) Inhibition by sodium nitrite, at increasing concentrations, of the basal and stimulated rates of [^{14}C]formate oxidation in adipocytes. Additions: insulin, 5×10^{-9} M; proinsulin and nerve growth factor, each at 10^{-8} M. Values are the averages of a typical experiment in triplicate. (B) Inhibition of basal and hormone-stimulated rates of [^{14}C]formate oxidation in adipocytes at increasing concentrations of sodium azide. The other details are the same as in panel A.

may be activated by the NADPH oxidase reaction (see Discussion).

Glutathione dynamics of the stimulated cells. To evaluate the relationship between H_2O_2 formation and glucose oxidation, changes in the cellular contents of glutathione (GSH) and its oxidized form (GSSG) in response to insulin, proinsulin or nerve growth factor were followed with time. The hormones appeared to cause an increased level of GSH during 1 hr of incubation, even in the absence of glucose, while this process was accelerated in the presence of an optimal concentration of D-glucose (0.5 mM). Evidently, glucose utilization through the hexose monophosphate shunt generated more NADPH which could support the glutathione reductase activity. We have found that in adipocytes the glutathione reductase is mostly dependent on NADPH. However, the GSSG content remained low, despite increases corresponding to the hormonal stimulations, so that the GSH/GSSG ratio remained nearly 80, under all the steady states examined. It is possible that a part of the glutathione disulfides were formed into mixed disulfides with intracellular proteins, or some of the GSH increase may have been due to *de novo* synthesis triggered by the mild oxidative stress of hormone-stimulated oxygen consumption, especially in the absence of glucose. Our present data do not resolve these alternative possibilities, but studies are in progress. A correlation between GSH regeneration and the active pentose phosphate pathway is evident from these results; insulin as well as NGF or proinsulin, in the presence of glucose, caused a profound increase in the cellular GSH level. This effect of the hormones was equipotent with their abilities to stimulate glucose oxidation. The data in Table 3 represent the effects of maximum concentrations of the respective hormones.

DISCUSSION

The evidence of hormone-stimulated H_2O_2 production in metabolically active peripheral tissue, especially in insulin-sensitive cells, increases our understanding (i) that the plasma membrane of such cells is a primary site of an electron transport system and (ii) of endogenous H_2O_2 production as a general

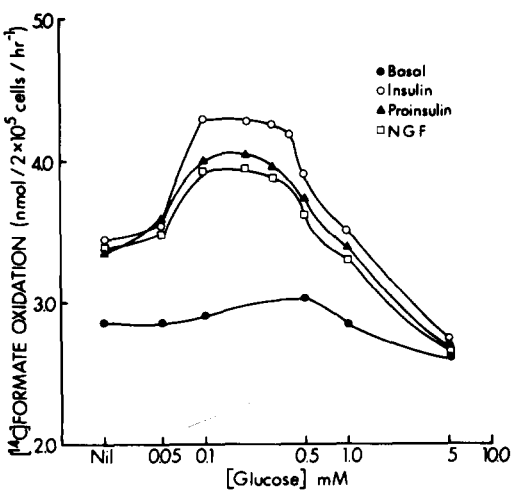

Fig. 5. Inhibition of hormone-stimulated formate oxidation in the presence of increasing D-glucose concentrations in the medium. Values are the averages of three experiments in duplicate.

Table 3. Changes in the net content of glutathione in adipocytes and the media in the presence of insulin, proinsulin and NGF*

Additions	Initial content (0 time) (nmoles/10^7 cells + medium) GSH	GSSG	Content at 60 min (nmoles/10^7 cells + medium) GSH	GSSG
Control	17.0	0.14	18.6	0.19
Control + D-glucose	18.5	0.16	24.0	0.22
Insulin	19.0	0.17	29.0	0.67
Insulin + D-glucose	21.0	0.16	52.0	0.24
Proinsulin	18.0	0.15	26.0	0.36
Proinsulin + D-glucose	16.0	0.15	38.0	0.3
NGF	19.0	0.2	28.0	0.42
NGF + D-glucose	21.0	0.17	36.0	0.28

* The total GSH and GSSG contents of the cells plus the medium at the initial (0 time) and 60-min time points were measured as described in Materials and Methods, in separate 1.0 ml aliquots of cell suspension which were immediately chilled, homogenized and centrifuged. The final concentration of insulin was 10^{-9} M; proinsulin 5×10^{-7} M; and nerve growth factor (NGF), 5×10^{-8} M. D-Glucose, where indicated, was 5×10^{-4} M. The values are the averages of two consecutive experiments in duplicate in which the detectable experimental variations were within 5–10%.

mechanism for cellular activation, rather than as a toxic effect. Although some of the insulin-like effects of NGF on its primary target tissues have been recognized previously, the mechanism of these actions was obscure. However, a striking homology in the amino acid sequence of the primary subunit of mouse NGF, human proinsulin and guinea pig insulin [17] reflects a probable common molecular ancestry of these polypeptide hormones in the process of evolution. The similarity in their mechanisms of action on the adipocyte as a model system reinforces our original conviction that H_2O_2 production is an important transmembrane signal of insulin [2–6, 8–13] or other mitogenic agents [4, 8]. It is possible that their structural homology is important in receptor recognition and transmission of their message within the cells. The structure–activity relationships amongst the insulin derivatives, viz. desalanine-insulin and desoctapeptide-insulin and insulin, proinsulin or NGF suggest that there is a correlation between their relative affinities towards the insulin receptors, as studied before [28], and their potencies in stimulating H_2O_2 formation. The coupling of plasma membrane NADPH oxidase with insulin receptors is evident also from the recent study showing that antibodies raised against the purified rat hepatocyte insulin receptors [29] can activate this oxidase in direct proportion to their stimulation of glucose transport and oxidation [30]. Thus, a specific ligand–receptor interaction has been found to generate this "messenger".

Subsequent studies [31–34] have, in general, confirmed our theory. Our hypothesis has been that endogenous H_2O_2 may be fundamental to growth promotion and ordered cell proliferation, in addition to being a mediator of insulin [3–6, 31]; this also may explain the *in vivo* nerve growth due to NGF [35].

In earlier studies by Lavis *et al.* [36], proinsulin was found to be nearly 20 to 25-fold less potent than insulin in its effect on glucose oxidation in adipose tissue. Their evidence agrees closely with the observation of Cuatrecasas [28] that proinsulin has about 20-fold less affinity than insulin toward the insulin receptors in rat adipocytes in competition binding studies employing [^{125}I]-insulin. Although nerve growth factor has not been so characterized before, Foppen *et al.* [16] demonstrated that NGF, at 0.02 μg/ml (approximately 10^{-9} M) *in vitro*, causes increased formation of various classes of phospholipids in the isolated sensory ganglia of chick embryo. The potency of NGF in adipocytes appears to be in the same range in our present studies.

Desoctapeptide-insulin, which was reported previously to be biologically inactive [3, 25, 28] was found to be without any appreciable effect on glucose oxidation in our studies (Table 1). One group of investigators [37] showed, however, that this peptide can stimulate glucose oxidation in fat cells nearly to the same extent as insulin, at a concentration of 10^{-9} M. From our own experience, as well as from the results of Bromer and Chance [25], it is difficult to explain such discrepant data [37]. However, we have observed a small but significant increase in formate oxidation in the presence of this peptide. This effect actually reflects the occurrence of a small concentration of H_2O_2 production, which diffuses into the peroxisomal sites, and is consistent with our previous observation that desoctapeptide-insulin has only a small stimulatory effect on plasma membrane oxidase [3]. These data also illustrate, therefore, the sensitivity of the formate oxidation assay in detecting H_2O_2, especially when it accumulates in low concentrations as catalase:H_2O_2 complex [6, 26, 27]. In adipocytes stimulated by insulin or analogous polypeptides, the formation of the catalase:H_2O_2 compound II, which is inactive catalytically but retains the peroxidatic function [26, 27], is evident from the relatively greater inhibition of formate oxidation in these stimulated cells than in the controls, in the presence of aminotriazole (Table 2). Under this condition, the glutathione peroxidase reaction appears to play a major role in the decomposition of the accumulating H_2O_2 [4–6]. Considered mechanistically, this type of control would enable cells to utilize

H_2O_2 for a beneficial effect on cell metabolism, viz. an increased hexose monophosphate shunt activity.

The ability of these cells to stimulate H_2O_2 production in fat cells is essentially independent of glucose in the medium and, therefore, of an operating glucose transport. With increasing availability of D-glucose, however, formate oxidation declines, apparently due to regeneration of cytosolic glutathione (GSH) as a likely electron donor system [38–43], and thus imposes a competitive inhibition. Such competitive inhibition can be observed by employing sodium nitrite. However, formate appears to be the preferred substrate for this reaction, since it is not completely inhibited in the presence of nitrite (Fig. 4A); this may be attributed to the resonance stabilization of the nitrite molecules. Complete inhibition of formate oxidation in the presence of sodium azide (Fig. 4B) indicates that the reaction is indeed catalyzed by the peroxidatic action of catalase which is inactivated by the azide.

Stimulation of glucose oxidation via the hexose monophosphate shunt is critically dependent upon the activation of glucose-6-phosphate dehydrogenase by the mass-action of an elevated $NADP^+$/NADPH ratio, but the estimated NADPH/$NADP^+$ ratio in the resting state of most actively metabolizing cells has been observed to remain near 80 [44]. A drastic reversal of this condition is, therefore, essential for the stimulation of this pathway activity. The NADPH oxidase activity in the plasma membrane [2, 3] as well as the glutathione dynamics of the cells, first shown by Mukherjee and Lynn [39–41], can conjointly fulfill the above requirement. Our estimation of the changes in the GSH level of these cells in response to insulin, proinsulin or NGF (Fig. 1 and Table 3) indicates its parallel relationship with the stimulation of [1-^{14}C]-D-glucose oxidation. The GSSG content of these cells did not account for the total change in the GSH level. As a result, the GSH/GSSG ratio remained over 80 under all the steady states examined, despite a corresponding rise in the GSSG content with a decline in GSH. It remains likely that some of the glutathione may be involved in the formation of mixed disulfides with cellular protein components and, therefore, is inaccessible to the glutathione (−NADPH) reductase reaction [45]. The high levels of GSH in these cells seem to have a regulatory role in many metabolic functions, especially in their hormonal response and adenylate cyclase activity [41, 42]. Specific oxidants and titrants of cellular GSH cause a profound stimulation of the hexose monophosphate shunt activity [3, 13, 41], deactivation of adenylate cyclase activity [41, 43], inhibition of epinephrine-stimulated lipase [6] and stimulation of the intramitochondrial enzyme, pyruvate dehydrogenase [8, 9, 12], in adipocytes. On the other hand, H_2O_2 is not an effective agent for non-enzymatic oxidation of NADPH [3]. While the evidence strongly supports a physiological role for hormone-stimulated H_2O_2 production as insulin's transmembrane signal, we recognize that this is a different kind of "second messenger" from the adenylate cyclase-cAMP system. In contrast to cAMP which acts catalytically, H_2O_2 can act through an alteration of the intracellular redox potential. This fact is exemplified in our data that insulin or concanavalin A, as well as low concentrations of exogenous H_2O_2 or *tert*-butyl peroxide, causes an early and rapid increase in intracellular pH in both adipocytes and thymocytes ([46] and our unpublished results). Thus, important cellular regulation by means of the pH gradient is also accomplished through H_2O_2 formation [46].

The correlation between hormone-stimulated H_2O_2 production and lipogenesis (Table 1), which was originally presented in 1976 [2], is of more than passing interest. It shows that the primary event in the plasmalemma, triggered by growth-promoting factors such as NGF, has a regulatory role in the distal intracellular effects. We now have the evidence, which will be published separately, that both exogenous H_2O_2 and the endogenous H_2O_2 production in response to insulin, oxytocin, Arg^8-vasopressin or NGF are involved in the activation of the lipogenic steps beyond the pyruvate dehydrogenase complex [8, 9, 12, 31], viz. acetyl CoA carboxylase and fatty acyl CoA ligase (C. Mukherjee and S. P. Mukherjee, unpublished results). Some authors have considered previously [47, 48] that the stimulation of the hexose monophosphate shunt by insulin is only secondary to the activation by the hormone of lipid synthesis pathways. Although this conclusion is partly valid only under the insulin-deficient conditions which they studied, it is difficult to reconcile the acute stimulation by insulin of the HMP shunt with the idea of a primary effect of the hormone on lipogenesis and, therefore, a "pull" from increased fatty acid synthesis. In point of fact, however, the plasma membrane-linked NADPH oxidase–H_2O_2 tandem is the only defined mechanism through which a primary change in the cytoplasmic redox potential can be achieved [2–4, 46]. In consonance with this is the observation that the insulin-refractory state of the large adipocytes of obese rats is accompanied by a reversible impairment of the plasma membrane oxidase [49, 50]. Another study [51] of lipogenesis during insulin deficiency [47] essentially followed and echoed the previous studies [47, 48] on the HMP shunt pathway.

A preeminent site of the effect of the H_2O_2 generated within the plasma membrane may be the saturable D-glucose transport component, the activation of which by insulin has been presumed by many authors to involve conversion of some reactive sulfhydryl groups in the hexose "carriers" to the disulfide state [52–57]. This model of activation of the sugar transport system has been applied to various cell types which are either dependent on, or independent of, insulin [57]. Although evidence in support of this model has been presented in our studies employing the non-permeating sulfhydryl oxidant, *o*-iodosobenzoate, and the -SH blocker, *p*-chloromercuribenzoate [5], it appears that the net transport activity depends on a distinct enzymatic process, viz. the NADPH oxidase [38, 39]. This inference was deduced from the findings [58, 59] that stimulation of the hexose monophosphate shunt by insulin and several insulin-mimicking agents is essentially independent of their effects on the redox state of the saturable D-glucose transport system, under the defined experimental conditions which permit only negligible non-specific diffusion. It is apparent

that the NADPH oxidase activity may furnish the necessary free energy for net glucose translocation, although the physiological action of insulin is presumably the result of both this process and the activation of the saturable transport component(s). Indeed, transport mechanisms such as the one regulated by the NADPH oxidase in our system, are reckoned in biology as "secondary active transport" which is independent of synthesis or breakdown of high-energy bonds. We have obtained more definitive evidence in support of this theory (S. P. Mukherjee, unpublished results).

At this time some evidence clearly suggests a wide spectrum of metabolic roles that endogenous H_2O_2 may fulfill. While on the cell surface it may cause activation of the saturable glucose transport component, H_2O_2 also appears to be involved in the coupling of catecholamine and glucagon receptors with the catalytic unit of adenylate cyclase in fat cells [40, 42]. On the other hand, intracellular accumulation of H_2O_2 may have such diverse effects as deactivation of adenylate cyclase [40–43] and hormone-stimulated lipase [6], stimulation of the hexose monophosphate shunt and activation of the lipogenic pathways [3–6] as discussed above. Since mammalian brain is known to be dependent on glucose utilization for its energy supply, the implication of the present findings in brain or other neural tissue metabolism and growth is suggestive. The generality of the primary role of the NADPH oxidase-catalyzed H_2O_2 production in the plasma membrane, in the activation of quiescent cells, is emerging from our findings that the same activity is stimulated in thymocytes by mitogenic stimuli [10] or in human neutrophils by the chemotactic peptides [60], apparently to serve a regulatory role. Although some details of these controls remain to be explored, a fundamental mechanism of cell regulation is becoming clear from these studies.

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