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MEDIATION OF THE ANTILIPOLYTIC AND LIPOGENIC EFFECTS OF INSULIN IN ADIPOCYTES BY INTRACELLULAR ACCUMULATION OF HYDROGEN PEROXIDE*

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Abstract—Exposure of rat adipocytes to insulin causes activation of a pyridine nucleotide oxidase at the internal side of the plasma membrane, generating hydrogen peroxide [S. P. Mukherjee and W. S. Lynn, *Fedn Proc.* **35**, 1694 (1976); S. P. Mukherjee and W. S. Lynn, *Archs Biochem. Biophys.* **184**, 69 (1977)]. Evidence was also presented that intracellular utilization of H_2O_2 by the peroxidative pathways of glutathione peroxidase and catalase is coupled with glucose oxidation via the pentose phosphate pathway [S. P. Mukherjee, R. H. Lane and W. S. Lynn, *Biochem. Pharmac.* **27**, 2589 (1978)]. The relationship between the glucose-independent insulin effect on H_2O_2 production and its metabolic role is evaluated on the basis of formate oxidation, lipogenesis, antagonism of lipase activity and lowering of cellular levels of cyclic 3′,5′-adenosine monophosphoric acid (cAMP). These measures of the effects of insulin, observed at low concentrations of glucose, were reversed at higher concentrations of glucose (over 0.3 mM). Exogenous H_2O_2 had metabolic effects similar to insulin. Addition of H_2O_2 (10^{-4} M and higher) to the extracellular medium caused a substantial inhibition of epinephrine-stimulated and adrenocorticotropin-stimulated depot-fat lipase activity in these cells, measured by net glycerol production. H_2O_2 also increased lipogenesis by increasing the provision of the substrates and cofactor (NADPH) and activating pyruvate dehydrogenase in the same manner as insulin. Exogenous catalase (16 μg/ml) abolished the insulin-like effects of H_2O_2 (but not of insulin itself) on glucose oxidation, the contribution of glucose carbons to glyceride fatty acids and glyceride glycerol, the inhibition of lipolysis, and the time-dependent decline in the cAMP content of the cells. The data suggest that, while insulin-stimulated H_2O_2 production from the plasma membrane may bring about some glucose-independent metabolic effects through a lowering of cytoplasmic redox potential, increasing availability of D-glucose may counterbalance these effects by replenishing the reducing equivalents. Endogenous H_2O_2 appears, therefore, to satisfy the manifold criteria for a 'second messenger' of insulin.

The manifold metabolic effects of insulin, which are, in general, antagonistic to those of catecholamines in target cells such as adipocytes, raise the possibility of generation of a common regulatory molecule in response to insulin [1, 2]. Intense efforts are directed at the identification of such a distinct molecule or cell component, and several metabolites or chemical agents are found to mimic some, but not all of the biological effects of insulin. To understand the molecular mechanism of the actions of insulin, a biochemical process has to be revealed which represents an integral regulation of the various responses, e.g. glucose transport and intracellular enzymatic activities which are apparently independent of glucose uptake [1–3].

A possible mechanism involving sulfhydryl oxidation in the plasma membrane components was indicated by the finding that insulin-dependent activation of glucose transport in isolated cells [4] or tissues [5–7] requires reactive sulfhydryl (−SH) groups. Attempts to confirm this model using H_2O_2 and the *S*-alkylating agent *N*-ethylmaleimide [8] had the limitation that both of these agents alter intracellular enzyme activities. For example, H_2O_2 activates and *N*-ethylmaleimide inhibits cytosolic glutathione peroxidase and glutathione reductase, among others [9]. Thus, it is impossible to conclude from such data [8] whether the effects represent a primary action of insulin on the plasma membrane or are secondary to intracellular changes. A new insight developed more recently with the finding that an enzyme, NADPH oxidase, in the plasma membrane of adipocytes is activated when these cells are exposed to insulin or to various agents mimicking some of the actions of insulin, e.g. sulfhydryl reagents, thiols [10, 11] and concanavalin A [12], yielding H_2O_2 as the product. Further evidence has also been presented that these cells have efficient metabolic routes for H_2O_2 catabolism [13] for the final control of altered cytosolic redox potential and enhanced glucose utilization via the hexose monophosphate shunt and adenylate cyclase activity [13–15]. In addition, this may account for the differential saturation of the hexose monophosphate shunt independently of the effect of insulin on carrier-mediated glucose transport [16, 17]. Thus, a redox phenomenon in regulating glucose transport and utilization, as well as other plasma membrane functions, e.g. adenylate cyclase, responding to hormonal

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stimulation, was identified for the first time and characterized. On the basis of the unique metabolic roles played by the product of this reaction, viz. hydrogen peroxide (H_2O_2), I suggested at the Federation meetings of 1976 that it may serve as the second messenger for insulin. This hypothesis interested Dr. C. deHaen who subsequently furnished some supportive evidence for intracellular accumulation of H_2O_2 [18] as was shown previously by us [13].

The present studies preceded our work at Duke University Medical Center [10–17] and were undertaken: (i) to investigate if adipocytes have a H_2O_2 generating system comparable to that of other cells, e.g. inflammatory cells [19, 20] which have a predominant hexose monophosphate shunt activity characteristic of insulin-stimulated adipocyte metabolism [21, 22]; and (ii) to examine if such a mechanism might influence hormone-responsive metabolic events such as lipolysis. The data suggest that intracellular accumulation of H_2O_2 in response to insulin may account for the antagonism by insulin of epinephrine-stimulated and ACTH-stimulated depot fat lipase activity. A correlation between enhanced glucose oxidation and lipogenesis in the presence of exogenous H_2O_2, together with other recent data cited above [13], strongly suggest that endogenous H_2O_2 may be a natural 'messenger' for the various metabolic effects of insulin.

MATERIALS AND METHODS

Adipose cells were obtained from male Wistar rats (150–200 g) of an inbred colony of the CDRI (Lucknow) strain. The cells were isolated by digesting the pooled fractions of epididymal fat pads with collagenase (*Clostridium histolyticum*) at a concentration of 1 mg of crystalline enzyme/ml of medium (3% bovine serum albumin in Krebs–Ringer bicarbonate buffer) according to the method of Rodbell [23]. An optimum digestion of the tissue, measured by the response of adipocytes to insulin, was attained by incubating for 50 min at 37° on a Dubnoff metabolic shaker. The composition of the buffer was: 126 mM NaCl, 5.2 mM KCl, 1.4 mM $MgSO_4$, 4 mM Na_2HPO_4 and 12 mM $NaHCO_3$. The buffer was pre-gassed with a mixture of O_2–CO_2 (95:5, v/v). At the end of the digestion, the cells were filtered through a double layer of cheesecloth, washed three times with approximately 4 vol. of 1% albumin-buffer, and suspended in this medium. Glucose oxidation was assayed in polyethylene tubes fitted with rubber stoppers, employing [U-^{14}C]-D-glucose at final concentration of 0.2 mM. The $^{14}CO_2$ which was generated over a time course of 1–3 hr was absorbed into the strip of filter paper in the center well which was soaked previously with 0.2 ml hyamine hydroxide. The reaction was stopped by injecting 0.2 ml of 1 N H_2SO_4. The media were filtered and the insoluble fractions on the filter papers were extracted for lipids according to the method of Folch *et al.* [24], as described previously [25]. Briefly, the insoluble materials 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* and redissolved in 8.0 ml of 5% ethanolic KOH and heated at 60° for 3 hr. To the cooled fractions, 3.0 ml water were added and the samples were extracted two times with 10 ml of light petroleum ether (b.p. range 40–60°). The petroleum fraction was separated and the remaining ethanolic KOH layer was acidified with 1.0 ml of 10 N H_2SO_4 and extracted three 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 fraction. The remaining aqueous portion was measured directly for radioactivity in the glyceride glycerol fraction, using Bray's cocktail as the scintillation fluor.

Oxidation of sodium [^{14}C]formate, as the index of the intracellular catalase: H_2O_2 complex, was measured using the same procedure as for D-glucose oxidation and as described previously [11], with the exception that the formate concentration was 0.5 mM. Unlabeled D-glucose was added to the assay medium at 0.1 mM in order to provide substrate for the alternative peroxidative pathway, glutathione peroxidase, which appears to be coupled to the pentose phosphate shunt pathway of glucose oxidation via NADPH-GSSG reductase activity. Blank corrections were made for any possible carbonate contamination by measuring oxidation of H[^{14}C]OONa, in the absence of cells, as described previously [13]. The blank values also indicated the presence of a Cu^{2+}-dependent peroxidase activity in some preparations of bovine albumin, which could be inhibited by adding 1 mM 1,10,-diphenyl *o*-phenanthroline.

Lipase activity was assayed for measuring the net production of glycerol enzymatically, according to the method of Wieland [26], in the neutralized perchlorate homogenates of cells and media. The differences between the glycerol content of the cells at O time and the sum of the free glycerol in the cells and the media at various time periods of incubation are expressed as depot fat lipase activity, on the assumption that these cells have a very weak glycerol kinase activity to reutilize the released glycerol [27].

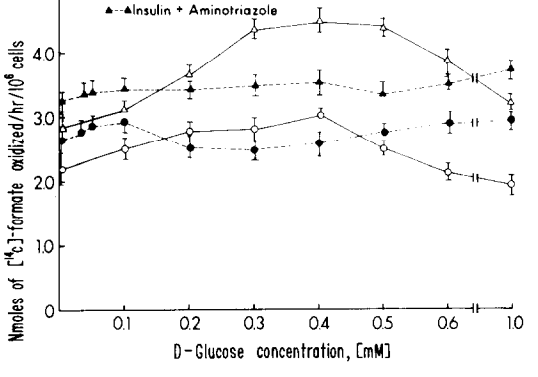

Fig. 1. Sodium [^{14}C]formate oxidation and its inhibition in the presence of 4-amino-1,2,4-triazole (50 mM) in the presence or absence of insulin, at varying concentrations of D-glucose in the medium. Insulin concentration: 1.2 nM.

The tissue concentrations of cyclic 3′,5′-adenosine monophosphoric acid (cAMP) were estimated according to Brown *et al.* [28], using assay kit TRK432 from Amersham, Arlington Heights, IL.

The radiochemicals, [U-^{14}C]-D-glucose and H[^{14}C]OONa, were purchased from the Amersham Nuclear Corp., Amersham, U.K. The reagents used for the enzymatic assay of glycerol, viz. adenosine 5′-triphosphate (ATP), nicotinamide adenine dinucleotide (DPN^+), glycerol kinase and α-glycerophosphate dehydrogenase were purchased from the Sigma Chemical Co., St. Louis, MO. Crystalline crude collagenase was from the Worthington Biochemical Corp., Freehold, NJ. 4-Amino-1,2,4-triazole was purchased from the Aldrich Chemical Co., Milwaukee, WI. The inorganic chemicals, all of analytical grade, were the preparations of E. Merck & Co., Germany and British Drug House.

RESULTS

Increased production of H_2O_2 and its intracellular accumulation in adipocytes in response to insulin are evident from the data on $^{14}CO_2$ production from H[^{14}C]OONa in these cells (Fig. 1). Oxidation of formate is a catalase-dependent peroxidative reaction, and represents the amount of H_2O_2 that diffuses into the intracellular peroxisomes [13, 29, 30]. The inhibition of this reaction by the catalase inhibitor 4-amino-1,2,4-triazole was also greater in the insulin-treated cells than in the untreated controls, as illustrated in Fig. 1. This indicates an increased occurrence of the catalase : H_2O_2 complexes [29, 30]. The formation of H_2O_2 in these cells is independent of glucose in the medium, as shown previously [11]. However, an optimal concentration of D-glucose appeared to support the insulin-stimulated formate oxidation. This effect of glucose was observed to be maximal at about 0.3 mM. Increasing the glucose concentration had a gradual inhibiting effect on insulin-stimulated formate oxidation. This effect of glucose was noticed at the formate concentration of 0.5 mM, which is much lower than its maximal (saturating) level [18]. Aminotriazole, the inhibitor of the catalase-dependent peroxidase activity, permitted a small but significant ($P < 0.01$) increase in formate oxidation in the absence of glucose. This can be explained by a possible competition for substrates between catalase and glutathione peroxidase. At optimal glucose concentration, permitting the regen-

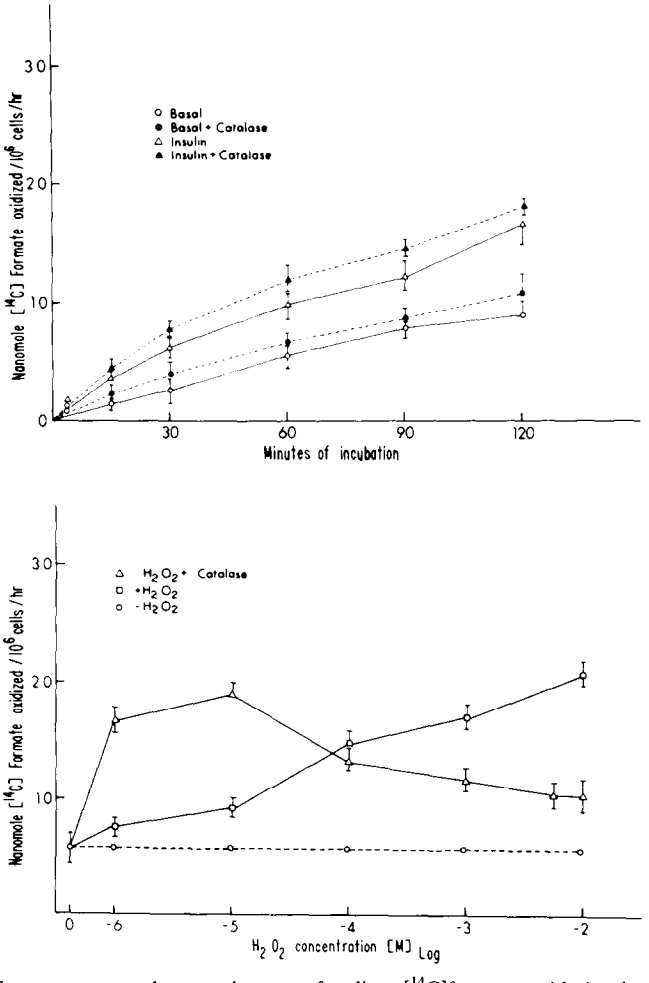

Fig. 2. Effects of exogenous catalase on the rate of sodium [^{14}C]formate oxidation in adipocytes in the presence or absence of insulin (top panel) and of H_2O_2 at different concentrations (bottom panel). The final effective conc. of H_2O_2 was estimated to be 0.6 times the indicated log values.

Table 1. Effects of insulin and exogenous H_2O_2 on glucose utilization and lipogenesis in adipocytes*

Additions	$^{14}CO_2$ from [U-^{14}C]-D-glucose	Total lipids (nmoles/hr/10^6 cells)	Glyceride glycerol	Glyceride fatty acid
None (control)	8.6 ± 1.4	21.0 ± 3.2	6.6 ± 0.7	14.0 ± 2.4
Catalase	9.4 ± 1.3	20.6 ± 2.5	5.9 ± 1.2	14.6 ± 1.7
Insulin	34.5 ± 2.2	46.6 ± 3.7	11.4 ± 0.94	34.0 ± 2.6
Insulin + catalase	36.0 ± 3.1	44.3 ± 4.1	11.0 ± 1.6	32.0 ± 2.0
H_2O_2 (10^{-4} M)	31.0 ± 2.6	38.0 ± 3.6	10.2 ± 1.7	28.0 ± 0.6
H_2O_2 (10^{-4} M) + catalase	9.3 ± 1.4	22.0 ± 2.7	6.4 ± 1.7	16.0 ± 1.4
H_2O_2 (10^{-3} M)	37.4 ± 3.6	42.4 ± 4.6	11.7 ± 0.5	31.0 ± 2.6
H_2O_2 (10^{-3} M) + catalase	10.6 ± 2.1	24.7 ± 3.6	7.0 ± 1.2	16.0 ± 1.3

* Values are averages ± S.E. of three experiments in duplicate, using uniformly labeled D-glucose (0.5 mM). Catalase was added at 16 μg/ml.

eration of glutathione through the glutathione reductase pathway (NADPH-GSSG reductase), the glutathione peroxidase is possibly saturated with its preferred substrate, GSH. Aminotriazole inhibition of catalase-dependent formate peroxidation is best revealed under these conditions. At a relatively high but physiological glucose concentration, increasing availability of GSH in the cells may compete with formate for the catalase reaction, which should be released in the presence of aminotriazole, an inhibitor of catalase.

Most of the formate oxidation occurred inside the cells, as is evident from the effect of exogenous catalase on this process (Fig. 2). Catalase, which is presumed not to enter the cells, failed to abolish the insulin-dependent increase in formate oxidation, while it inhibited the effect of an optimal concentration of exogenous H_2O_2 (10^{-3} M) (Fig. 2, bottom panel). At low concentrations of H_2O_2, however, catalase can make a peroxidative complex to utilize formate, as shown in the bottom panel of Fig. 2.

The net utilization of glucose is increased in the presence of H_2O_2 in the extracellular medium, as evident from the $^{14}CO_2$ production and incorporation of the glucose carbons into glyceride fatty acids and glyceride glycerol (Table 1). The latter effect of H_2O_2 is similar to that of insulin. With catalase added to the medium, the stimulatory effect of H_2O_2 on glucose utilization almost completely disappeared, while that of insulin remained unimpaired. These data suggest that the intracellular accumulation of H_2O_2, as is shown to occur in response to insulin, is responsible for the increase in glucose utilization and lipogenesis. A small amount of H_2O_2 can be detected, however, in the extracellular medium, as represented by the data on formate oxidation in the absence of added H_2O_2 (Fig. 2, top panel), apparently due to leakage.

The comparable effects of insulin and H_2O_2 on hormone-stimulated lipase activity are illustrated in Table 2 and Fig. 3. Insulin or H_2O_2 added to the medium together with epinephrine or adrenocorticotropin (ACTH) had identical antilipolytic effects. The effect of H_2O_2 was observed at a concentration as low as 10^{-4} M and was abolished in the presence of exogenous catalase (16 μg/ml). Catalase did not alter the antilipolytic effect of insulin. As shown in Fig. 3, the addition of catalase 5 min after the addition of H_2O_2 and epinephrine failed to alter the antagonistic effect of H_2O_2 on epinephrine-stimulated depot fat lipase activity.

The antilipolytic effect of H_2O_2 against both epinephrine and ACTH was observed in the absence of glucose in the medium (Fig. 3), a condition which is characteristic of the effect of insulin [31]. In the presence of glucose, both insulin and H_2O_2 failed to antagonize the lipolytic stimulus of epinephrine and adrenocorticotropin, and above a concentration of

Table 2. Effects of insulin and H_2O_2 on the lipolytic response of adipocytes to epinephrine and adrenocorticotropin*

Addition to incubation	Glycerol production (μmoles/hr/10^6 cells)	Addition to incubation	Glycerol production (μmoles/hr/10^6 cells)
Control	0.63 ± 0.06	Control	0.56 ± 0.11
Insulin	0.48 ± 0.14	ACTH	2.24 ± 0.14
H_2O_2	0.44 ± 0.13	ACTH + insulin	0.76 ± 0.15
Epinephrine	2.74 ± 0.11	ACTH + insulin + catalase	0.79 ± 0.04
Epinephrine + insulin	0.86 ± 0.07	ACTH + H_2O_2	0.67 ± 0.1
Epinephrine + insulin + catalase	0.94 ± 0.06	ACTH + H_2O_2 + catalase	0.71 ± 0.09
Epinephrine + H_2O_2	0.72 ± 0.14		
Epinephrine + H_2O_2 + catalase	0.76 ± 0.14		

* Concentrations: insulin, 10^{-9} M; epinephrine or adrenocorticotropin (ACTH), 10^{-5} M; H_2O_2, 10^{-4} M; and catalase, 16 μg/ml. The values are averages ± S.E. of four experiments in duplicate.

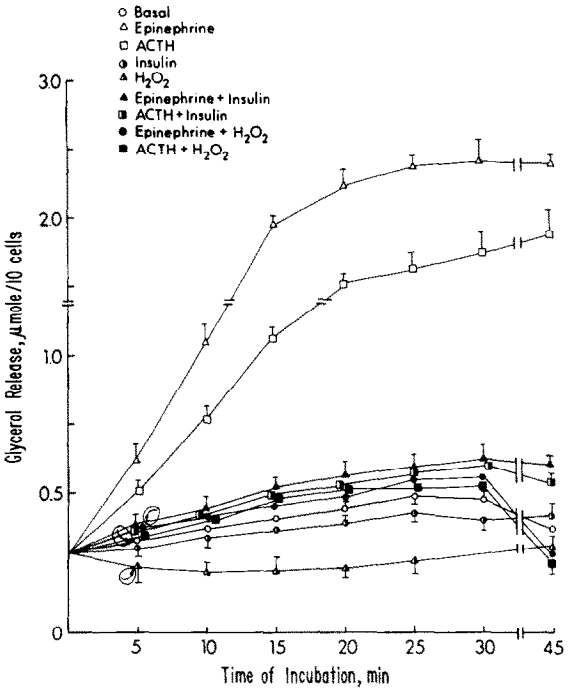

Fig. 3. Antilipolytic effects of insulin or H_2O_2 upon the lipolytic stimulus of epinephrine (10^{-5} M) in adipocytes. The concentration of catalase, where added (as indicated by the arrows), was 16 μg/ml.

about 0.3 mM appeared to increase the lipolytic response to some extent (Fig. 4). This effect may be due to an increased precursor pool size for glyceride glycerol (α-glycerophosphate) as well as an elevated cytoplasmic redox potential.

The changes in the cellular content of cyclic 3′,5′-AMP with time, in the presence of H_2O_2 or insulin, are shown in Table 3. The cAMP content of the cells gradually declined to an appreciably lower level in response to H_2O_2 only after 30 min of incubation. Insulin produced an identical effect, but to a smaller degree. However, in the presence of glucose (maximal at 0.3–0.5 mM), both insulin and H_2O_2 failed to lower the AMP level. Exogenous catalase abol-

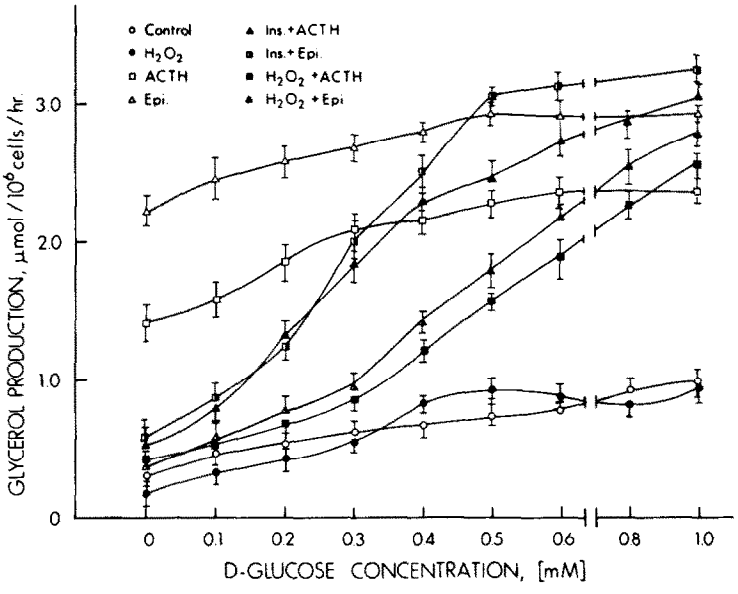

Fig. 4. Effect of varying concentrations of D-glucose upon the response of adipocytes to epinephrine (10^{-5} M) or adrenocorticotropin (ACTH) (10^{-5} M) and the effect of insulin (0.5 mU/ml) or H_2O_2 (1 mM). The maximal lipase-supporting concentration of glucose varied from 0.3 to 0.6 mM, apparently depending on the net intracellular utilization glucose. These cells were prepared in the presence of 2 mM glucose.

Table 3. Changes in the content of cyclic 3′,5′-adenosine monophosphoric acid in adipocytes treated with insulin or H_2O_2*

Addition to cell incubation	cAMP (pmoles/10^7 cells) 0 min	10 min	20 min	30 min
None	26	21	18	17
D-Glucose				26
H_2O_2	24	22	16	14
H_2O_2 + D-glucose	31	27	24	34
Insulin	24	26	19	14
Insulin + D-glucose		32	37	44
Epinephrine		63	86	118
Epinephrine + D-glucose		77	124	132
Epinephrine + insulin		66	43	37
Epinephrine + insulin + D-glucose		86	112	144
Epinephrine + H_2O_2		54	46	32
Epinephrine + H_2O_2 + D-glucose			94	106

* Values are averages of two experiments expressed as the nearest integers. Concentrations to additions of cell incubation. D-glucose, 10^{-3} M; H_2O_2, 10^{-3} M; insulin, 1.2 nM; and epinephrine, 10^{-5} M. The incubations were stopped by addition of 3% perchloric acid and tubes were transferred to ice. The cAMP contents were assayed in the neutralized samples using assay kit TRK 432, according to Brown *et al.* [28].

ished the effect of H_2O_2, but not of insulin, upon the cAMP level in these cells.

DISCUSSION

With the discovery that insulin activates a pyridine nucleotide oxidase in the plasma membrane of adipocytes with the production of H_2O_2 [10–12], a major role of this pathway in some of the physiological actions of insulin was envisioned. It was suggested that the site of insulin-stimulated NADPH oxidase in fat cells may be on the cytoplasmic side of the plasma membrane [10, 11]. We also suggested [10] that the intracellular utilization of the endogenous H_2O_2 may represent a unique 'second messenger' system for the actions of insulin. Evidence was presented subsequently [13] that this H_2O_2 is utilized intracellularly via glutathione peroxidase and the catalase: H_2O_2 complex in adipocytes for funneling glucose to the pentose phosphate pathway. These studies were confirmed more recently by May and deHaen [18].

The increase in formate oxidation in these cells in response to insulin and its inhibition by aminotriazole is an index to the intracellular accumulation of a catalase: H_2O_2 complex and the rate of peroxidative activity [29, 30]. The data in Fig. 2 clearly document that insulin-stimulated H_2O_2 production primarily leads to intracellular utilization, since the exogenous catalase added to the extracellular medium (which may not enter the cells) did not abolish the effect of the hormone on formate oxidation, but instead increased it to a small extent, indicating accumulation of a small quantity of H_2O_2 in the extracellular accessible medium. Catalase, added to the cell medium where exogenous H_2O_2 was also added, affected the formate oxidation in a manner dependent on the concentration of H_2O_2 present (Fig. 2, bottom panel). The peroxidative function of catalase is normally expressed in a lower concentration of H_2O_2, observed as between 0.01 and 0.1 mM. At higher concentrations of H_2O_2, the catalatic activity predominates, to decompose the excess H_2O_2 present.

Exogenous catalase also inhibited the insulin-like effect of extracellular H_2O_2 to increase glucose oxidation and lipogenesis, but did not alter these effects of insulin itself, which now appears to alter the intracellular redox potential. This is consistent with previous evidence that the NADPH oxidase activity in adipocytes is located at the internal side of the plasma membrane [11], in contrast to the situation in polymorphonuclear leukocytes.

Two major routes for catabolism of H_2O_2 in these cells have been recognized, viz. glutathione peroxidase and catalase. The glutathione peroxidase system appears to be coupled to glucose-6-phosphate dehydrogenase activity through the generation of both $NADP^+$ and GSSG, as discussed previously [13], both of which are limiting factors for optimum activity of the hexose monophosphate shunt pathway [21, 22]. The concept of a peroxidative function of catalase in the presence of low concentrations of H_2O_2, as monitored by formate oxidation [29, 30], has remained a fascinating yet unresolved question. It is possible, on the other hand, that glutathione peroxidase may be the sole peroxidative system to account for the formate oxidation. Alternatively, another distinct enzyme, formate dehydrogenase (NAD^+-formate oxidoreductase), which reversibly yields *s*-formyl glutathione, has been characterized in bacteria [32] and eukaryocytes e.g. yeast and pea seed [33]. Whether this enzyme may also occur in animal systems, and especially in adipocytes, is not yet known. However, several aspects of these data clarify some of these questions: (i) formation of H_2O_2 in isolated plasma membranes has been clearly shown [11, 12], as well as the occurrence of catalase in these cells [13]; and (ii) inhibition of formate oxidation in the presence of aminotriazole is greater in insulin-treated cells than in control cells (Fig. 1), a condition which measures the peroxidative catalase: H_2O_2 complex. As this inhibition in the presence of the catalase inhibitor is only partial, the

remainder of the formate oxidation may actually be catalyzed by glutathione peroxidase. It is possible, however, that in the absence of exogenous formate and at a physiological concentration of glucose, H_2O_2 is preferentially metabolized by the glutathione peroxidase pathway and thereby the reducing equivalents are channeled to lipogenesis. The possibility of oxidation of formate by other active forms of oxygen, e.g. [O_2^-] or OH^- radicals, in these cells seems quite unlikely in the face of a better orchestrated metabolic activity compared to the inflammatory cells, and we could not detect any lipid peroxide formation under these conditions. The H_2O_2 formation in adipocytes in response to insulin is, therefore, a metabolic control distinct from that associated with phagocytosis or oxygen toxicity.

The antilipolytic effect of insulin is also mimicked by exogenous H_2O_2 at a concentration as low as 10^{-4} M. Both epinephrine-stimulated and ACTH-stimulated depot fat lipase activities were antagonized by H_2O_2 as well as by insulin. It is remarkable that insulin is just as effective at a much smaller concentration range, which is another indication of a second messenger role for H_2O_2 as generated in response to insulin. The finding that the antilipolytic effect of H_2O_2 in the medium, but not of insulin, can be prevented by simultaneous addition of catalase suggests that any such effect of metabolically generated H_2O_2 is due to intracellularly available H_2O_2. The minute amounts of detectable H_2O_2 in the extracellular medium of insulin-treated cells reported earlier [11] and evident from the data in Fig. 2 (top panel) may appear due to cell leakage. The antilipolytic effect of H_2O_2, observed in the absence of glucose, may result from an oxidative change in the depot fat lipase, or from an increased rate of re-esterification of fatty acids, which was shown by us to be an effective regulator of hormone-sensitive lipase activity [34]. Although the present data do not permit a distinction between these possibilities, there is an apparent correlation between the cytoplasmic redox potential and the depot fat lipase activity. Reversal of the antilipolytic effect of H_2O_2 by increasing concentrations of D-glucose in the medium suggests a potentially important role of glutathione regeneration through the NADPH–GSSG reductase shuttle operating in conjunction with the pentose phosphate pathway [11, 13]. The antilipolytic effect of H_2O_2 may be due to a lowering of cAMP level, as shown in Table 3. These data are consistent with previously published evidence that H_2O_2 has an inhibitory effect on adenylate cyclase activity in isolated membranes [11, 14, 15]. I have also observed that treatment of adipocytes [12, 14, 15], as well as leukocytes [35], with H_2O_2 or with agents which trigger endogenous H_2O_2 production through the activation of the plasma membrane NADPH oxidase and effect a lowering of the cellular reduced glutathione leads to depressed adenylate cyclase activity. However, a possible site of the antilipolytic effect of H_2O_2, independently of changes in cAMP level, is indicated by the fact that this effect is initiated within 5 min, whereas an appreciable decline in the cAMP level is noted only after 20–30 min. D-Glucose prevents this decline in cAMP with a maximal effect at 0.3–0.5 mM (Table 3). The antagonism by H_2O_2 of the epinephrine- or ACTH-stimulated cAMP level does not seem to result from an oxidative denaturation of the hormones, because in that case glucose would not be able to restore the potential effects of these hormones.

Hydrogen peroxide was used previously as a chemical tool to study glucose utilization by the pentose phosphate shunt [36] on the consideration that it may acutely reverse the cytoplasmic mass-action ratios of GSH/GSSG and NADP/$NADP^+$. Also, it was employed by Czech *et al.* [8] and Mukherjee *et al.* [13, 16] in studies on hexose transport to confirm the earlier suggestion that activated transport of sugar involves an exchange of plasma membrane sulfhydryl groups with the interchain disulfide of insulin [4–7]. The reports by Czech *et al.* [8] did not suggest, however, that H_2O_2 could be a physiological regulator of insulin-mediated action. The demonstration of an intra-plasma membrane NADPH oxidase as a definitive process for insulin-dependent H_2O_2 production [10–13] underscored the potential role of H_2O_2 as the putative 'second messenger' for the cellular effects of insulin, as originally proposed [10]. Other possible sources of H_2O_2 such as the traces of amine oxidase activity [37] remain to be explored further. The present evidence that H_2O_2 mimics the major biological effects of insulin in these sensitive cells, viz. glucose transport and oxidation, lipogenesis, antagonism of hormone-sensitive lipolysis and lowering of cAMP content as glucose-independent effects, together with the finding that it can deactivate adenylate cyclase activity [10, 11, 15], fulfils the criteria for insulin-stimulated endogenous H_2O_2 to be reckoned as the second messenger. I have also found, in collaboration with Dr. Chhabirani Mukherjee, that preincubation of intact cells with H_2O_2 at a concentration as low as 0.5 mM causes an immediate (2–3 min) and sustained activation of mitochondrial pyruvate dehydrogenase [38], another key locus of the stimulatory effect of insulin on lipogenesis. This effect is due to a rapid dephosphorylation [2] of pyruvate dehydrogenase in consequence of decreased lipolysis and increased esterification of fatty acids, as well as of a drastic lowering of the cytoplasmic redox potential (manuscript in preparation). At this point it should be noted that the consummate effects of insulin on lipogenesis involve both glucose transport and H_2O_2 metabolism. Thus, agents which fail to activate glucose transport (as assayed with tracer methyl D-glucose at concentrations $\ll K_m$) or those which inhibit transport, e.g. thiols and sulfhydryl inhibitors, yet activate the NADPH oxidase in the plasma membrane, actually reduce the net fatty acid synthesis, apparently due to the lack of substrate (pyruvate) for the pyruvate–dehydrogenase complex (S. P. Mukherjee, C. Mukherjee and W. S. Lynn, manuscript submitted for publication). In all such instances, however, a glucose-independent antagonism of lipase activity due to endogenously accumulated hydrogen peroxide was observed. Further studies are in progress to elucidate the relationship between the effects of insulin on glucose transport and H_2O_2 production. Other measures of insulin's action which are identically modified by H_2O_2 are the activation of the

microsomal fatty acyl coA ligase (S. P. Mukherjee and C. Mukherjee, unpublished) and the regulation of phosphorylation of some plasma membrane proteins of adipocytes which are susceptible to insulin action [39].

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