Counterregulatory Control of Intracellular Hydrogen Peroxide Production by Insulin and Lipolytic Hormones in Isolated Rat Epididymal Fat Cells: A Role of Free Fatty Acids†

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ABSTRACT: Exogenously added H_2O_2 is known to mimic a large number of the actions of insulin in isolated rat epididymal fat cells. Further, insulin is known to stimulate intracellular H_2O_2 production, raising the possibility that H_2O_2 may mediate insulin action. The present study was undertaken to further define the regulation of intracellular H_2O_2 production. The specific aim was to determine if insulin-stimulated H_2O_2 production is an event proximate to the site of the insulin–receptor interaction. Under a variety of conditions, a consistent inverse relationship between lipolysis and H_2O_2 production was observed. Thus corticotropin–(1–24)-tetracosapeptide stimulated glycerol release with the same concentration dependence as it inhibited formate oxidation, a measure of H_2O_2 production. Similar results were obtained with $N^6,O^{2'}$-dibutyryladenosine cyclic 3′,5′-phosphate or 1-methyl-3-isobutylxanthine as lipolytic agents. Conversely, (*R*)-*N*-(2-phenylisopropyl)adenosine, an agent that inhibits fat cell adenylate cyclase, and thus lowers adenosine cyclic 3′,5′-phosphate (cAMP) levels and consequent lipolysis, resulted in stimulation of H_2O_2 production with the same concentration dependence. The linkage between lipolytic rates and H_2O_2 production was further substantiated in experiments that exploited the biphasic insulin concentration effect relationship on submaximally stimulated lipolysis; again H_2O_2 formation was seen to mirror lipolysis as measured by both glycerol and free fatty acid release. So that one could determine if the observed modulation of H_2O_2 production was due to a proximal factor in the lipolytic system (e.g., cAMP itself) or to a lipolytic end product, the effects of exogenous glycerol and fatty acids were studied. Glycerol exhibited no activity whereas palmitate and oleate produced concentration-dependent decrements in measured H_2O_2 production. Definite evidence for a primary role of endogenous free fatty acids in the regulation of H_2O_2 production was obtained by inhibiting lipolysis with maximally effective concentrations of (*R*)-*N*-(2-phenylisopropyl)adenosine and then exposing cells to various concentrations of insulin in the presence of medium glucose. Under these conditions glycerol production and, most likely, cAMP levels as well were unaffected by insulin, but free fatty acid release was inhibited because of increased reesterification resulting from insulin-stimulated glucose uptake. The observed fall in free fatty acids again was mirrored by increased H_2O_2 production. The data best fit the hypothesis that hormone effects on H_2O_2 production in fat cells are mediated via changes in free fatty acid levels, thus placing regulation of this production at a site distant from the insulin–receptor interaction and rendering a role for H_2O_2 as second messenger for insulin unlikely.

Insulin has been shown to stimulate release of H_2O_2 into the medium by rat epididymal adipocytes (Mukherjee & Lynn, 1977) and also to stimulate formate oxidation by these cells, a process that is believed to reflect intracellular H_2O_2 production (Mukherjee et al., 1978; May & Haën, 1979a). Other hormones that possess insulin-like activity in adipocytes, i.e., ocytocin, vasopressin, gastric inhibitory polypeptide, and nerve growth factor β subunit, also stimulated H_2O_2 production (de Haën et al., 1980; Muchmore et al., 1981). Exogenously added H_2O_2 mimics many of the effects of insulin, e.g., stimulation of glucose transport (Czech et al., 1974a), preferential stimulation of glucose C-1 oxidation over glucose C-6 oxidation (Czech et al., 1974b), enhancement of glucose incorporation into glycogen (Lawrence & Larner, 1978), stimulation of glucose incorporation into lipids (Cascieri et al., 1979; May & de Haën, 1979b) accompanied by activation of pyruvate dehydrogenase (May & de Haën, 1979b), inhibition of hormone-stimulated lipolysis (Cascieri et al., 1979; Little & de Haën, 1980), and down-regulation of insulin receptors (Caro & Amatruda, 1980). On the basis of these findings, the possibility that endogenous H_2O_2 may play a role in the mechanism of action of insulin was considered (May & de Haën, 1979a). This paper shows that lipolytic hormones, which are known to elevate endogenous adenosine cyclic 3′,5′-phosphate (cAMP) levels, exogenously added $N^6,O^{2'}$-dibutyryl-cAMP, and inhibitors of phosphodiesterase, all inhibit intracellular H_2O_2 production whereas inhibitors of adenylate cyclase stimulate it. Moreover, evidence is presented that free fatty acids may be important regulators of intracellular H_2O_2 production.

Experimental Procedures

Materials

Materials for the preparation of epididymal fat cells from ad libitum fed Sprague-Dawley rats (130–180 g) and for assaying formate oxidation were those specified by Muchmore et al. (1981). Materials for measuring glycerol were those given by Little & de Haën (1980). Regular Cohn fraction V bovine serum albumin, which had been selected for low insulin-like activity in a variety of metabolic assays, was purchased from Reheis Chemical Co. (lot S11709). Crystallized bovine serum albumin, similarly screened, was obtained from Miles Laboratories (lot 48), and essentially fatty acid free Cohn fraction V bovine serum albumin (lot 80F9340) was from Sigma. Adenosine deaminase from calf intestinal mucosa

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(Type I) and N^6,$O^{2'}$-dibutyryl-cAMP also were purchased from Sigma. (*R*)-*N*-(2-Phenylisopropyl)adenosine, henceforth referred to simply as phenylisopropyladenosine, was bought from Boehringer Mannheim Biochemical, Indianapolis, IN, under the name of N^6-(L-2-phenylisopropyl)adenosine and prepared as a stock solution (0.1 M) in dimethyl sulfoxide. Dimethyl sulfoxide in the concentrations used was shown not to interfere with metabolic assays. Palmitic acid (lot LA02347) purchased from Supelco, Inc., Bellefonte, PA, was dissolved in chloroform to 100 mM and stored as a stock solution. A portion was evaporated to dryness, dissolved at 100 °C in an equimolar amount of 100 mM KOH, and diluted in assay buffer to the specific concentrations. Oleic acid was neutralized with an equimolar quantity of KOH and then diluted in Krebs–Ringer bicarbonate buffer to 0.11 M. This solution was heated to 80 °C, and then the pH was brought to 8 with HCl. Further dilutions of this stock were made in assay buffer. Synthetic adrenocorticotropic hormone (ACTH)–(1–24)-tetracosapeptide was obtained from Organon Pharmaceuticals, and 1-methyl-3-isobutylxanthine was a product of Aldrich.

Bovine insulin was purified to homogeneity as described by May et al. (1978). A 5×10^{-5} M stock solution in 0.01 M HCl was prepared by using a molar absorptivity coefficient at 277 nm of 5530 L mol^{-1} cm^{-1} (Harrison & Garratt, 1969). The insulin concentration was confirmed by diluting the stock to 2×10^{-9} M and assaying by radioimmunoassay; the results of these separate assays agreed within 3%.

Methods

Fat Cells. Isolated epididymal fat cells were prepared as previously described (May & de Haën, 1979a) by using the technique of Rodbell (1964), with the modification that the digestion and wash media were 0.275 mM in glucose for all experiments.

Buffers. Krebs–Ringer bicarbonate buffer, which contained half the calcium concentration recommended by Cohen (1957) and which additionally was 10 mM in *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid and 0.275 mM in glucose, was used in all studies. In early experiments (Figures 1–5) crystallized bovine serum albumin at 0.1% (w/v) concentration was used in the formate oxidation assay buffer whereas in the early lipolysis experiments (Figures 1–6) regular Cohn fraction V bovine serum albumin was used at 2% (w/v) concentration. In later experiments (Figures 7 and 8), when the relationship between fatty acid levels and formate oxidation came to light, the albumin was changed to essentially fatty acid free Cohn fraction V bovine serum albumin. In these later experiments the same albumin was used at 2% (w/v) concentration in simultaneously performed formate oxidation and lipolysis experiments. Early experiments in this work were performed without adenosine deaminase present during cell incubations, whereas later experiments employed this reagent at a final concentration of 0.28 IU/mL as specified in corresponding figure legends.

Formate Oxidation. Isolated adipocytes [(1–3) $\times 10^5$/2 mL] were incubated with test reagents for 1 h at 37 °C in a Dubnoff shaking metabolic incubator (1 s^{-1}), and intracellular H_2O_2 production as assessed by formate oxidation was measured according to May & de Haën (1979a), including the improvements reported by Muchmore et al. (1981).

Lipolysis. Isolated adipoctyes [(0.5–2) $\times 10^5$/2 mL] were incubated with shaking (1 s^{-1}) for 2 h at 37 °C. For glycerol determinations, the aliquots were transferred to iced plastic test tubes (12 × 75 mm) and centrifuged at 1500 rpm for 10 min, following which the cells were aspirated away. The infranatant was assayed for glycerol by the method of Chernick (1969). For free fatty acid determinations, the cells and medium were extracted by using the single-extraction and microtitration methods as described by Dole & Meinertz (1960).

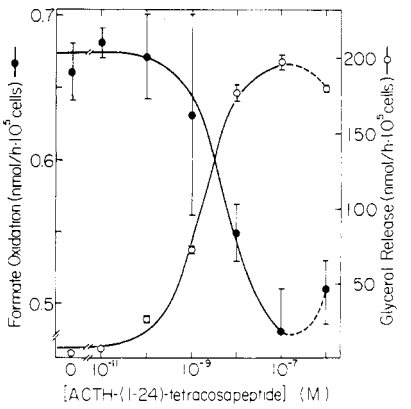

FIGURE 1: Concentration-effect relationship for ACTH–(1–24)-tetracosapeptide on formate oxidation and glycerol release as determined simultaneously on the same cell batch under conditions that varied only as to the albumin composition of buffers (see Methods). No adenosine deaminase was present. Data shown are $\bar{x} \pm$ SE of five replicates for formate oxidation and three for glycerol release from a representative experiment. Solid lines are computer generated (see Methods) and dashed lines are hand-drawn extensions.

Calculations. Simple dose–response curves were analyzed by weighted nonlinear least-squares fitting of the Hill equation $y = (y_{max} - y_{min})/[(EC_{50}/x)^{n_0} + 1] + y_{min}$ to the data, with the Levenberg–Marquart modification of the Gauss–Newton iterative procedure (Magar, 1972). Statistical weights were SE^{-2}; n_0 is the Hill coefficient, x is the hormone concentration, and y_{min}, y_{max}, and y are the minimal, maximal, and intermediate responses, respectively. Biphasic dose–response curves (Figure 5) were analyzed by fitting two terms of the kind used in the above equation to the data, with the Hill coefficient set at 1 for both.

Results

Lipolytic hormones were found to inhibit formate oxidation, and thus intracellular H_2O_2 production, with the same concentration dependence as that for stimulation of glycerol release, as illustrated for ACTH–(1–24)-tetracosapeptide in Figure 1. Similar data were obtained with glucagon (de Haën et al., 1980) and β-adrenergic agents (data no shown). Since lipolytic polypeptide hormones and β-adrenergic agents are thought to stimulate lipolysis via activation of adenylate cyclase, the effect of the permeant cAMP analogue N^6,$O^{2'}$-dibutyryl-cAMP on formate oxidation was measured. Figure 2 shows that, again, adipocyte formate oxidation responded to this lipolytic stimulus in an inverse fashion and with similar concentration dependence as glycerol release. An alternative method for raising intracellular cAMP levels is to treat cells with a phosphodiesterase inhibitor, such as 1-methyl-3-isobutylxanthine (Fain, 1973). Figure 3 depicts the concentration-effect relationship for 1-methyl-3-isobutylxanthine inhibition of formate oxidation and stimulation of glycerol release. Results are comparable to those in Figures 1 and 2, the rise in lipolysis mirroring the fall in formate oxidation.

Having thus shown that adenylate cyclase activators, phosphodiesterase inhibitors, and cAMP derivatives inhibited intracellular H_2O_2 production, it was pertinent to examine the effect of an agent that inhibits cAMP production. Phenylisopropyladenosine is such an agent that inhibits adenylate cyclase (Londos & Wolff, 1977; Londos et al., 1978, 1980) and lowers cAMP levels (Fain, 1973; Stock & Prilop, 1974;

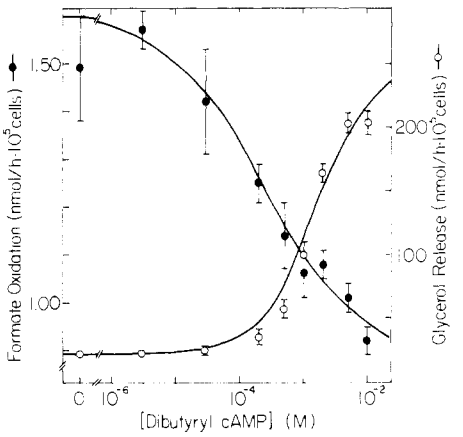

FIGURE 2: Concentration-effect relationship for $N^6,O^{2'}$-dibutyryl-cAMP effects on formate oxidation and glycerol release as determined simultaneously on the same cell batch under conditions that varied only as to the albumin composition of buffers (see Methods). Adenosine deaminase was present. Data shown are $\bar{x} \pm$ SE of five replicates for formate oxidation and three for glycerol release from a representative experiment. Solid lines are computer generated (see Methods).

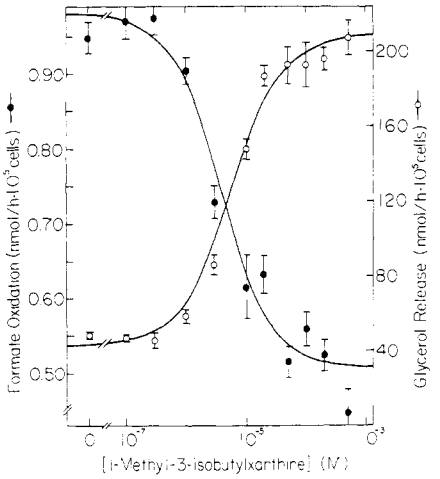

FIGURE 3: Concentration-effect relationship for 1-methyl-3-isobutylxanthine effects on formate oxidation and glycerol release as determined simultaneously on the same cell batch under conditions that varied only as to the albumin composition of buffers (see Methods). Adenosine deaminase was present. Data shown are $\bar{x} \pm$ SE of five replicates for formate oxidation and three for glycerol release from a representative experiment. Solid lines are computer generated (see Methods).

Prilop, 1975; Wieser & Fain, 1975; Wieser & Pendleton, 1979; Garcia-Sainz et al., 1981) even below basal levels (Prilop, 1975; Fain & Malbon, 1979). The high potency of this agent ($EC_{50} = 2 \times 10^{-10}$ M) helps assure a high specificity action. Shown in Figure 4 are dose–response relationships for phenylisopropyladenosine effects on formate oxidation, glycerol release, and free fatty acid accumulation. Again, an inverse relationship with similar concentration dependence was seen when comparing lipolysis to H_2O_2 production: in this case formate oxidation rose and lipolysis was depressed with increasing reagent concentrations. It should be noted that demonstration of these effects of phenylisopropyladenosine on cells in the basal state is dependent on the use of adenosine deaminase to prevent accumulation of medium adenosine. The same inverse relation between formate oxidation and lipolysis was also observed when phenylisopropyladenosine was used to inhibit adenylate cyclase in cells in which lipolysis was submaximally stimulated with ACTH–(1–24)-tetracosapeptide at 3×10^{-9} M (data not shown).

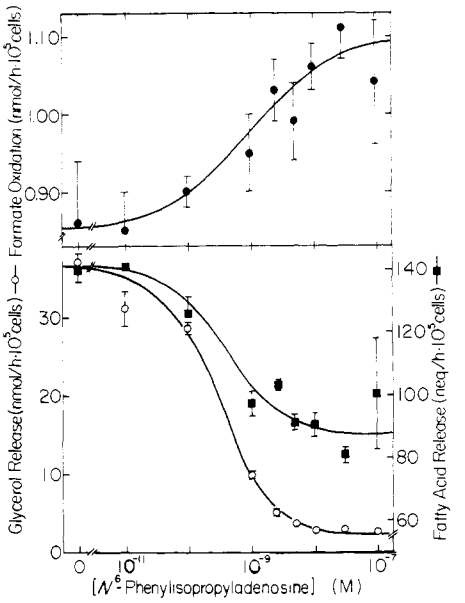

FIGURE 4: Concentration-effect relationship for phenylisopropyladenosine effects on formate oxidation, and glycerol and free fatty acid release, as determined simultaneously on the same cell batch under conditions that varied only as to the albumin composition of buffer (see Methods). Adenosine deaminase was present. Data shown are $\bar{x} \pm$ SE of five replicates for formate oxidation and three for glycerol release from a representative experiment. Solid lines are computer generated (see Methods).

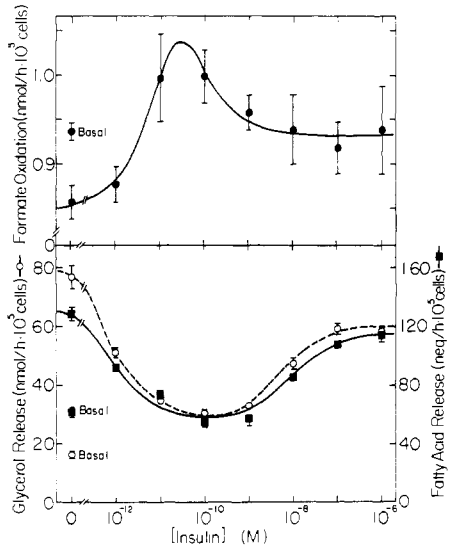

FIGURE 5: Concentration-effect relationships for insulin effects on lipolysis stimulated by and formate oxidation inhibited by ACTH–(1–24)-tetracosapeptide (3×10^{-9} M). All determinations were made simultaneously on the same cell batch under conditions that varied only as to the albumin composition of buffers (see Methods). Basal refers to values obtained without ACTH stimulation. Data are $\bar{x} \pm$ SE of five replicates for formate oxidation and three for glycerol and free fatty acid release. Lines are computer generated (see Methods).

For further substantiation of the relationship between lipolysis and intracellular H_2O_2 production, insulin dose–response curves for glycerol and free fatty acid release and formate oxidation by fat cells submaximally stimulated with ACTH–(1–24)-tetracosapeptide (3×10^{-9} M) were measured in the same cell batch under identical incubation conditions except for previously noted differences in albumin type and concentration (see Methods). Figure 5 shows the typical multiphasic effect of insulin, with inhibition of both glycerol and free fatty acid accumulation at concentrations below 10^{-10}

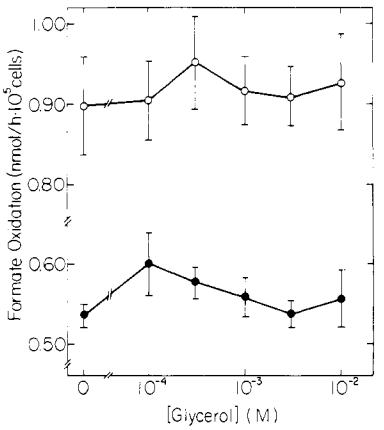

FIGURE 6: Concentration-effect relationship for exogenous glycerol effects on basal (●) and insulin-stimulated (5×10^{-9} M) (○) formate oxidation. Date are $\bar{x} \pm$ SE ($n = 5$) from a representative experiment.

M and a progressive loss of inhibition above this concentration. Notably, the rate of formate oxidation again mirrored the other complex dose–response curves.

The preceding experiments documented an inverse relationship between lipolytic rates, and thus cAMP levels, and formate oxidation. cAMP could regulate intracellular H_2O_2 production directly through interaction with its production site, less directly if mediation by cAMP-dependent protein kinase were to be required or very indirectly if H_2O_2 production were to be sensitive to the end products of lipolysis, i.e., glycerol or fatty acids. This latter possibility was tested by performing glycerol and exogenous fatty acid dose–response curves for formate oxidation. Figure 6 shows the effect of exogenously provided glycerol on basal and insulin-stimulated formate oxidation. There was no evident trend for glycerol effects on formate oxidation in either basal or insulin-stimulated cells for glycerol concentrations up to 10 mM. In contrast, addition of fatty acids did inhibit formate oxidation. The results of experiments with exogenous palmitic and oleic acids are seen in Figure 7. It is notable that greater inhibition was achieved with oleate, largely because of the solubility limit imposed by palmitate. Further, there is approximately a 2-fold higher concentration of oleic acid required to inhibit insulin-stimulated formate oxidation when compared to its effects on basal activity. Since glucose is required for the assay of formate oxidation (May & de Haën, 1979a), it seems likely that insulin-enhanced intracellular glucose availability resulted in increased fatty acid esterification and thus functionally decreased fatty acid levels. The lack of effect of exogenous palmitate on insulin-stimulated formate oxidation (Figure 7) may then reflect the combination of this dose–response shift and the solubility limit of palmitate.

An additional approach to the role of endogenous fatty acids in regulation of formate oxidation utilized a combination of phenylisopropyladenosine and insulin in the presence of glucose in order to dissociate lipolytic rates from free fatty acid levels. A maximally effective concentration of phenylisopropyladenosine (10^{-7} M) was used to inhibit adenylate cyclase and thus lipolysis, and the concentration-dependent curves for insulin effects on glycerol and free fatty acid release as well as on formate oxidation in the presence of medium glucose were determined. As seen in Figure 8, insulin had no effect on lipolysis as assessed by glycerol release. However, insulin lowered free fatty acid release, presumably due to increased fatty acid reesterification promoted by insulin-stimulated glucose influx. Again, formate oxidation increased with falling free fatty acid levels. This was interpreted to mean that free

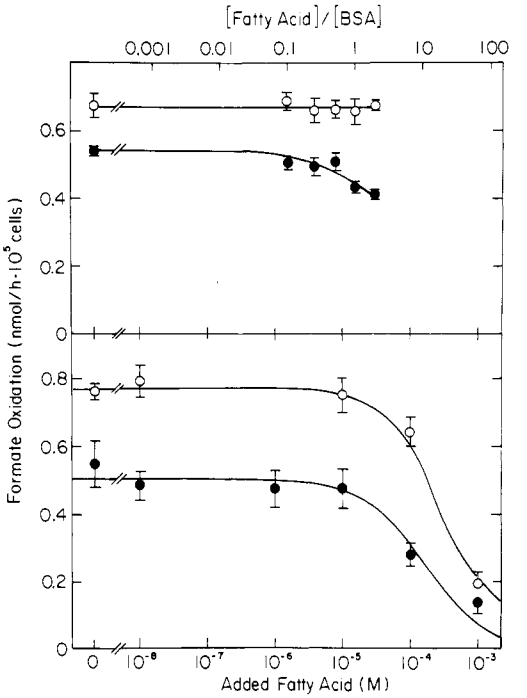

FIGURE 7: Concentration-effect relationships for exogenously added fatty acid effects on formate oxidation. Results for palmitate (upper panel) and oleate (lower panel) are shown in the absence (●) and presence (○) of insulin (10^{-8} M). Fatty acid concentrations given are total added fatty acids, and bovine serum albumin (BSA) was present at 0.1% (w/v) concentration, corresponding to [fatty acid]/[BSA] ratios as shown. Data are $\bar{x} \pm$ SE ($n = 5$). Solid lines are computer generated (see Methods).

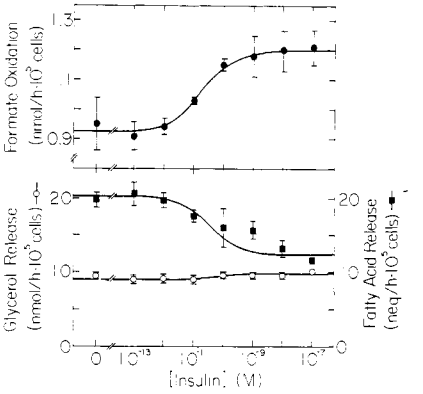

FIGURE 8: Concentration-effect relationships for insulin effects on formate oxidation, glycerol production, and free fatty acid release in the presence of phenylisopropyladenosine (10^{-7} M). All determinations were made simultaneously on the same cell batch. Adenosine deaminase was present. The buffer differs from other experiments in that essentially fatty acid free Cohn fraction V bovine serum albumin at 2% (w/v) concentration was used for both formate oxidation and lipolysis. Data are $\bar{x} \pm$ SE of four replicates for formate oxidation and three for lipolytic studies. Lines are computer generated with Hill coefficients set at 1 (see Methods).

fatty acids and not glycerol or cAMP are important regulators of formate oxidation and thus H_2O_2 production.

Discussion

One of the many metabolic effects of insulin is the recently described stimulation of adipocyte formate oxidation, which is believed to reflect intracellular H_2O_2 production (Mukherjee et al., 1978; May & de Haën, 1979a; de Haën et al., 1980). This finding was of interest to us in view of the fact that exogenously provided H_2O_2 mimicked such a wide variety of insulin effects (see the introduction), thus suggesting the

possibility that H_2O_2 may be a mediator of some of insulin's action (May & de Haën, 1979a). Thus, the current study was undertaken to further define the hormonal regulation of intracellular H_2O_2 production in adipocytes. We have now shown that insulin and lipolytic hormones act in a counterregulatory fashion to respectively stimulate and depress H_2O_2 production. So that the mechanism involved could be analyzed, cellular cAMP levels were elevated by means of exogenously added $N^6,O^{2'}$-dibutyryl-cAMP or by means of the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine, or cAMP levels were lowered by the adenylate cyclase inhibitor phenylisopropyladenosine (Londos & Wolff, 1977; Londos et al., 1978, 1980). Phenylisopropyladenosine had previously been shown to efficiently lower cAMP levels in fat cells (Fain, 1973; Stock & Prilop, 1974; Prilop, 1975; Wieser & Fain, 1975; Wieser & Pendleton, 1975; Garcia-Sainz et al., 1981) even below basal levels and under conditions identical with ours (Prilop, 1975; Wieser & Fain, 1975; Fain & Malbon, 1979). We have recently added additional evidence that phenylisopropyladenosine (10^{-7} M) lowered cAMP production in intact cells to such a degree that the selective phosphodiesterase inhibitor *d*-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724/1) had lost its ability to stimulate lipolysis (Beat U. Raess, Douglas B. Muchmore, and Christoph de Haën, unpublished results). In the present experiments, we have not directly measured cAMP levels but rather infer them from the rates of glycerol release, based on the accumulated evidence that has demonstrated close interdependence of these variables under conditions of less than maximally stimulated lipolysis (Robison et al., 1971; Kono & Barham, 1973; Desai et al., 1973; Hjemdahl & Fredholm, 1976; Burns et al., 1979; Schimmel et al., 1980; Arner & Östman, 1980; Wong & Loten, 1981; Fan & Ho, 1981; Sengupta et al., 1981). In fact, since rates of lipolysis are related to cellular cAMP levels by a saturable dose–response curve (Robison et al., 1971; Burns et al., 1979; Sengupta et al., 1981; Wong & Loten, 1981; Fan & Ho, 1981), it appears legitimate to conclude that phenylisopropyladenosine in our experiments indeed lowered cAMP levels as a result of inhibition of adenylate cyclase.

The present studies showed that whenever cAMP, and thus lipolysis, was elevated, H_2O_2 production was inhibited (Figures 1–3), whereas lowering of cAMP levels and rates of lipolysis resulted in stimulation of H_2O_2 production (Figure 4). Further confirmation of the link between the lipolytic process and H_2O_2 production was obtained in experiments that took advantage of the biphasic dose–response curve for insulin effects on submaximally stimulated lipolysis (Kono & Barham, 1973; Lavis & Williams, 1973; Desai et al., 1973). These studies confirmed an inverse relationship between lipolysis and H_2O_2 production even in the presence of both lipolytic hormone and insulin (Figure 5). Because lipolysis and cAMP levels depend in a biphasic manner on the insulin concentration under these conditions (Kono & Barham, 1973; Desai et al., 1973), none of the above experiments allowed discrimination of the possibility that cAMP was regulating H_2O_2 production from the possibility that the products of lipolysis were responsible.

To make this distinction, we performed two kinds of experiments. First, the effects of the end products of lipolysis, glycerol, and fatty acids on H_2O_2 production were investigated, and it could be shown that whereas glycerol had no effect, free fatty acids were inhibitory (Figures 6 and 7). In these experiments we have not directly measured intracellular free fatty acid levels, but time-course studies of intracellular and extracellular free fatty acid levels show that, following a short (5-min) lag phase during which intracellular free fatty acid levels rise quickly, the release of fatty acids to the medium is a good reflection of intracellular levels until such time as the medium albumin becomes saturated with fatty acids (Angel et al., 1971). This latter situation does not obtain in the present studies since the rate of lipolysis under our conditions is linear for at least 2 h. In the second type of experiment the rate of lipolysis was held constant while the rate of fatty acid reesterification was increased. This was achieved by depressing lipolysis maximally with the adenylate cyclase inhibitor phenylisopropyladenosine; the resulting level of lipolysis could not be further affected by insulin. Glucose transport, and thus *sn*-glycerol 3-phosphate availability, was then increased by insulin, resulting, as could be expected (Chlouverakis, 1967), in enhanced fatty acid reesterification. In the absence of significant effects of insulin on the suppressed rates of glycerol production, there was a concentration-dependent fall in free fatty acid levels that was associated with an inverse rise in H_2O_2 production (Figure 8). Taken together, the foregoing results showed that insulin and counterregulatory hormones act reciprocally to modulate intracellular H_2O_2 production, likely by means of their effects on cellular levels of free fatty acids.

Additional modulators of H_2O_2 production may be operative under certain circumstances. Ocytocin stimulation of H_2O_2 production provides a case in point. Ocytocin, which in the lower concentration range has many insulin-like effects, also stimulates H_2O_2 production (de Haën et al., 1980; Muchmore et al., 1981). However, in the higher concentration range, where ocytocin acts as a weak lipolytic agonist, no inhibition of H_2O_2 production was observed. It should be noted, however, that in these experiments lipolysis was assessed only by glycerol release, and the possibility still remains that a lack of elevation of free fatty acids, due to reesterification, accounts for the lack of inhibition of H_2O_2 production at high ocytocin concentrations.

Mukherjee & Lynn (1977) ascribed the H_2O_2 production to an insulin-sensitive plasma membrane bound NADPH oxidase, and preliminary work in our hands confirms the presence of such an activity (Beat U. Raess and Christoph de Haën, unpublished observations). Other potential sources of intracellular H_2O_2 may also have to be considered, foremost among them peroxisomes and mitochondria (Loschen et al., 1971; Boveris et al., 1972; Boveris & Chance, 1973). Since it is unlikely that hormones affect cellular levels of urate, glycolate, or D-amino acids, oxidation of these substrates in peroxisomes by respective oxidases appears to constitute an unlikely source for the hormone-modulated H_2O_2. H_2O_2 is also produced by the first enzyme in peroxisomal β-oxidation of fatty acids (Lazarow & de Duve, 1976). However, palmitate and oleate inhibited H_2O_2 production rather than stimulating it, thus again rendering this peroxisomal source improbable.

A more likely candidate for the source of the observed H_2O_2 production is the mitochondrion in state-4 respiration, i.e., when substrate is plentiful and ADP is rate limiting (Loschen et al., 1971; Boveris et al., 1972; Boveris & Chance, 1973). Uncouplers of oxidative phosphorylation functionally induce a state-4 to state-3 transition, state 3 being a state in which electron flow rather than ADP availability is rate limiting. This is associated with inhibition of H_2O_2 production (Loschen et al., 1971; Boveris & Chance, 1973; Loschen, 1975). Free fatty acids have long been considered to act functionally as uncouplers of oxidative phosphorylation [e.g., Bihler & Jeanrenaud (1970)], and thus our finding in the present work

that free fatty acids inhibit intracellular H_2O_2 production could be explained on this basis. It should be noted the Boveris and co-workers (Boveris et al., 1972; Boveris & Chance, 1973) described a weak stimulation by octanoate or palmitoylcarnitine of H_2O_2 production by isolated mitochondria in state 1, state 1 being a state of substrate deficiency. We believe that these observations in isolated mitochondria under conditions not likely to occur in whole cells do not speak against a mitochondrial origin of the free fatty acid regulated intracellular H_2O_2 production demonstrated in the present study, and the exact site of this production remains to be elucidated.

If H_2O_2 production occurred at a site distant from the plasma membrane (e.g., mitochondria) and if it were primarily regulated by free fatty acid levels, then it would appear that the mechanisms underlying the insulin-like effect of exogenously added H_2O_2 are unrelated or only distantly related to the mechanism involving generation and metabolism of intracellularly generated H_2O_2. Evidence has been presented that suggested that exogenous H_2O_2 may make plasma membranes leaky to cAMP (Little & de Haën, 1980). Direct inhibition of adipocyte adenylate cyclase by oxidants, including H_2O_2, has also been reported (Mukherjee & Lynn, 1977). Alternatively, H_2O_2 may lower cellular ATP levels, as described in blood platelets (Holmsen & Robkin, 1977), and it may thereby decrease cAMP levels. Such lowering of cAMP levels by these or other mechanisms would reproduce in part the glucose transport independent actions of insulin (B. U. Raess, D. B. Muchmore, and C. de Haën, unpublished results). Also H_2O_2 has been found to cause loss of pyridine nucleotides and Ca^{2+} from mitochondria (Lötscher et al., 1980). Coupled with the known action of H_2O_2 and other oxidants to activate glucose transport (Czech, 1976), these activities could well explain the striking ability of exogenous H_2O_2 to mimic insulin. Studies to clarify some of these issues are under way.

In conclusion, intracellular H_2O_2 production as assessed by formate oxidation in fat cells is under counterregulatory control by lipolytic and antilipolytic hormones. Although a direct role of cAMP in this regulation remains a possibility, the evidence favors an important role of free fatty acids, which are among the end products of lipolysis. This suggests that H_2O_2 production is a metabolic consequence of insulin action distal to the receptor. Thus, despite the insulin-like activities of exogenously added H_2O_2 and its insulin-stimulated intracellular production, H_2O_2 would now seem to be an unlikely candidate for principal mediator of insulin action in the adipocyte.

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Biosynthesis, Nonenzymatic Synthesis, and Purification of the Intermediate in Synthesis of Sepiapterin in *Drosophila*†

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ABSTRACT: The enzymatic conversion of the D-*erythro*-dihydroneopterin triphosphate [H_2-neopterin-$(P)_3$] to sepiapterin occurs via a nonphosphorylated intermediate as shown by others. We have developed a high-performance liquid chromatography assay for this intermediate and have found that the intermediate (X) and two related compounds (X1 and X2) can be formed nonenzymatically under certain conditions from H_2-neopterin-$(P)_3$. The reaction is catalyzed by tris(hydroxymethyl)aminomethane, dependent upon H_2-neopterin-$(P)_3$ concentration, significant at temperatures greater than 80 °C, and maximal between pH 8.5 and 9.5 (as determined at 25 °C). All three compounds were purified, and it was found that both X and X1 can serve as substrates for the enzymatic, NADPH-dependent synthesis of sepiapterin. From the kinetics of formation from H_2-neopterin-$(P)_3$ and the similarity of the ultraviolet spectra, it is clear that X, X1, and X2 are closely related compounds. None of the three compounds is reduced by $NaBH_4$; only X1 is sensitive to periodate oxidation. All three can be oxidized with iodine to give rise to highly fluorescent compounds that in turn can be reduced by $NaBH_4$ to give rise to the respective parent compounds. These latter observations indicate that X, X1, and X2 are dihydropterins. These results are discussed relative to the proposed structures for enzymatically produced X. The methods described for the nonenzymatic synthesis of X and its purification should allow preparation of large amounts of X for future study.

The enzymatic conversion of the D-*erythro*-dihydroneopterin triphosphate [H_2-neopterin-$(P)_3$][1] to 7,8-dihydro-6-lactoylpterin (sepiapterin) occurs in the presence of Mg^{2+} and NADPH. It has been demonstrated that sepiapterin synthase from *Drosophila melanogaster* (Dorsett et al., 1979; Krivi & Brown, 1979) and chicken kidney (Tanaka et al., 1981) consists of two enzymes, the first requiring Mg^{2+} as a cofactor and the second requiring NADPH. The separation of these two activities from *Drosophila* (Krivi & Brown, 1979) and from chicken kidney (Tanaka et al., 1981) has been reported. It has also been proposed that the product of the Mg^{2+}-dependent first enzyme is a precursor of the *Drosophila* red eye pigments, the "drosopterins", as well as of sepiapterin (Dorsett et al., 1979; Dorsett & Jacobson, 1982).

Following an earlier report (Dorsett et al., 1980), we describe here an assay for the sepiapterin synthase intermediate (the product of the Mg^{2+}-dependent first enzyme) using high-performance liquid chromatography (HPLC). A nonenzymatic method for the conversion of H_2-neopterin-$(P)_3$ to the intermediate (X) and related compounds (X1 and X2) is described, as well as the purification and partial characterization of these compounds. The implications of these studies with regard to the structure of the intermediate and the mechanism of sepiapterin synthesis are discussed.

Materials and Methods

Chromatography columns were obtained from Waters Associates (C_{18} μBondapak) and Du Pont (C8 Zorbax). Thin-layer cellulose sheets (no. 13255) were from Eastman Kodak Co.; methanol was purchased from Burdick and

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; H_2-neopterin-$(P)_3$, 3′-triphosphoester of 7,8-dihydro-6-(D-*erythro*-1,2,3-trihydroxypropyl)-pterin; sepiapterin, 7,8-dihydro-6-lactoylpterin; xanthopterin, 6-hydroxypterin; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; CE, crude enzyme extract; PFE, pteridine-free extract.

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