Evidence for Electron Transfer Reactions Involved in the Cu\(^{2+}\)-dependent Thiol Activation of Fat Cell Glucose Utilization*

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SUMMARY

Previous studies indicated that the ability of thiols to mimic insulin action on fat cell glucose oxidation and lipolysis was dependent on Cu\(^{2+}\). Evidence now presented indicates that H\(_2\)O\(_2\) formed by the reaction of thiols, Cu\(^{2+}\), and O\(_2\) mediates the effects of these agents on isolated fat cells. Oxidation of thiols to the respective disulfides in the presence of Cu\(^{2+}\) accompanied the stimulatory effects on glucose metabolism. Diphenyl-1,10-phenanthroline, which binds tightly to Cu\(^{2+}\) but not Cu\(^{2+}\), blocks copper-catalyzed thiol oxidation and the stimulatory effect on fat cell glucose utilization, indicating that reduction of Cu\(^{2+}\) is an obligatory step for thiol action. The thiol-Cu\(^{2+}\) effect on fat cells is readily reversible since stimulated rates of glucose oxidation returned to control levels following addition of the chelator. Catalase inhibited the stimulatory effect of thiols but not insulin on fat cell glucose oxidation, whereas H\(_2\)O\(_2\) mimicked the action of thiols and insulin on this process. As little as 10 \(\mu M\) H\(_2\)O\(_2\) stimulated glucose utilization, whereas 1 mm H\(_2\)O\(_2\) was maximally effective. Higher concentrations were less effective. The stimulatory effect of various mercaptoethanol and Cu\(^{2+}\) concentrations on labeled CO\(_2\) production from 3-[\(\text{\textsuperscript{14}C}\)]glucose by fat cells paralleled the peroxide formed under these conditions. Other oxidants such as MnO\(_4^-\) and diamide (diazeneicarboxylic acid bis-N,N-dimethylamide) also stimulated glucose utilization in isolated fat cells.

Among the large number of agents which have been reported to mimic the ability of insulin to stimulate glucose metabolism and inhibit lipolysis in isolated fat cells are the thiols (1, 2). We have previously reported that the effects of thiols on fat cell function were dependent on Cu\(^{2+}\), a contaminant of albumin preparations used in fat cell incubation media (2). The data presented in this report indicate that H\(_2\)O\(_2\) which is produced nonenzymatically by the combination of thiols, Cu\(^{2+}\), and O\(_2\) under the conditions used, mediates the biological actions of these agents. Further, other oxidants such as MnO\(_4^-\) and diamide (diazeneicarboxylic acid bis-N,N-dimethylamide) also stimulated glucose utilization in isolated fat cells.

METHODS

White fat cells were obtained by enzymatic digestion of the parametral adipose tissue from 130 to 150-g female rats (Charles River C\(_3\) strain) fed laboratory chow ad libitum (3). For each experiment the parametral adipose tissue from two or more rats was pooled and then cut into small pieces with scissors and blotted, and the desired amount added to 1-ounce polyethylene bottles. Depending on the requirements of the particular experiments 1 to 3 g of tissue were incubated in each bottle with 3 to 8 ml of 3% albumin in phosphate buffer and 1 mg of crude collagenase per ml (Clostridium histolyticum, Worthington) at 37\(^\circ\) for 1 hour. The phosphate buffer contained 128 mm NaCl, 1.4 mm CaCl\(_2\), 1.4 mm MgSO\(_4\), 5.2 mm KCl, 10 mm Na\(_2\)HPO\(_4\), pH 7.4, and 3% bovine serum albumin, Fraction V (Armour).

At the end of the digestion period cells were filtered through cheesecloth and washed twice with 6 ml of 1% albumin buffer at 37\(^\circ\). The fat cells were resuspended in albumin buffer and incubated in plastic culture tubes (16 mm \(\times\) 100 mm) at 37\(^\circ\) in a shaking water bath. The final incubation mixture volume in each tube was 1.2 ml and contained 0.1 or 0.2 mm 3-[\(\text{\textsuperscript{14}C}\)]glucose. The reaction was stopped by addition of 0.2 ml of 0.5 mm H\(_2\)SO\(_4\). Glucose conversion to CO\(_2\) was determined as described by Fain et al. (4). The values for each experiment are the averages of duplicate tubes and are based on changes during the incubation period over those of initial controls incubated for 5 min. The data presented in this report for each experimental design are the averages of two or more experiments performed on different days.

Reduction of Cu\(^{2+}\) by thiols (Fig. 3) was measured by the method of Wharton (5) using diphenyl-1,10-phenanthroline.
Cu²⁺ at a concentration of $2 \times 10^{-5}$ M in 1 ml of Krebs-Ringer phosphate buffer containing diphenyl-1,10-phenanthroline (1 mM), was reduced by 1 mM cysteine, and the time course of reduction of Cu²⁺ was measured at 479 nm. In other tubes 1 mM cysteine was added to 1 ml of 3% albumin in Krebs-Ringer buffer and the reduction of copper which contaminated the albumin and was accessible to cysteine was measured similarly.

Oxidation of mercaptoethanol was monitored using the sulfhydryl blocking reagent 6,6'-dithiodinitrolic acid by following the absorbance at 344 nm (6). Mercaptoethanol was incubated in 1.2 ml of buffer under the conditions studied (Figs. 4 and 6), and 30-μl aliquots were withdrawn at the appropriate times and added to 10 μl of 10 mM 6,6'-dithiodinitrolic acid. After dilution with 1 ml of H₂O the absorbance was measured at 344 nm. Essentially no difference was observed in the rate of mercaptoethanol oxidation in the presence or absence of fat cells.

H₂O₂ was assayed as follows. One-milliliter aliquots of buffer with appropriate additions of thiols and Cu²⁺ (Fig. 6) were added to the assay mixture. This mixture contained 30 μl of 0.1 M N-ethylmaleimide, 50 μl of Worthington Glucostat chromagen (diluted with 3 ml of H₂O per bottle), and 2 μg of lactoperoxidase (Calbiochem). This mixture was allowed to stand at room temperature for 30 min before adding 1 drop of 4 N HCl. After an additional 30 min the absorbance was measured at 420 nm. For the experiments described in Fig. 7 aliquots of fat cell incubation medium were filtered on glass fiber filters (Whatman GF/C) to free the aliquots from cells before assay.

Crystalline porcine insulin (Lot P-5589) with less than 0.005% glucagon was generously supplied by Eli Lilly. N-[¹-¹⁴C]glucose was obtained from New England Nuclear Corp. Diphenyl 1,10-phenanthroline was obtained from the G. Frederick Smith Chemical Co., Columbus, Ohio, and 30% H₂O₂ from Allied Chemical. Diamide was obtained from Calbiochem and 6,6'-dithionicotinic acid from Newcell Biochemicals, Berkeley, Calif.

RESULTS

We have previously demonstrated that albumin preparations which were freed of heavy metal contaminants by reaction with o-phenanthroline and passage through Sephadex G-50 were unable to support the thiol effect on fat cell glucose oxidation; addition of divalent copper to the treated albumin restored the potentiation of cysteine action (2). Fig. 1 shows the results of experiments designed to test the effect of various concentrations of Cu²⁺ on mercaptoethanol-enhanced glucose oxidation in the presence of a very low albumin concentration (0.1%): This amount of albumin was able to potentiate the thiol effect only slightly. Under these conditions 30 μM Cu²⁺ maximally potentiated the effects of 0.2 mM and 1 mM mercaptoethanol on fat cell glucose utilization. In contrast to our earlier observations using higher albumin levels, 0.1 mM Cu²⁺ itself markedly stimulated glucose oxidation by fat cells incubated in 0.1% albumin. Mercaptoethanol at both concentrations inhibited this effect of Cu²⁺ by about 50%.

In order to determine whether the permissive effect of Cu²⁺ on thiol action involves reduction of the metal to the monovalent form we tested the effect of diphenyl-1,10-phenanthroline. Fig. 2 demonstrates the potent inhibitory effect of this agent, which binds to Cu²⁺ but not Cu²⁺, with an extremely high affinity, on the actions of cysteine, glutathione, and mercaptoethanol in 1% albumin buffer. These results suggested that an obligatory step in the action of thiols and Cu²⁺ on fat cell glucose metabolism involved the oxidation of thiols to the disulfide form with concomitant reduction of Cu²⁺. When the oxidation state of either added copper or that present in albumin preparations was...
monitored by diphenyl-1,10-phenanthroline absorbance at 479 nm, rapid reduction of Cu²⁺ by thiols was found to occur (Fig. 3). The amount of Cu²⁺contaminating albumin which is accessible to reduction by cysteine was approximately 6.6 nm per mg of albumin.

Fig. 4 (top panel) presents the time course of labeled CO₂ production by fat cells incubated in the presence of 1 mM mercaptoethanol. Diphenyl-1,10-phenanthroline added at 15 min reversed thiol-activated glucose oxidation back to control levels. The chelator had essentially no effect on control levels of fat cell glucose oxidation. Oxidation of mercaptoethanol accompanied the stimulation of glucose utilization over the 60-min incubation period and this oxidation was also blocked by diphenyl-1,10-phenanthroline. Under the conditions of this series of experiments about 65% of the initial amount of mercaptoethanol was oxidized (Fig. 4). The presence of fat cells had little or no effect on the rate of mercaptoethanol oxidation in 2% albumin buffer.

We have previously presented evidence consistent with the concept that some substance formed by the reaction of thiols and Cu²⁺ mediates the stimulatory effect on fat cell glucose oxidation (2). The data presented in Table I indicate that this mediator appears to be H₂O₂ formed by the interaction of Cu²⁺, thiol, and O₂. The effect of cysteine and mercaptoethanol on fat cell glucose utilization was blocked by catalase, but there was no effect of catalase on insulin action nor basal glucose oxidation. Further, 1 mM H₂O₂ like insulin and thiols, enhanced labeled CO₂ production by fat cells and this effect was also abolished by catalase (Table I). When H₂O₂ was added to fat cells at zero time and glucose oxidation monitored for 15 min, a biphasic effect of the agent was observed (Fig. 5). As little as 10 μM H₂O₂ enhanced the conversion of both D-[1-¹⁴C]glucose and D-[6-¹⁴C]glucose to labeled CO₂. A maximum effect was found at around 1 mM, whereas 4 mM H₂O₂ was significantly less effective (Fig. 5).

Further evidence which suggests that H₂O₂ mediates the effect of thiols is presented in Fig. 6. Fat cell glucose oxidation in the presence of 0.2 mM and 1 mM mercaptoethanol was enhanced to a greater extent with 30 μM Cu²⁺ than with 3 μM. Both the amount of thiol oxidized and the amount of H₂O₂ formed during this 15-min incubation period paralleled the magnitude of the response under conditions identical to those used to monitor CO₂ production except without cells, since in their presence H₂O₂ was difficult to assay due to its rapid disappearance (Fig. 7). The rate of mercaptoethanol oxidation is the same in the presence and absence of cells.¹ Fig. 7 presents the results of an experimental design where H₂O₂ generated by 1 mM cysteine, 30 μM Cu²⁺, and O₂ was assayed in the presence and absence of fat cells over a 15-min incubation period. In the absence of fat cells the maximum net amount of H₂O₂ produced was about 110 nmoles and occurred at about 3 min. At this point, the net amount of H₂O₂ produced in the presence of fat cells was only about 35 nmoles and this dropped to about 10 nmoles by 5 min. This enhanced rate of H₂O₂ decomposition by fat cells also occurred when the H₂O₂ was added directly rather than generated.

**Table I**

**Inhibition of the effect of thiols on fat cell glucose metabolism by catalase**

| Additions | Control | Catalase, 16 μg/ml |
|-----------|---------|------------------|
| None      | 0.67    | 0.65             |
| Increment due to added agents |
| Insulin, 400 microunits/ml | +12 | +12 |
| Cysteine, 1 mM | +20 | +0.44 |
| Mercaptoethanol, 1 mM | +28 | +2.4 |
| H₂O₂, 1 mM | +28 | +0.53 |

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¹ Further evidence which suggests that H₂O₂ mediates the effect of thiols is presented in Fig. 6. Fat cell glucose oxidation in the presence of 0.2 mM and 1 mM mercaptoethanol was enhanced to a greater extent with 30 μM Cu²⁺ than with 3 μM. Both the amount of thiol oxidized and the amount of H₂O₂ formed during this 15-min incubation period paralleled the magnitude of the response under conditions identical to those used to monitor CO₂ production except without cells, since in their presence H₂O₂ was difficult to assay due to its rapid disappearance (Fig. 7). The rate of mercaptoethanol oxidation is the same in the presence and absence of cells. Fig. 7 presents the results of an experimental design where H₂O₂ generated by 1 mM cysteine, 30 μM Cu²⁺, and O₂ was assayed in the presence and absence of fat cells over a 15-min incubation period. In the absence of fat cells the maximum net amount of H₂O₂ produced was about 110 nmoles and occurred at about 3 min. At this point, the net amount of H₂O₂ produced in the presence of fat cells was only about 35 nmoles and this dropped to about 10 nmoles by 5 min. This enhanced rate of H₂O₂ decomposition by fat cells also occurred when the H₂O₂ was added directly rather than generated.

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**Fig. 4.** Reversibility of the effect of mercaptoethanol oxidation on fat cell glucose utilization. Isolated fat cells (2 × 10⁶ cells per tube) were incubated in Krebs-Ringer phosphate buffer containing 2% albumin and 0.2 mM D-[1-¹⁴C]glucose at 37° for 30 min. The concentrations of H₂O₂ indicated were added to incubation tubes after the addition of cells and after labeled CO₂ was monitored.

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**Fig. 5.** The stimulatory effect of H₂O₂ on D-[1-¹⁴C]glucose and D-[6-¹⁴C]glucose conversion to CO₂ in isolated fat cells. Fat cells (2 × 10⁶ cells per tube) were incubated in Krebs-Ringer buffer containing 0.2 mM D-[1-¹⁴C]glucose or D-[6-¹⁴C]glucose and 2% albumin at 37° for 15 min. The concentrations of H₂O₂ indicated were added to incubation tubes after the addition of cells and after labeled CO₂ was monitored.
Fig. 6. The relationship between the effectiveness of mercaptoethanol on fat cell glucose utilization and the formation of \( \text{H}_2\text{O}_2 \) in the presence of \( \text{Cu}^{2+} \). Fat cells (3 X 10^6 cells per tube) were incubated in Krebs-Ringer phosphate buffer containing 0.1% albumin and 0.1 mM \( \text{n-[1-14C]} \text{glucose} \) in the presence or absence of mercaptoethanol and \( \text{Cu}^{2+} \). The incubation was carried out at 37° for 15 min, and labeled CO\(_2\) production and mercaptoethanol oxidized were monitored. \( \text{H}_2\text{O}_2 \) formed under the appropriate conditions was determined in duplicate tubes treated as above, but in the absence of fat cells.

![Graph showing the relationship between mercaptoethanol and \( \text{H}_2\text{O}_2 \) formation](image)

by the thiol-Cu\(^{2+}\) mixture. The amount of \( \text{H}_2\text{O}_2 \) formed by the reaction of cysteine with Cu\(^{2+}\) and O\(_2\) in 15 min exceeds that produced by mercaptoethanol under the same conditions (Fig. 6). We have also found that the rate of \( \text{H}_2\text{O}_2 \) formation by cysteine oxidation is much more rapid than that of mercaptoethanol.

![Graph showing decomposition of \( \text{H}_2\text{O}_2 \) formed by cysteine and Cu\(^{2+}\)](image)

The effect of other oxidants on fat cell glucose metabolism is shown in Table II. \( \text{MnO}_4^- \) and diamide markedly stimulated the conversion of \( \text{n-[1-14C]} \text{glucose} \) to labeled CO\(_2\) by isolated fat cells. The stimulatory effects of these agents as well as those of \( \text{H}_2\text{O}_2 \) and insulin were inhibited by 2 mM phlorizin and cytochalasin B, which are glucose transport inhibitors (Table II). We also tested whether the effect of Cu\(^{2+}\) on fat cell glucose oxidation was mediated by peroxide. This stimulatory effect of Cu\(^{2+}\) was much more marked at 0.1 mM than at 0.3 mM (Fig. 8). Neither diphenyl-1,10-phenanthroline nor catalase significantly inhibited the effect of Cu\(^{2+}\) on glucose utilization, whereas 2 mM EDTA abolished its effect. Thus, these data indicate that large amounts of Cu\(^{2+}\) do not produce \( \text{H}_2\text{O}_2\); rather it seems that divalent copper at these high concentrations acts directly on fat cells to stimulate glucose utilization.

**DISCUSSION**

One approach to the study of insulin action has been the study of chemically simple or specific agents which either mimic or inhibit the effects of insulin on fat cell metabolism. The general experience has been that a large number of agents with very different chemical specificities can mimic some of these insulin effects. For example, thiols (1, 2), mild proteolysis (7, 8), polyamines (9), hypertonicity (10), sulhydryl reagents (11),...
prostaglandins (12), vitamin K₃ (13, 14), lectins (15), and others have been reported to stimulate glucose oxidation and inhibit lipolysis in fat cells. However, none of these have yet been shown to mimic all of the effects of insulin, and it is unlikely that any act exactly as does insulin. A major disappointment in this approach has been that despite the existence of so many active agents little or no progress has been made at identifying the specific cell components involved in the action of any of these agents.

Several of the above agents have been tested on trypanized cells which no longer respond to insulin and have been found to be still active (16-19); therefore, if their actions do share any common pathways with those of insulin, they probably occur at steps beyond the hormone-receptor interaction. The greatest utility of studies with these agents may be to provide clues to the chemical events which occur subsequent to insulin binding and which lead to the enhancement of glucose transport. Our previous finding that the effects of thiols were dependent on divalent copper (2) prompted us to further characterize the basis for this dependence. The present findings (Fig. 1) demonstrate the potentiation of thiol action by Cu²⁺ at a very low albumin concentration (0.1%). Attempts to demonstrate this effect in the complete absence of albumin have been variable, probably due to the markedly diminished viability of fat cells under these conditions (20). Furthermore, the data presented show that albumin preparations used in the cell incubation buffer contain sufficient amounts of Cu²⁺ to catalyze production of H₂O₂ from thiols (Fig. 2).

Under no circumstances have we found an effect of thiols on fat cell glucose utilization without the concomitant oxidation of a portion of the sulfhydryl groups to the disulfide (Figs. 4 and 6). Divalent copper is well known for its catalytic role in this oxidation reaction and its subsequent reduction of molecular oxygen (21). We therefore propose that the Cu²⁺-dependent thiol activation of fat cell glucose oxidation is mediated by peroxide formed by the following reactions.

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\begin{align*}
2 \text{RSH} + 2 \text{Cu}^{2+} & \rightarrow \text{RSSR} + 2 \text{Cu}^{+} + 2 \text{H}^+ \quad (1) \\
2 \text{H}^+ + 2 \text{Cu}^{+} + \text{O}_2 & \rightarrow 2 \text{Cu}^{2+} + \text{H}_2\text{O}_2 \quad (2)
\end{align*}
\]

This scheme predicts that the absence of molecular oxygen should abolish the ability of thiols and Cu²⁺ to mimic insulin action. We have thus tested the effects of 1 mM cysteine and 200 μunits per ml of insulin on fat cells incubated anaerobically for 20 min in 2% albumin buffer and found that the fat cell response to the thiol was inhibited by about 95% whereas that to insulin was unaffected.¹

¹ That the resulting H₂O₂ formed from these electron transfers is the species which actually interacts with the fat cells is strongly supported by the present data. Catalase which catalyzes the rapid decomposition of H₂O₂, inhibited the effect of thiols but not insulin on fat cell glucose metabolism, whereas added H₂O₂ mimicked the effect of the thiols (Table I). Further, the amount of peroxide formed by various concentrations of mercaptoethanol and Cu²⁺ was proportional to the magnitude of the fat cell response (Fig. 6).

Of interest was the finding that the enhanced fat cell glucose oxidation rates which occurred in response to thiol-Cu¹ returned to control levels upon inhibition of further peroxide formation by diphenyl-1,10-phenanthroline (Fig. 4). This ready reversibility of thiol action argues against the involvement of irreversible damage to cells due to, for example, lipid peroxidation. In support of this conclusion, Lavis et al. (22) showed that ascorbate initiated extensive lipid peroxidation when added to fat cells in the absence of effects on glucose oxidation.

The effectiveness of the oxidants MnO₄⁻, Cu²⁺, and diamide as well as previously reported effects of phenazine methosulfate (23) and vitamin K₃ (13, 14) suggest that these agents as well as H₂O₂ act by catalyzing the oxidation of a fat cell component or components. It is known that diamide, MnO₄⁻, and H₂O₂ can rapidly catalyze the oxidation of sulfhydryls. Diamide has been reported to exhibit a relative selectivity towards reduced glutathione (24-26). Cu²⁺ is also a potent oxidant of sulfhydryl groups (21). High levels of medium albumin (27-29) or EDTA inhibit this effect of Cu²⁺ by binding the metal.

It is not clear whether the actions of these oxidants reside at the level of glucose transport or intracellular glucose metabolism or both. Lavis and Williams found that the effect of thiols was additive to that of submaximal, but not maximal, doses of insulin (1). These workers also showed that the thiols were ineffective at stimulating glucose oxidation of fat cell homogenates and suggested that the primary effect may be at the level of glucose transport (1). On the other hand, Jacob and Jandl (30) concluded from their careful studies that H₂O₂ stimulated red cell d-[3H]glucose conversion to labeled CO₂ secondary to lowering intracellular reduced nicotinamide adenine dinucleotide phosphate levels. This effect was found to be mediated through oxidation of intracellular glutathione which is then reduced at the expense of reducing equivalents from the dinucleotide-phosphate in a reaction catalyzed by glutathione reductase. The increased level of oxidized nicotinamide adenine dinucleotide phosphate is then thought to stimulate reactions in the pathway of glucose metabolism which form the reduced state of the dinucleotide phosphate. That this series of events also occurs in fat cells upon addition of H₂O₂ is likely, since diamide, which is a rather specific agent for oxidizing glutathione, is also a potent stimulator of glucose utilization (Table II).

Whether oxidants also directly or indirectly modulate the β-glucose transport system in the fat cell plasma membrane is the subject of further studies. We have recently developed a method to monitor 2-deoxy-β-glucose transport in fat cell ghosts which may be useful in answering this question (31).

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