Insulin-like effects of dithiothreitol on isolated rat adipocytes

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(Received 13 July 1981/Accepted 28 July 1981)

The effects of dithiothreitol on basal glucose oxidation, hormone-induced lipolysis and insulin receptors in isolated rat adipocytes were studied. Dithiothreitol produced a dose-dependent stimulation of basal glucose oxidation and inhibition of adrenaline-induced lipolysis. Dithiothreitol also inhibited corticotropin-induced lipolysis, but failed to inhibit dibutyl cyclic AMP-induced lipolysis. Dithiothreitol did not inhibit the binding of the β -adrenergic antagonist [3 H]dihydroalprenolol to adipocytes. Neither catalase (100 μ g/ml) nor EDTA (2 mM) abolished the antilipolytic effect of dithiothreitol. Treatment of isolated adipocytes with 1 mM-dithiothreitol for 20 min at 37°C also caused stimulation of basal glucose oxidation and inhibition of adrenaline-induced lipolysis. A Scatchard plot of insulin binding to control adipocytes was curvilinear. However, treatment of cells with 1 mM-dithiothreitol decreased the curvilinearity of the plot, indicating that only a low-affinity state of the insulin receptors exists in the dithiothreitol-treated adipocytes. These findings suggest that the insulin-like activities of dithiothreitol are mediated through the interaction of dithiothreitol with insulin receptors.

Many chemicals are known to mimic the actions of insulin on adipocytes. Vitamin K₅ (Kuo *et al.*, 1969) and vanadate (Dubyak & Kleinzeller, 1980; Shechter & Karlsh, 1980) only stimulate glucose oxidation, whereas concanavalin A, wheat-germ agglutinin (Cuatrecasas & Tell, 1973) and antibodies to insulin receptors (Kahn *et al.*, 1977; Jacobs *et al.*, 1978), which interact with the insulin receptors, stimulate glucose oxidation and inhibit adrenaline-induced lipolysis. Lavis & Williams (1970) reported that dithiothreitol stimulates glucose oxidation and inhibits lipolysis. However, the mechanisms of the insulin-like actions of this chemical are still unknown.

Binding of insulin to solubilized avian erythrocyte receptors induces dissociation of the receptors into subunits (Ginsberg *et al.*, 1976). Jacobs *et al.* (1979) have found that treatment of solubilized rat liver insulin receptors with dithiothreitol also induces dissociation of the receptors into subunits. These results suggest that dithiothreitol alters the insulin receptors, and the interaction of this chemical with the insulin receptors may induce the insulin-like activities of dithiothreitol.

Experimental

Materials

Bovine serum albumin (non-esterified fatty acid-

free, fraction V), crude collagenase (type I), pig insulin, catalase (EC 1.11.1.6), adrenaline, alprenolol, dibutyl cyclic AMP, dithiothreitol and cytochalasin B were purchased from Sigma. Corticotropin (synthetic 1–24 fragment) was obtained from Daiichi Seiyaku Co., Tokyo, Japan. Hyamine 10X was from Nakarai Chemicals, Kyoto, Japan. Aquasol-2 was from New England Nuclear. 125 I-labelled insulin (224 μ Ci/ μ g) was from Dainabot Radioisotope Laboratory, Tokyo, Japan. [3 H]Dihydroalprenolol (59 Ci/mmol) and D-[U- 14 C]glucose (255 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Isolation of adipocytes. Adipocytes were isolated from the epididymal fat-pads of Wistar male rats (160–240 g) by the method of Rodbell (1964). The fat-pads were incubated for 60 min at 37°C in Krebs–Ringer bicarbonate medium (119 mM-NaCl/4.8 mM-KCl/1.3 mM-CaCl₂/1.2 mM-KH₂PO₄/1.2 mM-MgSO₄/25 mM-NaHCO₃/30 mg of bovine serum albumin/ml) with 2 mg of collagenase/ml. After digestion, the cells were filtered through nylon mesh and washed three times with buffer.

Glucose oxidation. Isolated adipocytes were incubated for 60 min at 37°C with 0.1 μ Ci of D-[U- 14 C]glucose (0.5 mM) and various concentrations of dithiothreitol. The incubations were terminated by

injecting 0.5 ml of 3M-H₂SO₄ and the CO₂ formed was collected in 1 ml of Hyamine 10X.

Lipolysis. Isolated adipocytes were incubated for 60 min at 37°C with various concentrations of dithiothreitol in the presence of adrenaline, corticotropin or dibutyryl cyclic AMP. Lipolysis was determined by measuring glycerol release into the medium.

[³H]Dihydroalprenolol binding. Isolated adipocytes were incubated for 20 min at 25°C with 0.2 μCi of [³H]dihydroalprenolol and 0, 1 mM- or 3 mM-dithiothreitol. Non-specific binding was calculated from [³H]dihydroalprenolol binding in the presence of 10 mM-alprenolol. At the end of the incubation, 2 ml of ice-cold Krebs–Ringer bicarbonate buffer was added to each vial. The adipocytes were rapidly filtered under vacuum through Millipore SC filters (pore size 8.0 μm), followed by washing with 3 × 1 ml of ice-cold Krebs–Ringer bicarbonate buffer. Each filter was added to 9 ml of Aquasol-2 and counted for radioactivity in a liquid-scintillation counter.

Dithiothreitol treatment of isolated adipocytes. Isolated adipocytes were incubated for 20 min at 37°C with 1 mM-dithiothreitol. After incubation, adipocytes were washed with 3 × 10 vol. of dithiothreitol-free Krebs–Ringer bicarbonate buffer.

Insulin binding. Control and dithiothreitol-treated adipocytes were incubated with ¹²⁵I-labelled insulin (0.3 ng) and various concentrations of unlabelled insulin for 30 min at 25°C in a total volume of 0.8 ml. Incubation was terminated by centrifuging 300 μl samples of cell suspension through dinonyl phthalate in plastic tubes as described by Gliemann *et al.* (1972). The tubes were cut through the oil layer and the cell-bound radioactivities were determined. Specific binding was calculated by subtracting non-specific binding (cell-bound ¹²⁵I-labelled insulin in the presence of 10 μg of unlabelled insulin) from total binding.

Results

Insulin-like effects of dithiothreitol

Fig. 1 shows the dose–response relationships of dithiothreitol in the inhibition of adrenaline-induced lipolysis and stimulation of basal glucose oxidation. At 0.1 mM-dithiothreitol, lipolysis was slightly stimulated and basal glucose oxidation was slightly suppressed. Both inhibition of lipolysis and stimulation of glucose oxidation began at about 0.3 mM-dithiothreitol. The shapes of these two dose–response curves were very similar. The concentrations of the chemical required for half-maximal inhibition of lipolysis and half-maximal stimulation of glucose oxidation were 1 mM and 0.78 mM respectively. The stimulation of basal glucose oxidation was not due to the increase of passive glucose transport by dithio-

Fig. 1. Effects of dithiothreitol on adrenaline-induced (1 μM) lipolysis (●) and basal glucose oxidation with (Δ) or without (▲) cytochalasin B (50 μM).

Isolated adipocytes were incubated for 60 min at 37°C with various concentrations of dithiothreitol in the presence of 1 μM-adrenaline to induce lipolysis, or in the presence of 0.1 μCi of D-[U-¹⁴C]glucose (0.5 mM) with or without 50 μM-cytochalasin B. Each point represents the mean ± S.E.M. of two sets of triplicate determinations.

threitol, since this effect was diminished by the presence of 50 μM-cytochalasin B (Fig. 1).

Dithiothreitol could exert its antilipolytic effect through the β-adrenergic receptor. In order to know whether adrenaline binding to adipocytes is affected by dithiothreitol, the binding of the β-adrenergic antagonist [³H]dihydroalprenolol to isolated adipocytes was measured. Dithiothreitol (up to 3 mM) did not inhibit [³H]dihydroalprenolol binding. Antagonist binding to adipocytes in the presence of 3 mM-dithiothreitol was 125 ± 3.7% (n = 6) of the control value. This stimulation may be due to the protective effect of dithiothreitol on oxidation of [³H]dihydroalprenolol.

Next we examined the effect of dithiothreitol on lipolysis induced by corticotropin and dibutyryl cyclic AMP. As shown in Table 1, lipolysis induced by corticotropin (50 ng/ml) was inhibited by 1 mM-dithiothreitol, whereas 1 mM-dithiothreitol failed to inhibit the lipolysis induced by 3 mM-dibutyryl cyclic

Table 1. *Effects of adrenaline, catalase, EDTA, dibutylryl cyclic AMP and corticotropin on the antilipolytic action of dithiothreitol*

Isolated adipocytes were incubated with the additions noted below in the presence or absence of 1 mM-dithiothreitol at 37°C for 1 h. The results are expressed as nmol of glycerol released/h per mg of triacylglycerol, means $\pm$ S.E.M. for two sets of triplicate determinations.

Additions	Glycerol release	
	Control	+Dithiothreitol
None	0.16 $\pm$ 0.03	0.64 $\pm$ 0.09
Adrenaline (1 μ M)	8.85 $\pm$ 0.12	5.96 $\pm$ 0.19
+catalase (100 μ g/ml)	9.67 $\pm$ 0.50	5.38 $\pm$ 0.16
+EDTA (2 mM)	8.85 $\pm$ 0.36	4.89 $\pm$ 0.16
Dibutylryl cyclic AMP (3 mM)	11.85 $\pm$ 0.47	11.62 $\pm$ 0.26
Corticotropin (50 ng/ml)	9.60 $\pm$ 0.23	4.41 $\pm$ 0.29

AMP. This indicates that dithiothreitol does not exert its action on post-cyclic AMP processes in lipolysis.

Czech *et al.* (1974) have found that thiols such as cysteine or glutathione stimulate glucose oxidation in adipocytes and that this stimulation results from H₂O₂ generated by thiols, Cu²⁺ and O₂, since EDTA or catalase abolishes this effect. In order to determine whether EDTA or catalase has any effect on the antilipolytic action of dithiothreitol, isolated adipocytes were incubated with or without 2 mM-EDTA, 100 μ g of catalase/ml and 1 mM-dithiothreitol in the presence of 1 μ M-adrenaline. Neither EDTA nor catalase diminished the antilipolytic effect of dithiothreitol (Table 1).

Effects of dithiothreitol treatment of adipocytes on lipolysis, basal glucose oxidation and insulin binding

Isolated adipocytes were treated with 1 mM-dithiothreitol for 20 min at 37°C, then washed with 3 $\times$ 10 vol. of dithiothreitol-free buffer. Basal glucose oxidation and adrenaline-induced lipolysis were measured in control and in dithiothreitol-treated adipocytes. Basal glucose oxidation in dithiothreitol-treated adipocytes was increased 64% in comparison with control values (Table 2). Lipolysis induced by 1 μ M-adrenaline in dithiothreitol-treated cells was 37% less than in control cells (Table 2). Thus dithiothreitol need not exist in the medium to exert its insulin-like effects.

As shown in Fig. 2, the Scatchard plot of insulin binding to control adipocytes was curvilinear, whereas that to dithiothreitol-treated cells was only slightly curvilinear. This disappearance of the high-affinity state may be due to inactivation of

Table 2. *Effects of dithiothreitol treatment of adipocytes on lipolysis and glucose oxidation*

Isolated adipocytes were incubated with 1 mM-dithiothreitol at 37°C for 20 min and then washed with 3 $\times$ 10 vol. of buffer. Control and dithiothreitol-treated cells were incubated with 1 μ M-adrenaline for lipolysis, or with 0.5 mM-D-[U-¹⁴C]glucose for glucose oxidation, at 37°C for 1 h. The results are expressed as nmol/h per mg of triacylglycerol, means $\pm$ S.E.M. for two sets of triplicate determinations. Levels of significance: **P* < 0.002, ***P* < 0.001.

Conditions	Glycerol release	Glucose oxidation
Control	9.17 $\pm$ 0.43	0.192 $\pm$ 0.008
Dithiothreitol-treated	5.74 $\pm$ 0.63*	0.314 $\pm$ 0.014**

Fig. 2. *Scatchard plot of insulin binding to control (●) or dithiothreitol-treated (○) adipocytes*

Isolated adipocytes were treated with 1 mM-dithiothreitol for 20 min at 37°C, then washed with 3 $\times$ 10 vol. of dithiothreitol-free buffer. Control and dithiothreitol-treated adipocytes were incubated with ¹²⁵I-labelled insulin and various concentrations of unlabelled insulin for 30 min at 25°C. Each point represents the mean of two sets of triplicate determinations.

high-affinity receptors or diminution of negative co-operativity. If the former is the case, the number of high-affinity receptors was decreased from 4500 to 1100 sites per cell by treatment with 1 mM-dithiothreitol. If the latter is the case, the dissociation constant at the high-affinity state was

increased from $5.7 \times 10^{-10} \text{ M}$ to $2.7 \times 10^{-9} \text{ M}$. The Hill coefficient for insulin binding to adipocytes was increased from 0.88 to 0.97, indicating that negative co-operativity was almost diminished by treatment with 1 mM-dithiothreitol.

Discussion

The results of this study show that dithiothreitol has insulin-like effects on adipocytes. This is consistent with the observations of Lavis & Williams (1970) that thiols stimulate basal glucose oxidation and inhibit adrenaline-induced lipolysis. It is possible that inhibition of adrenaline-induced lipolysis might result from the alteration of the adrenaline receptor to decrease the affinity for adrenaline. This possibility was ruled out, since the binding of the β -adrenergic antagonist [^3H]dihydroalprenolol was not inhibited by dithiothreitol. Furthermore, dithiothreitol, like insulin, inhibited corticotropin-induced lipolysis, but not that induced by dibutyryl cyclic AMP. Dose-response curves of inhibition of adrenaline-induced lipolysis and of stimulation of basal glucose oxidation were essentially parallel (Fig. 1). These observations suggest that both the insulin-like effects of dithiothreitol are mediated through the same site.

Czech *et al.* (1974) reported that stimulation of basal glucose oxidation by thiols was due to H_2O_2 formed by O_2 , Cu^{2+} and thiols, since EDTA or catalase abolished this effect. However, our results showed that neither EDTA nor catalase abolished the antilipolytic effect of dithiothreitol (Table 1). May & de Haën (1979) have found that insulin stimulates intracellular H_2O_2 production in rat epididymal fat-cells, suggesting that H_2O_2 may act as a second messenger of insulin. Thus it cannot be ruled out that dithiothreitol stimulates endogenous H_2O_2 formation; in that case externally added EDTA or catalase would not prevent H_2O_2 formation.

Even in the absence of dithiothreitol, basal glucose oxidation was enhanced and adrenaline-induced lipolysis was inhibited in washed dithiothreitol-treated adipocytes to the same extent as in the presence of dithiothreitol (Table 2), indicating that dithiothreitol need not be present in the incubation medium to exert its insulin-like effects. At the same time, Scatchard analysis of insulin binding to dithiothreitol-treated adipocytes revealed that treatment of isolated adipocytes with 1 mM-dithiothreitol for 20 min at 37°C caused the disappearance of the

high-affinity receptors (Fig. 2). This alteration of the insulin receptors by dithiothreitol could be closely related to the insulin-like effects of dithiothreitol.

Ginsburg *et al.* (1976) have reported that detergent-solubilized avian erythrocyte insulin receptor is dissociated into four subunits by the addition of insulin. Jacobs *et al.* (1979, 1980) have found that solubilized rat liver insulin receptors are composed of four subunits, two 135 000-dalton components and two 45 000-dalton components which are joined by disulphide bonds and significant non-covalent interactions. Pilch & Czech (1980) showed that high-affinity fat-cell and liver plasma-membrane insulin-receptor subunits are linked by disulphide bonds. These results and our observations suggest that treatment of adipocytes with dithiothreitol may result in the dissociation of the insulin receptors into subunits that have low affinity for insulin. Such dissociation of the insulin receptors might be a necessary step in evoking the intracellular responses.

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