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EFFECTS IN ADIPOCYTES OF DIAMIDE ON GSH LEVELS, GLUCOSE UPTAKE AND CELL INTEGRITY

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Summary

Concentrations of insulin and chemical agents (H_2O_2, vitamin K-5) which stimulate hexose transport in fat cells do not alter the cellular levels of glutathione (reduced form; GSH). Diamide, another agent used in studies of insulin action, markedly reduces GSH levels and increases the movement of sugar into the cell. However, unlike insulin, H_2O_2 or vitamin K-5, diamide causes a change in the permeability of fat cells that allows entry of compounds (inulin, sucrose, L-glucose) which are normally excluded by the plasma membrane. Moreover, the accelerated rate of methylglucose uptake produced by diamide treatment is not inhibited by cytochalasin B, an agent that blocks basal and insulin-stimulated methylglucose transport. These results indicate that diamide does not cause a stimulation of the glucose transport system and should not be used (or used with caution) in transport studies. Furthermore, oxidation of GSH does not appear to be necessary for the stimulation of hexose transport in adipocytes by insulin, H_2O_2 or vitamin K-5.

Introduction

It is well established that the first step in the action of insulin is association of the hormone with a membrane-bound receptor [1]. Unfortunately, very little is known of the events that follow this interaction which couple an effector system like glucose transport to the receptor system. Recently, the oxidants H_2O_2, vitamin K-5, and diamide have been used to investigate post-receptor events since they enhance the entry of glucose into fat cells by acting at sites other than the insulin receptor [2—4]. From studies using oxidants and

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sulfhydryl reagents, Czech and coworkers [5,6] have postulated that control of glucose transport activity resides in the transition of this system between a less active reduced (thiol) form and an active oxidized (disulfide) form. Since diamide oxidizes glutathione (GSH), it was further suggested that GSH levels may play a role in the regulation of hexose entry.

The present study was undertaken to test the importance of GSH in the activation of the glucose transport system by insulin and the chemical agents mentioned above. Special attention was paid to the action of diamide because of its known effects on cellular GSH levels.

Materials and Methods

Materials. Crude collagenase (type I) was supplied by Worthington Biochemical Corp. Bovine albumin fraction V was obtained from Metrix. Silicone oil was purchased from Arthur H. Thomas Co. Uniformly labeled D-[^{14}C]-glucose, 3-*O*-[^{14}C]methylglucose, [^{14}C]sucrose, [*Me*-^{3}H]inulin and L-[1-^{14}C]-glucose were supplied by New England Nuclear. Crystalline porcine insulin was a gift of Eli Lilly. Phlorizin was purchased from K&K Laboratories, vitamin K-5 from Nutritional Biochemicals and H_2O_2 from Fisher Scientific Co. Diamide, *O*-phthalaldehyde, glutathione and cytochalasin B were supplied by Sigma Chemical Co. All other chemicals were of reagent grade.

Isolation of fat cells. Adipocytes were prepared from the epididymal fat pads of male Sprague-Dawley rats (175—200 g) by a slight modification of the method of Rodbell [7] as previously reported [8]. The cells were suspended in a phosphate Krebs-Ringer buffer, pH 7.4, containing 30 mg/ml bovine albumin fraction V [8]. Fat cell content was estimated by lipid weight [9].

D-Glucose uptake. The rate of D-glucose and 3-*O*-methylglucose uptake was assayed with previously reported methods [8]. Briefly, 0.3-ml aliquots of a cell suspension (50 mg lipid/ml) were incubated at 37°C in the presence or absence of insulin or various chemical agents under an atmosphere of 95% O_2/5% Co_2 for the indicated period of time. The rate of D-glucose uptake was estimated by incubating the cells for 30 s at 37°C with a 0.1 mM concentration of D-[U-^{14}C]glucose (4 Ci/mol). The assay was considered terminated when the cells were isolated from the incubation medium by the oil-centrifugation method previously outlined [8]. Following the oil-centrifugation step, the adipocytes are located in a cell pellet over the silicone oil layer. In certain experiments the data for sugar uptake are reported as the amount of hexose contained in this cell pellet.

3-O-methylglucose uptake. Uptake of 3-*O*-methylglucose was measured as previously described using the oil-centrifugation technique [8]. The assay was begun by the addition of 0.1 mM 3-*O*-[U-^{14}C]methylglucose (19 Ci/mol) to the cells and terminated after a 20 s incubation by the addition of an ice-cold phlorizin-saline solution [8]. Cytochalasin B was dissolved in ethanol for use in transport studies; the quantity of ethanol (final concentration of 0.7%, v/v) added to fat cell suspensions had no independent effects on sugar transport activity.

Cell pellet water spaces. The total water space in the pellet was measured by determining the distribution of methylglucose under steady-state conditions

[9]. Following a 60 min treatment of the cells with insulin or the chemical agents, 25 μl of a 1.25 mM 3-*O*-[U-^{14}C]methylglucose solution (4 Ci/mol) was added to 0.3 ml of the cell suspension and the incubation continued for an additional 60 min at 37°C. The cells were then isolated by the oil-centrifugation technique and the radioactivity in both the cell pellet and in the cell-free incubation medium was determined. The methylglucose distribution space in the cell pellet was calculated from the amount of radioactivity per unit volume present in the incubation medium. Extracellular volume in the cell pellet was determined in a similar fashion by the addition of 0.6 μCi of [*methoxy*-^{3}H]-inulin (1 μCi/mg) or 0.14 μCi of [U-^{14}C]sucrose (4 Ci/mol) [9].

L-Glucose distribution. The distribution space for L-[1-^{14}C]glucose (2.3 Ci/mol) was also measured following treatment of the adipocytes with diamide. The cells were incubated for 15 s at 37°C with a 0.1 mM concentration of L-glucose and then isolated from the incubation medium by the oil-centrifugation procedure. The distribution of L-glucose was calculated as described above for the methylglucose space.

Glutathione levels. The intracellular concentration of glutathione (reduced form; GSH) was estimated by a modification of the fluorimetric method described by Hissin and Hilf [10]. Cells (160 mg lipid/ml) were incubated in the usual manner with insulin or the chemical agents. After the incubation, 1 ml of silicone oil was added and the suspension was spun gently to separate the cells from the aqueous medium. The aqueous and oil phases were removed by aspiration, then 0.5 ml of a 25% HPO_3 solution and 1 ml of an ice-cold 0.1 M sodium phosphate/5 mM EDTA buffer, pH 8.0, were added to the cell pellet and maintained at 4°C. The preparation was subjected to sonication for 1 min at 100 W using a cuphorn sonifier (Heat Systems Ultrasonics). The suspension was then centrifuged at 31 000 × *g* in a Sorvall SS-34 rotor for 30 min at 4°C. The aqueous phase was carefully removed and the assay for GSH was performed according to the fluorometric method using *o*-phthalaldehyde exactly as described [10]. Standard curves were linear with GSH concentrations up to 150 ng/ml which allowed an accurate assessment of fat cell GSH levels. The quantity of GSH present in the incubation medium after treatment of cells with the various agents was found to be insignificant.

Results and Discussion

The effects of insulin and oxidants on the glutathione levels (reduced form; GSH) in adipocytes are shown in Table I. It is evident that concentrations of insulin, H_2O_2 or vitamin K-5 which maximally stimulate hexose transport [3,4] do not alter the GSH levels following either a 10 min (data not shown) or 90 min treatment. In contrast, treatment of the cells with 1.0 mM diamide markedly reduces GSH levels within 10 min and the levels remained low for at least 90 min. These results confirm previous reports that diamide causes a reduction in the GSH level of cells [11].

Further studies were conducted with diamide to determine if its effect on GSH could be correlated with its reported effects on glucose transport [4]. Fig. 1 shows that diamide in a dose-dependent fashion increases the apparent rate of glucose uptake by adipocytes. A time course for this effect on sugar

TABLE I

EFFECT OF INSULIN OR OXIDANT TREATMENT OF FAT CELLS ON THE INTRACELLULAR CONTENT OF GSH

Treatment	Incubation period (min)	GSH content * (μg/g lipid)
None (basal)	90	13.2 ± 0.9 (4)
Insulin (1000 μU/ml)	90	13.3 ± 1.5 (4)
Diamide (1 mM)	10	0.8 ± 0.2 (3)
Diamide (1 mM)	90	1.0 ± 0.3 (4)
H_2O_2 (3 mM)	90	12.2 ± 0.5 (3)
Vitamin K5 (48 μM)	90	12.1 ± 1.1 (3)

* Each value represents the mean ± S.E. for the number of experiments shown in parentheses.

uptake is shown in Fig. 2. Using a 1.0 mM diamide concentration, maximum enhancement during the incubation was obtained between 60 and 90 min. These findings indicate that the reduction in GSH levels, which occurs rapidly with diamide treatment (within 10 min), precedes the increase in glucose uptake. Based on the dose-response and time-course studies, subsequent experiments with diamide employed a 1.0 mM concentration and a 90—120 min incubation period.

In the experiments described above, we have reported the rate of glucose uptake as the amount of sugar present in the cell pellet following a 30 s assay period. No correction was made for the amount of sugar trapped between cells (extracellular sugar) which is usually calculated from a measurement of the inulin distribution space [8]. In Table II, the distribution spaces in the fat

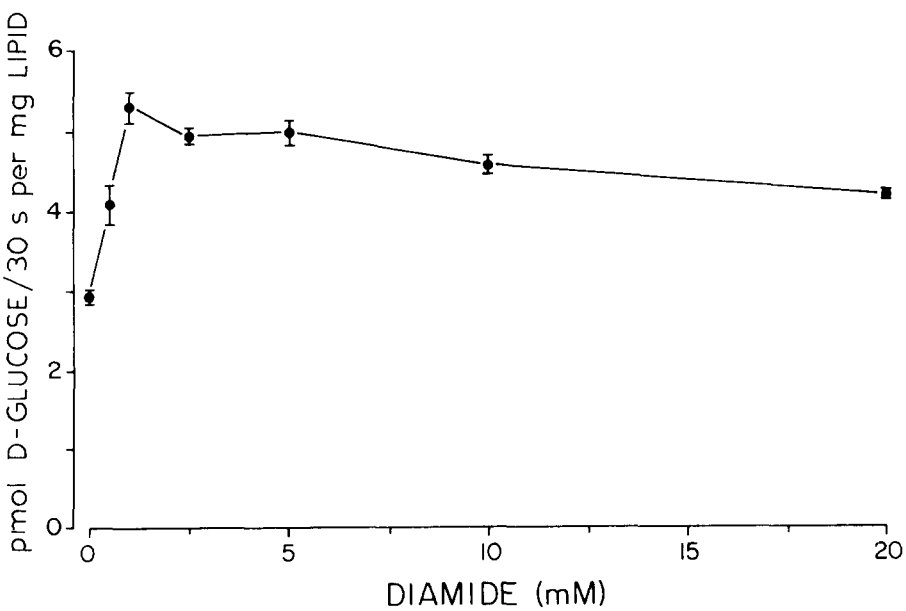

Fig. 1. Effect of various concentrations of diamide on glucose uptake. Fat cells were incubated with diamide for 90 min at 37°C before glucose uptake was measured as described in Materials and Methods. The results are the mean ± S.E. of four determinations.

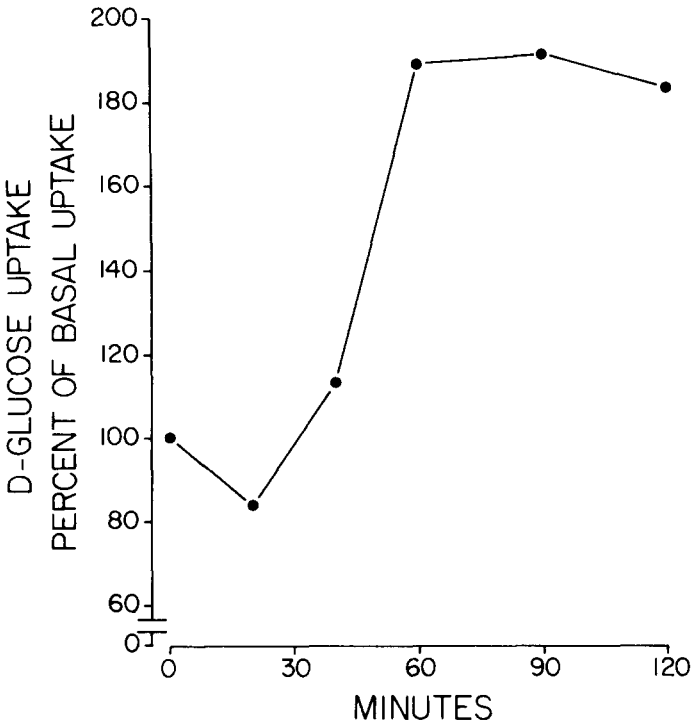

Fig. 2. Effect of time on diamide enhancement of glucose uptake. Fat cells were incubated for various periods of time at 37°C with 1.0 mM diamide. Glucose uptake was measured as described in Materials and Methods. The results are the mean of two separate experiments.

cell pellet are given for methylglucose, measured at steady-state (which gives total water space), and for inulin and sucrose (both of which measure extracellular water space) [9]. Treatment of fat cells with insulin, H_2O_2 or vitamin K-5 did not cause a change from control values for the methylglucose and inulin spaces. In contrast, diamide treatment caused an increase in the methylglucose space and a dramatic increase in the inulin space. The increase in the total water space suggests that diamide treatment causes the cells to swell. However, expansion of this space was not the only cause of the increase in the

TABLE II

DISTRIBUTION SPACES OF INULIN, METHYLGLUCOSE, SUCROSE AND L-GLUCOSE IN FAT CELL PELLETS AFTER VARIOUS TREATMENTS

Treatment	Distribution space (μl/g lipid) *			
	Inulin	Methylglucose	Sucrose	L-Glucose **
None	16 ± 0.4 (4)	55 ± 4 (4)	23 ± 1 (4)	23 ± 1 (3)
Diamide (1.0 mM)	37 ± 3 (4)	71 ± 7 (4)	53 ± 1 (4)	62 ± 4 (3)
Insulin (1000 μU/ml)	15 ± 1 (3)	53 ± 5 (3)	—	—
H_2O_2 (3.0 mM)	21 ± 2 (3)	53 ± 4 (3)	—	—
Vitamin K5 (48 μM)	15 ± 1 (3)	50 ± 3 (3)	—	—

* Values are the mean ± S.E. for the number of experiments shown in the parentheses. Dashes indicate that the experiments were not done.

** L-Glucose space was measured using a 15 s incubation period with the sugar.

TABLE III

EFFECT OF CYTOCHALASIN B ON 3-O-METHYLGLUCOSE UPTAKE

Cells were incubated with the indicated treatments for 90 min at 37°C before the uptake assay was performed as described in Materials and Methods. Values represent the mean ± S.E. of three experiments.

Treatment	3-O-Methylglucose uptake (pmol/20 s per mg lipid)
None (basal)	1.72 ± 0.04
Cytochalasin B (20 μM)	0.95 ± 0.05
Diamide (1 mM)	2.43 ± 0.11
Diamide (1 mM) + Cytochalasin B (20 μM)	2.12 ± 0.14
Insulin (1000 μU/ml)	2.93 ± 0.23
Insulin (1000 μU/ml) + Cytochalasin B (20 μM)	1.16 ± 0.13

inulin space. For example, the inulin space for the treated cells is approx. 53% of the total water space (intracellular plus extracellular water) compared to a calculated control value of 29%. This marked expansion of the 'extracellular' space is also evident when sucrose distribution is measured. In addition, the L-glucose spaces for diamide-treated and untreated cells are given in Table II. This sugar slowly enters adipocytes and has been used to assess the entry of hexoses by non-transport mechanisms [12]. As shown, treatment with diamide causes a marked increase in the L-glucose space. These findings indicate an alteration in the permeability of cells treated with diamide which allows entry of substances (inulin, sucrose, L-glucose) that are normally excluded.

Results from the above studies suggest the possibility that diamide increases the entry of glucose by changing the integrity of the cell membrane and not by a specific stimulatory action on the glucose transport system. To directly test this possibility, cytochalasin B, a potent inhibitor of the glucose transport system [4], was used in an effort to inhibit methylglucose uptake (Table III). Treatment of adipocytes with diamide or insulin increased the rate of methylglucose uptake above basal (unstimulated) levels. As expected, the addition of cytochalasin B markedly reduced both basal and insulin-stimulated uptake; the amount not inhibited reflects primarily sugar that is trapped extracellularly. In contrast to these findings, the inhibitor failed to significantly reduce the increased rate of methylglucose uptake in diamide-treated cells. Thus, unlike insulin, the stimulatory effect of diamide treatment on hexose uptake results from a nonspecific increase in the 'leakiness' of the cell.

An effort was made to protect the cells from the adverse effects of diamide by providing an energy source (5 mM D-glucose) during the treatment period. Following a 90 min incubation with 1 mM diamide and 5 mM D-glucose, methylglucose and sucrose distribution spaces in the cell pellet were measured. Under these conditions, diamide treatment resulted in a total water space of 61 ± 0.7 μl per g lipid compared to a control value of 47 ± 2 μl (mean ± S.E. of three experiments). The extracellular spaces for treated and control cells as measured by sucrose distribution were 56 ± 3 and 30 ± 2 μl per g lipid, respectively (mean ± S.E. of three experiments). These results show that the diamide-induced change in permeability found in the preceding experiments was not in part caused by the absence of a substrate in the incubation buffer.

The findings reported in this study conflict with the results reported by

Czech [4] who showed that adipocytes treated for 15 min with 20 mM diamide had an accelerated rate of methylglucose uptake which could be blocked by cytochalasin B. When these incubation conditions were used, we did not find a stimulation of methylglucose or glucose uptake; instead, an incubation period of 30 min or more with 20 mM diamide was required to obtain a significant increase in sugar uptake, which was not inhibited by cytochalasin B (data not shown). Furthermore, a comparison of the time courses required for the increase in glucose uptake and for the increase in sucrose space, all of which occurred following 20 mM diamide treatment, demonstrate a close correlation between the two phenomena (data not shown). This again supports the concept that the increase in sugar movement caused by diamide is mediated by a change in cell permeability.

We have shown in this report that agents (insulin, H_2O_2 and vitamin K-5) which activate the glucose transport system do not alter the intracellular GSH levels of fat cells. These results suggest that the action of these agents on the glucose transport system does not require the oxidation of GSH. Diamide, another agent which increases glucose entry into fat cells, rapidly lowers the GSH content of adipocytes. However, the effect of this agent on sugar movement was not mediated by a stimulation of the glucose transport system but instead was caused by a non-specific change in the permeability of the cell. It is evident from these studies that diamide should not be used as an insulin 'mimicker' nor should it be used in other studies of the glucose transport system. Moreover, an effect of diamide on other cellular processes should be examined in light of its effect on cell permeability.

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