Evidence for the Involvement of Sulfhydryl Oxidation in the Regulation of Fat Cell Hexose Transport by Insulin

(brown fat cells/3-O-methylglucose uptake/insulin effector system/membranes/insulin receptors)

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ABSTRACT Previous studies have shown that the oxidants Cu++, H2O2, and diamide mimic the stimulatory effect of insulin on 3-O-methylglucose transport in isolated fat cells. The present experiments were designed to determine whether sulfhydryl oxidation plays a key role in the activation of the glucose transport system. It was found that reductants such as dithiothreitol inhibited 3-O-methylglucose transport rates and that this effect was reversible when cells were washed free of reducing agent. Treatment of cells with 1 mM N-ethylmaleimide for 5 min completely blocked the actions of insulin and oxidants on hexose transport without affecting control transport system activity. Under these conditions, binding of 125I-labeled insulin to fat cell surface receptors was inhibited by only about 50%. Addition of insulin or oxidants to fat cells for 10 min before addition of N-ethylmaleimide completely prevented the inhibitory effect of N-ethylmaleimide on the activated transport system. This protective effect on transport rates appears to reside at a site that is altered by insulin subsequent to hormone–receptor interaction, since prior treatment of fat cells with insulin did not prevent the partial inhibitory effect of N-ethylmaleimide on insulin receptors. Furthermore, treatment of cells with N-ethylmaleimide after incubation with insulin prevented the elevated transport rates from returning to control levels when either the cells were washed free of hormone or insulin binding to its receptors was disrupted by trypsin digestion. However, transport rates in these cells treated with N-ethylmaleimide remained sensitive to cytochalasin B, phlorizin, and reductants. These data suggest that a component of the glucose transport system in isolated fat cells must be maintained in its disulfide state for expression of transport activity. Furthermore, the results are consistent with the concept that the binding of insulin to cell surface receptors triggers sulfhydryl oxidation in this component, which prevents its reaction with N-ethylmaleimide.

The well-known stimulatory effects of insulin on transport processes are thought to result from events triggered by the interaction of insulin with specific cell surface receptors. Although considerable information about the hormone–receptor interaction has become available in the last few years, nothing is known about the subsequent steps that lead to the physiological response. Studies on the actions of chemically specific agents, such as certain oxidants (1, 2) which mimic effects of insulin on fat cell glucose transport, may be useful in this regard since they appear to act at steps subsequent to the hormone–receptor interaction (3). A major obstacle in these studies has been that it is technically difficult to assay hexose transport directly in white fat cells due to their extremely small intracellular water space. We, therefore, use isolated brown fat cells as a model system since they respond to virtually all agents that modulate white fat metabolism and contain a relatively large cytoplasmic space. 3-O-Methylglucose transport into these cells is readily monitored, occurs by facilitated diffusion, and is inhibited by n-glucose, phlorizin, and cytochalasin B (4).

Using this rationale we found that agents such as H2O2, Cu++, and diamide, which readily oxidize sulfhydryls to disulfides, markedly enhanced 3-O-methylglucose uptake in brown fat cells (4). These agents did not alter uptake of L-glucose, which enters these cells by simple diffusion. The presence experiments demonstrate that activation of the hexose transport system by these oxidants or insulin is blocked by prior treatment of cells with N-ethylmaleimide (MalNEt), while control or previously stimulated transport rates are unaltered by this agent. It is further shown that MalNEt stabilizes insulin- or oxidant-activated transport rates at elevated levels even when the stimulatory agents are removed from the medium. These data represent substantial evidence for the concept that oxidation of key fat cell sulfhydryls in response to insulin–receptor interaction plays a role in mediating the activation of hexose transport.

MATERIALS AND METHODS

Dorsal interscapular brown adipose tissue was obtained from 180- to 230-g female Sprague-Dawley rats (Charles River CD strain) fed laboratory chow ad libitum (5). For each experiment the brown adipose tissue from 4 to 12 rats was dissected away from muscle and cut into small pieces with scissors. Brown fat cells were isolated by digestion of fat pads for 1 hr with crude bacterial collagenase (Clostridium histolyticum, Worthington) at a concentration of 1 mg/ml in Krebs-Ringer phosphate buffer (6 ml) containing 3% (w/v) albumin. The buffer was prepared fresh daily and adjusted to pH 7.4 with NaOH after addition of bovine serum albumin (Armour Fraction V). The phosphate buffer contained: 128 mM NaCl; 1.4 mM CaCl2; 1.4 mM MgSO4; 5.2 mM KCl; 10 mM Na2HPO4 (pH adjusted to pH 7.4 with 1M HCl). At the end of the digestion period, the brown fat cells were filtered through two layers of cheesecloth and washed twice with 6 ml of albumin buffer (37°C). The number of fat cells obtained was estimated by counting an aliquot of cells on a hemacytometer.

Transport of 3-O-[3H]methylglucose was assayed essentially as described (4). Briefly, 0.1-ml aliquots of brown fat cells in

Abbreviation: MalNEt, N-ethylmaleimide.
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Krebs-Ringer phosphate buffer containing 3% albumin were incubated in plastic culture tubes (17 × 100 mm) at 23° in the presence or absence of the indicated additions. Ten microliters of 3-O-[3H]methylglucose (1–2 μCi per tube) were added, and at the appropriate times 3 ml of ice-cold Krebs–Ringer buffer containing 0.1% albumin was added and the cells were decanted onto a Whatman GF/C filter. The filters were washed under pressure with 2 aliquots (5 ml each) of ice-cold buffer and dried; the radioactivity retained was determined (4). The total time taken to filter and wash the cells was less than 20 sec. The values presented are corrected for radioactivity found on filters containing cells to which 3-O-[3H]methylglucose and 3 ml of cold buffer were added together. All experiments were performed in triplicate on 2–5 separate days, and the values presented are either the results from representative experiments or the means of the several experiments performed.

125I-Labeled insulin was bound to brown fat cells essentially according to the method of Cuatrecasas (6). Cells (0.1 ml) in Krebs–Ringer phosphate buffer containing 3% albumin were incubated for 30 min with 125I-labeled insulin. The reaction was stopped by addition of 3 ml of ice-cold Krebs–Ringer buffer containing 0.1% albumin, and the mixture was decanted onto NRWP Millipore filters and washed with 2 aliquots (5 ml each) of ice-cold buffer. The filters were dried and the radioactivity retained was determined in a Triton–Omnifluor–toluene scintillation fluid (4). Specific binding was determined by subtracting the radioactivity found when 30 μg of native insulin was present during the incubation of cells with labeled hormone.

Crystalline porcine insulin was a gift of Eli Lilly and contained less than 0.005% glucagon by weight. H2O2 was obtained from Allied Chemical; dithiothreitol, N-ethylmaleimide trypsin, and trypsin inhibitor were from Sigma. 3-O-[3H]-methylglucose was purchased from New England Nuclear and 125I-labeled insulin from Cambridge Nuclear.

RESULTS

H2O2 markedly stimulated 3-O-methylglucose uptake by brown fat cells, and the maximal effect observed at 8 mM was not additive to that elicited by insulin (Fig. 1). That this effect is not due to a general increase in membrane permeability is supported by our previous demonstration that H2O2 does not influence uptake of L-glucose, which in fat cells occurs by simple diffusion (4). The magnitude of the response to insulin has varied in over 50 experiments, from about 50 to 200% over control transport rates. In contrast, dithiothreitol markedly inhibited 3-O-methylglucose transport in isolated brown fat cells (Fig. 1). Cells in this series of experiments were incubated in buffer containing albumin freed of heavy metal contaminants (2), which prevents production of H2O2 from the interaction of thiol and O3 (1). Mercaptoethanol (13–15 mM) was also an effective inhibitor of hexose transport. The inhibitory effect of these reducing agents was readily reversible when cells were washed free of reagent (not illustrated), indicating these reductants were not destroying a fraction of the cell population. Furthermore, cells that had been washed free of reductant responded to insulin or H2O2 to the same extent as control cells.‡

‡ M. P. Czech, J. C. Lawrence, Jr., and W. S. Lynn, unpublished observation.

![Fig. 1. The effect of various concentrations of H2O2 and dithiothreitol on insulin-stimulated 3-O-methylglucose transport.](image)

Brown fat cells were isolated as described in Materials and Methods, except that they were washed and resuspended in 1,10-phenanthroline-treated albumin buffer. Cells (0.6 × 10⁶ cells per tube) were incubated at 23° with or without the indicated concentrations of dithiothreitol, and after 15 min the cells in tubes containing dithiothreitol were incubated with or without insulin (2.4 nM/ml). The remaining cells were incubated with or without the indicated concentrations of H2O2 or H2O2 and insulin. After 10 min, 3-O-[3H]methylglucose was added at a concentration of 50 μM, and transport was monitored at 4 min.

Incubation of isolated fat cells with various concentrations of MalNEt for 5 min before addition of an equivalent concentration of mercaptoethanol (Fig. 2), which reacts with excess MalNEt, resulted in little or no effect on control 3-O-methylglucose transport, but markedly inhibited the stimulatory effect of insulin (Fig. 2). Complete inhibition of insulin action occurred at 1 mM MalNEt, which also blocked the effects of H2O2 on hexose transport (Table 1), while up to 5 mM MalNEt has been found to be without effect on control transport rates (Fig. 2). Surprisingly, when brown fat cells were incubated with 2.4 μM/ml of insulin for 10 min before a 5-min treatment with 1 mM MalNEt, the sulphydryl reagent was without effect on the elevated transport rates (Table 1). Prior treatment of cells with H2O2 (8 mM) also completely prevented the inhibitory action of MalNEt on the stimulated rates of fat cell 3-O-methylglucose transport. Under the conditions of these experiments, 1 mM MalNEt added before insulin or H2O2 abolished the effects of these agents on hexose transport (Table 1).

In order to determine whether the interactions between insulin and MalNEt on hexose transport were occurring at the level of cell-surface insulin receptors, 125I-labeled insulin binding to brown fat cells was studied. Incubation of 1 mM MalNEt with isolated fat cells for 5 min before addition of mercaptoethanol and washing reduced the amount of 125I-labeled insulin specifically bound by about 50% under the conditions of our experiments (Table 2). Moreover, treat-
Fig. 2. The effect of various concentrations of N-ethylmaleimide on the stimulation of 3-O-[3H]methylglucose transport by insulin. Brown fat cells (0.7 ml) were incubated at 23° with or without the indicated concentrations of N-ethylmaleimide. After 5 min, 2-mercaptoethanol was added at a concentration equal to that of the N-ethylmaleimide. Fat cells (0.1 ml) were then dispensed into separate tubes (0.5 × 10⁷ cells per tube) and incubated at 23° with or without 2.4 μU/ml of insulin. After 10 min, 3-O-[3H]methylglucose was added at a final concentration of 50 μM, and transport was measured at 5 min.

Table 1. Effect of N-ethylmaleimide on insulin and H₂O₂ stimulated 3-O-methylglucose transport

<table>
<thead>
<tr>
<th>First treatment (10 min)</th>
<th>Second treatment (5 min)</th>
<th>3-O-[3H]Methylglucose uptake (pmoles/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td>MalNEt, 1 mM</td>
<td>6.1 ± 0.0</td>
</tr>
<tr>
<td>Insulin, 2.4 μU/ml</td>
<td>MalNEt, 1 mM</td>
<td>6.5 ± 0.1</td>
</tr>
<tr>
<td>H₂O₂, 8 mM</td>
<td>MalNEt, 1 mM</td>
<td>10.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.7 ± 0.4</td>
</tr>
</tbody>
</table>

Brown fat cells were incubated at 23° in four tubes (1.0 ml/tube) without (2 tubes) or with either insulin (2.4 μU/ml) or H₂O₂ (8 mM). After 10 min, MalNEt (1 mM) was added to tubes containing insulin, H₂O₂, or no addition (1 tube) and after 5 min, 2-mercaptoethanol (1.2 mM) was added to these tubes. Cells were then immediately incubated in new culture tubes (0.5 × 10⁷ cells per tube) with or without either insulin (2.4 μU/ml) or H₂O₂ (8 mM). After 10 min, 3-O-[3H]methylglucose was added at a concentration of 50 μM and uptake was monitored at 4 min. The values presented are the means ± standard errors of these observations.

Table 2. Effect of N-ethylmaleimide on the specific binding of ¹²⁵I-labeled insulin to brown fat cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>¹²⁵I-Labeled insulin bound (pg/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.2</td>
</tr>
<tr>
<td>MalNEt, 1 mM, 5 min</td>
<td>9.9</td>
</tr>
<tr>
<td>Insulin, 400 μU/ml, 15 min</td>
<td>19.5</td>
</tr>
<tr>
<td>Insulin, 400 μU/ml, 15 min</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Fat cells were incubated in 0.1 ml (4 tubes) of Krebs-Ringer phosphate buffer containing 3% albumin in the presence (2 tubes) or absence (2 tubes) of 400 μU/ml of insulin for 15 min at 23°. 1 mM MalNEt was added to control and insulin-treated cells where indicated, and 5 min later 1 M mercaptoethanol was added. All cell groups were washed twice in 3% albumin buffer and dispensed into new culture tubes (0.4 × 10⁷ cells per tube) before addition of 0.63 nM ¹²⁵I-labeled insulin and assay of insulin binding (see Materials and Methods). The values are the means of two experiments performed on different days.

DISCUSSION

We previously reported that several oxidants with various degrees of specificity for sulfhydryls stimulated glucose oxidation in white fat cells (1) as well as 3-O-methylglucose treated with 1 mM MalNEt for 5 min was also only slightly inhibited. Insulin stimulated fat cell hexose transport almost threefold in these experiments, whereas trypsinization of insulin-treated cells essentially abolished this effect. Interestingly, fat cells treated with MalNEt subsequent to incubation with insulin exhibited hexose transport activity that was as resistant as that of control or MalNEt-treated cells to trypsinization (Fig. 3). This observation suggested that MalNEt converted the insulin-activated transport system to a state independent of the hormone–receptor interaction, which is normally necessary to maintain the elevated rates of uptake. This conclusion was further supported by the observation that stimulated hexose transport activity that was observed in cells treated with MalNEt subsequent to incubation with insulin or H₂O₂ persisted when the cells were washed free of the hormone or oxidant. Furthermore, MalNEt-stabilized hexose transport activity either in control or insulin-stimulated cells was still sensitive to reductants and the inhibitor cytochalasin B (not illustrated).
transport in brown fat cells (4). These agents presumably act on the transport system independent of insulin receptors, since their actions are not sensitive to trypsinization of fat cells (2, 3). These results, coupled with our present finding that reductants reversibly inhibit control transport rates, suggested the possibility that the transport system or a regulatory component thereof might exist in two states: an active, disulfide form and an inactive, sulphydryl form. According to this scheme, which is depicted in Fig. 4, the hexose transport activity observed under different conditions reflects the amount of these membrane components that are in the active, disulfide state. Further, our observation that diamide, which rather selectively oxidizes intracellular glutathione (11), also stimulated fat cell hexose transport (4) suggests that reducing equivalents from intracellular glutathione may be involved in the maintenance of the reduced (inactive) state of this regulatory component. Evidence for reduction of a membrane site involved in permeability properties by intracellular constituents has also been recently presented in work on erythrocytes (12). In addition, Morgan et al. have suggested the possible involvement of sulphydryls in the activation of glucose transport in muscle due to insulin (13).

The experiments reported here on the effects of MalNEt on brown fat cell hexose transport lend further support to our working model described above. MalNEt had no effect on control or insulin-stimulated 3-O-methylglucose transport, which is consistent with the notion that transport rates reflect the number of regulatory components in the oxidized, MalNEt-resistant, state. Accordingly, prior reaction of cells with MalNEt abolishes insulin stimulation, since oxidation of the reduced (sulphydryl form) components in response to the hormone is prevented by their covalent linkage with MalNEt. It is possible that MalNEt renders the insulin-stimulated rates of transport essentially irreversible (Fig. 3) due to (i) its ability to penetrate these cells and react with intracellular glutathione, which normally would be required to reduce (inactivate) the active disulfide form of the transport component, or (ii) direct inactivation of the enzyme(s) that may catalyze the reduction of this component. Clearly, the normal mechanism that reverses the action of insulin on transport subsequent to removal of the hormone or destruction of its receptors (Fig. 3) is destroyed by MalNEt treatment. Thus, MalNEt fixes the rate of glucose transport into fat cells at the level that exists just before its addition.

Interestingly, over a decade ago Cadenas et al. reported that MalNEt abolished insulin action on hexose transport in the perfused heart, but that prior treatment with insulin prevented the inhibition (14). These workers found that MalNEt inhibited insulin binding to the tissue and concluded that insulin protection against MalNEt inhibition of transport reflected competition at the level of insulin receptors (14). In our current studies, insulin binding to cell surface receptors was similarly inhibited by treatment of cells with MalNEt (Table 2). However, the data clearly demonstrate that the protective effect of insulin against MalNEt inhibition of transport involves processes that occur subsequent to initial insulin-receptor interaction (Fig. 3 and Table 2). It thus seems likely that the effects observed by Cadenas et al. may have also involved membrane structures that transmit the insulin-receptor interaction event to the site of activation of the hexose transport system.

Several previous studies have appeared on the effect of such sulphydryl reagents as Hg^{2+} (15), organomercurials...
(16), and MalNEt (17) on fat cell glucose metabolism. At low concentrations, all these agents were found to stimulate glucose utilization in white fat cells and the effects of MalNEt and Hg sup+ sub+ were not additive to that of maximal concentrations of insulin (15, 17). At higher concentrations these agents abolished insulin action with little effect on control rates of glucose oxidation, while still higher levels markedly inhibited basal metabolism. Carter and Martin suggested that the effect of intermediate concentrations of MalNEt on abolishing insulin action was due to effects of this reagent at the level of the fat cell surface membrane, since intracellular enzymes involved in glucose metabolism appeared relatively unimpaired (17). However, unequivocal interpretation of the above studies has been difficult since they have relied on an assay of intracellular metabolism which, in general, is susceptible to inhibition by the sulfhydryl reagents used, and further progress in definitively identifying the locus of MalNEt action has not been forthcoming. Thus, the utility of our method to directly monitor 3-O-methylglucose uptake in brown fat cells is that effects of agents on D-glucose transport activity can be determined independent of effects on the subsequent metabolic pathways for glucose. Furthermore, the method is simple and rapid and yields results that are highly reproducible.

It should also be noted that Cuatrecasas (18) and Pilakis et al. (19) have reported that MalNEt was without effect on the binding of 125I-labeled insulin to white fat cells and rat liver plasma membranes, respectively. It is not clear why, in the present studies, MalNEt substantially inhibited 125I-labeled insulin binding to brown fat cells under conditions similar to those used with white fat cells by Cuatrecasas (18).

Although the data obtained to date are consistent with the concept that a sulfhydryl-disulfide interchange plays a key role in the regulation of fat cell hexose transport, they certainly do not exclude other hypotheses. Rather, the utility of this working hypothesis is that it accommodates all the results we have obtained using oxidants, reductants, and MalNEt, and provides a rationale for further experiments in this area. Furthermore, the data presented clearly demonstrate that cellular component(s) that mediate hexose transport modulation by insulin and other agents are inactivated by MalNEt, while the operative transport system itself, even when stimulated by insulin, is completely resistant to the agent. This selective action of MalNEt may be useful in attempts to identify the fat cell components involved in these processes.

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