Trifluoperazine inhibits insulin action on glucose metabolism in fat cells without affecting inhibition of lipolysis

(calcium-binding regulatory protein/phenotriazine/sulfonamide/insulin action and dissociation/exocytosis and protein phosphorylation)

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ABSTRACT One of the specific inhibitors of calmodulin action, trifluoperazine, blocked the stimulating action of insulin on 2-deoxyglucose uptake and glucose metabolism. The inhibitory effect of insulin on lipolysis was not altered by the drug. The active (insulin-stimulated) state and the basal state of lipogenesis were inhibited half-maximally at 80 and 550 µM trifluoperazine, respectively. 2-Deoxyglucose uptake was inhibited half-maximally at a trifluoperazine concentration of 70 µM. Other less potent calmodulin inhibitors also inhibited glucose metabolism in fat cells but in a nonspecific manner. The inhibition was noncompetitive and was not inhibited in Ca²⁺-free medium. The stimulating activity of wheat germ agglutin and of sodium vanadate were also inhibited by trifluoperazine. The dose-dependent inhibitions were indistinguishable whether the active (stimulated) state was produced by insulin, wheat germ agglutinin, or vanadate. The data indicate that a late event in the sequence that ultimately leads to enhanced glucose transport activity in fat cells is specifically inhibited by trifluoperazine. The possible involvement of calmodulin or another related Ca²⁺-dependent regulatory protein in the exocytic (fusion) reaction that recruits glucose-transport activity from storage sites to the plasma membranes is discussed.

Recent reports from two different laboratories (1–4) suggest that glucose transport activity in fat cells recycles between the plasma membrane and unidentified intracellular storage sites. These studies also indicate that insulin facilitates translocation of glucose-transport activity (in an unknown manner) from the storage sites to the plasma membrane (1–4). It was further suggested that the glucose-transport activity is translocated by a mechanism that involves endocytic and exocytic (fusion) reactions (1–3). Most exocytic reactions in nature require Ca²⁺ (5). Fusion under in vitro conditions requires millimolar concentrations of calcium (6), whereas the free Ca²⁺ concentration in cells is 0.1–1 µM (7). It is therefore conceivable that additional factors play a role in the regulation of fusion processes. Although an insulin-dependent increase in intracellular Ca²⁺ level has been suggested (reviewed in refs. 8 and 9), this in itself does not seem to be sufficient to produce insulin-like effects, because an artificial increase in intracellular calcium level does not mimic insulin in fat cells (10, 11). In recent years it has become apparent that many of the biological effects of Ca²⁺ are directed to specific regions or to specific activities within the cell by the Ca²⁺-dependent regulatory protein calmodulin (for general reviews see refs. 12 and 13). Calmodulin is therefore seen as directing the effect of a general increase in, for example, cytoplasmic Ca²⁺ to certain molecules or activities within the cell. Other calcium-binding proteins, which are less characterized than calmodulin, are known to exist in various mammalian tissues (12, 13). Some may have conformational homologies to calmodulin because they also bind to fluphena-

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MATERIALS AND METHODS

Materials. Porcine insulin was purchased from Eli Lilly. D-[U-¹⁴C]Glucose (4–7 mCi/mmol; 1 Ci = 37 GBq) and 2-deoxy-D-[G-¹³C]Glucose (9 Ci/mmol) were obtained from New England Nuclear. Collagenase type I (134 units/mg) was obtained from Worthington. Adrenocorticotropic hormone (ACTH) was purchased from Sigma. W12, which is N-(4-aminobuty)-2-naphthalene sulfonamide, and W13, which is N-(4-aminobuty)-5-chloro-2-naphthalene sulfonamide, were kindly donated by P. Michael Conn (Duke University, Durham, NC). All other materials used in this study were of analytical grade.

Methods and Procedures. The following methods and procedures were used with no modification: preparation of isolated fat cells from epididymal fat pads (16), lipogenesis (17, 18), 2-deoxy-D-glucose (deoxyglucose) uptake (19), lipolysis, and its inhibition by insulin (20).

RESULTS

Differential Effect of Trifluoperazine (TFP) on the Active and Basal States of Lipogenesis. Addition of increasing concentrations of TFP to a fat cell suspension inhibited activation of lipogenesis by insulin (Fig. 1, Inset). Inhibition was detectable at 20 ± 5 µM. The basal rate of lipogenesis (Vb) was unaffected up to a concentration of 150 µM and only partially affected (15 ± 5%) at a concentration where the effect of insulin was nearly completely abolished. When the rate of lipogenesis was plotted as Vt = Vb versus TFP concentration for the active state (Vt represents rate of lipogenesis at a given concentration of TFP and insulin) or Vb as a function of TFP concentration for the basal state, values of ID₅₀ = 87 and 550 µM were derived for the active and the basal state, respectively (Fig. 1). It is apparent that the active state is inhibited at a 1/6.3 concentration of TFP.

TFP Inhibition of 2-Deoxyglucose Uptake. Increasing concentrations of TFP blocked the stimulatory effect of insulin on 2-deoxyglucose transport by the fat cells (Table 1). Fifty percent inhibition was obtained at 70 µM and complete inhibition was observed at 150 µM TFP. The basal rate of trans-

Abbreviations: TFP, trifluoperazine; CDR, Ca²⁺-dependent regulatory proteins; Vt, rate of lipogenesis at a given concentration of TFP and insulin; Vb, basal rate of lipogenesis; ACTH, adrenocorticotropic hormone; WGA, wheat germ agglutinin.

The figure and table are not provided in the text. The text describes the methodology and results of experiments involving the effects of trifluoperazine on glucose metabolism and lipolysis in fat cells, with a focus on the role of calmodulin and calcium in these processes. The results show that trifluoperazine inhibits insulin action on glucose metabolism and lipolysis in a Ca²⁺-dependent manner, with differential effects on active and basal states of lipogenesis.
port was only slightly affected by the latter TFP concentration. Thus, the drug inhibits the stimulatory action of insulin on hexose transport.

Type of TFP Inhibition. The dose-dependent inhibition of the active state of lipogenesis (v - v_0) as a function of TFP concentration was compared after stimulation with low (0.017 nM) and high (17 nM) concentrations of insulin with that produced by wheat germ agglutinin (WGA) (21, 22) and vanadate ions (refs. 23-25; Fig. 2A). TFP inhibited the active state produced by either low or high insulin concentrations, WGA, or vanadate with about the same efficiency. ID_50 values derived in this particular experiment were ≈90 ± 4 μM in all four cases (Fig. 2A). It therefore seems that the TFP inhibition is noncompetitive and occurs at a post-receptor site. The site of inhibition is the same or subsequent to that modulated by vanadate ions (24, 25).

Inhibition of Lipogenesis by TFP in Calcium-Free Medium. Comparison of the dose-dependent inhibition of the active state of lipogenesis by TFP in the routinely used Krebs-Ringer bicarbonate buffer (which contained 1.3 mM Ca^{2+}) and in Ca^{2+}-free Krebs-Ringer bicarbonate buffer that was supplemented with 4 mM EGTA is shown in Fig. 2B. The inhibition curves of the buffers overlap, indicating that the effect of the drug is not related to exchange of calcium between the fat cells and the external medium.

Specificity of TFP Inhibition in the Fat Cell System. The effects on lipogenesis of more than 30 low molecular weight substances that are known to interfere with various cell functions were tested. These agents fall into two clear categories: (i) those that do not affect lipogenesis, such as methylamine (lysozomotropic amine), vinblastin (microtubule poison), cytochalasin E (microfilament poison), bacitracin (proteolysis inhibitor), and many other compounds; (ii) inhibitors that affect basal and active states of lipogenesis equally well. The effects of several such agents—namely, cyanide ions (uncoupler of oxidative phosphorylation), nicotinamide (inhibits mono- and poly(ADP) ribosylation), propranolol (hypophagic agent), and cytochalasin B (reversible inhibitor of the glucose transport system)—are shown in Fig. 3. Of the calmodulin inhibitors, TFP has the highest affinity for calmodulin or other related Ca^{2+}-dependent regulatory proteins (CDR) (summarized in ref. 26). This may account for the selective inhibition of the active state by TFP (Fig. 1; Table 1). Other calmodulin inhibitors, however, are also expected to inhibit lipogenesis (at least in a nonspecific manner), and in a reasonable order to their relative affinities for calmodulin, in in vitro systems. Fig. 4 indicates that except for fluoperazine, which is less potent in the fat cell than expected, other drugs inhibit this process with approximately the same efficacy that they inhibit calmodulin-activated phosphodiesterase in vitro (26, 27). The activity sequence is TFP > W13 > propranolol > W12 > promethazine (values derived from Figs. 3 and 4). In the case of W13 and its dechlorinated derivative W12, these compounds are nearly identical in their hydrophobicity (and accordingly in their nonspecific action; ref. 28). W13 and W12, however, inhibit calmodulin-activated phosphodiesterase with IC_50 values of 68 and 260 μM, respectively (27). In agreement with those values, W13 is about 3 times more potent than W12 in inhibiting lipogenesis (Fig. 4).

Table 1. Effect of TFP on the uptake of 2-deoxyglucose by fat cells

<table>
<thead>
<tr>
<th>Addition(s)</th>
<th>2-Deoxyglucose uptake, nmol/10 min per 10^2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.74</td>
</tr>
<tr>
<td>Insulin (17 nM)</td>
<td>1.65</td>
</tr>
<tr>
<td>TFP (20 μM)</td>
<td>0.74</td>
</tr>
<tr>
<td>TFP (40 μM)</td>
<td>0.72</td>
</tr>
<tr>
<td>TFP (70 μM)</td>
<td>0.70</td>
</tr>
<tr>
<td>TFP (150 μM)</td>
<td>0.71</td>
</tr>
<tr>
<td>Insulin (17 nM)/TFP (20 μM)</td>
<td>1.53</td>
</tr>
<tr>
<td>Insulin (17 nM)/TFP (40 μM)</td>
<td>1.42</td>
</tr>
<tr>
<td>Insulin (17 nM)/TFP (70 μM)</td>
<td>1.2</td>
</tr>
<tr>
<td>Insulin (17 nM)/TFP (150 μM)</td>
<td>0.73</td>
</tr>
</tbody>
</table>

A suspension of adipocytes was incubated with insulin and the indicated concentration of TFP for 40 min at 37°C. 2-Deoxy-D-glucose uptake was then measured for 10 min at the same temperature.
Lack of Inhibitory Effect of TFP on Antilipolysis. TFP at 300 μM, a dose that inhibited the action of insulin in stimulating lipogenesis (Fig. 1), partially reduced (15–20%) the basal rate of lipolysis and that obtained by various lipolytic hormones (Table 2). In contrast to the inhibitory effect of the drug in blocking insulin-mediated lipogenesis, TFP did not affect at all the ability of insulin to antagonize lipolysis (Fig. 5). Neither the extent of the inhibition nor the range of insulin concentration for inhibiting lipolysis was altered in the presence of 300 μM TFP. Fifty percent inhibition of lipolysis is obtained at insulin concentrations of 0.2 ng/ml in both control and TFP-treated cells (values derived from Fig. 5). Thus the TFP-dependent event is restricted to the effect of insulin on hexose transport and (possibly) glucose metabolism, while the system that mediates the inhibition of lipolysis remains unaltered.

DISCUSSION

The present study shows that TFP, an inhibitor that binds to calmodulin and inhibits calmodulin-dependent activation of enzymes (12, 13, 29), blocks the stimulating effect of insulin on hexose transport and glucose metabolism. Kinetic analysis of the inhibition revealed that this drug inhibits the active state of this process with ID₅₀ values of 70–90 μM. In contrast, the basal (unstimulated) state is half maximally inhibited at 550 μM TFP.

The preferential inhibitory effect of TFP toward the active state seems to be unique to this drug. Among a large number of other low molecular weight substances, some were totally ineffective, whereas others were equally effective in inhibiting both the basal and the stimulated state. Further studies have revealed that the antilipolytic activity of insulin (both the extent and dose dependency) has not been altered by the drug, thus indicating that the binding of the hormone, the putative cytosolic second messenger(s), as well as all biochemical pathways that lead to lipolysis and antilipolysis in adipocytes are not affected by TFP. Inhibition of lipogenesis is noncompetitive and is at the same site or posterior to that modulated by vanadate ion (24, 25).

The results are most compatible with a role for calmodulin or another related CDR in one of the events that finally leads to insulin-mediated increase in glucose-transport activity.

Because the affinity of the drug to those proteins is calcium dependent (14, 29), the active state is associated with higher proportions of Ca²⁺/CDR. This is in agreement with many studies that postulate an insulin-dependent increase in cytoplasmic Ca²⁺ (reviewed in refs. 8, 30, and 31). TFP may possibly inhibit the endocytic and exocytic (fusion) reactions that lead to the recruitment of glucose-transport activity from the storage sites to the plasma membrane (1–4). By analogy to the chromaffin cells (reviewed in ref.
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Fig. 5. Inhibition of lipolysis by various concentrations of insulin in the presence (●, □) and absence (○, ◊) of 0.3 mM TFP for 3 hr at 37°C. Lipolysis was carried out at a fixed concentration (1 μg/ml) of ACTH (---) and at various concentrations of insulin (—).

15), the fat cell system is postulated also to contain an essential CDR that would enable aggregation and fusion at low concentrations of calcium.

Creutz has shown that the addition of cis-un saturated fatty acids to chromaffin granules, previously aggregated by syn exin, led to fusion in the region of contact formed by synexin with the subsequent formation of larger vesicles (32). The addition of cis-un saturated fatty acids to intact adipocytes at 37°C has been found to partially stimulate lipogenesis. The same treatment greatly increased (12- to 20-fold) the ability of the cells to bind 45Ca2+ (unpublished observations). It is concluded that cis-un saturated fatty acids induce a gross change in lipid organization, leading to a new calcium-binding phase, which favors translocation of glucose-transport activity. Taken together, the experimental evidence from this laboratory and the analogy to the in vitro fusion of the chromaffin granules (15, 32) suggest that CDR, Ca2+, and free cis-un saturated fatty acids present in the cytoplasm are the prerequisite components for the insulin-dependent endo cytic and exocytic reactions that lead to translocation of glucose-transport activity.

Alternatively, CDR may participate in phosphorylation and dephosphorylation of enzymes. Calmodulin was shown to alter the activity of several protein kinases (33–36) and, as proposed by Denton et al. (37), activation of membrane-associated insulin-dependent protein kinase may be an essential event in metabolic regulation by insulin. Recent data from different laboratories have indicated that the B-subunit of the insulin receptor can itself undergo an insulin-dependent phosphorylation in several target cells (38, 39) as well as in a cell-free system (40, 41). Although it would appear that the insulin-dependent receptor-mediated protein kinase activity is insufficient to directly regulate metabolism, a cascade mechanism that would amplify this signal is possible. Calmodulin may participate in such a cascade.

It is somewhat surprising that among the phenothiazines and the naphthalene sulfonamides only TFP inhibits the active state of hexose transport and lipogenesis in a specific manner and at a relatively high concentration. TFP, however, is the most potent inhibitor of calmodulin (and probably of other related CDR) as judged by its anticalmodulin activity in enzymic (cell-free) systems (summarized in ref. 26). The relatively high concentration of TFP needed to inhibit this process probably reflects a low ratio of Ca2+/CDR associated with the active state. The insulin-dependent increase in calcium is believed to originate from internal stores (Fig. 2B; see ref. 8 for review). It is therefore logical to assume that at steady state, the increased intracellular Ca2+ concentration in the activated cells is lower than that in most exocytic systems, in which the increase in cytoplasmic Ca2+ originates from the medium. Higher concentrations of TFP are therefore required to block glucose entry and metabolism in adipocytes.

The skilful technical assistance of Ms. B. Zarmi and the fruitful discussions with Drs. P. Cuatrecasas, S. Jacobs, and N. Sahyoun from Burroughs Wellcome (Research Triangle Park, NC) and Dr. V. Bennett from Johns Hopkins University (Baltimore) are gratefully acknowledged. This study was supported by grants from the Israel Academy of Science and Humanities and from the Israeli Ministry of Health. Y.S. is the incumbent of the Robert Edward and Roselyn Rich Manson Career Development Chair in Perpetuity.


Table 2. Effect of 0.3 mM TFP on basal and hormone-stimulated lipolysis in fat cells

<table>
<thead>
<tr>
<th>Addition(s)</th>
<th>Amount of glycerol released, nmol/3 hr per 3 × 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>TFP (0.3 mM)</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Isoproterenol (0.3 μM)</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>Isoproterenol (0.3 μM)/TFP (0.3 mM)</td>
<td>83 ± 4</td>
</tr>
<tr>
<td>Isoproterenol (4 μM)</td>
<td>150 ± 12</td>
</tr>
<tr>
<td>Isoproterenol (4 μM)/TFP (0.3 mM)</td>
<td>120 ± 6</td>
</tr>
<tr>
<td>ACTH (1 μg/ml)</td>
<td>153 ± 8</td>
</tr>
<tr>
<td>ACTH (1 μg/ml)/TFP (0.3 mM)</td>
<td>121 ± 8</td>
</tr>
<tr>
<td>ACTH (0.2 μg/ml)</td>
<td>140 ± 5</td>
</tr>
<tr>
<td>ACTH (0.2 μg/ml)/TFP (0.3 mM)</td>
<td>110 ± 4</td>
</tr>
<tr>
<td>Norepinephrine (10 μM)</td>
<td>140 ± 6</td>
</tr>
<tr>
<td>Norepinephrine (10 μM)/TFP (0.3 mM)</td>
<td>114 ± 3</td>
</tr>
</tbody>
</table>

Lipolysis was carried out for 3 hr. TFP was added to the cell suspension 10 min before the addition of the hormones.