Interrelationship of cyclic AMP, lipolysis, and respiration in brown fat cells

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The calorigenic action of catecholamines on brown fat cells is thought to involve activation of adenylate cyclase, resulting in an increased accumulation of cyclic adenosine 3',5'-monophosphate (cyclic AMP), which accelerates lipolysis (2, 8, 20). The marked increase in oxygen consumption seen after the addition of lipolytic agents to brown fat cells has been attributed to a K+-dependent uncoupling action of free fatty acids released as a result of activation of lipolysis by cyclic AMP. The present studies indicate that there was a good correlation between the ability of catecholamines and serotonin (5-hydroxytryptamine) to affect cyclic AMP accumulation, lipolysis, and respiration. Propranolol, a beta-adrenergic antagonist, blocked the effects of both scotinin and norepinephrine. High concentrations of ouabain reduced the stimulation of cyclic AMP accumulation, lipolysis, and oxygen consumption by catecholamines but did not inhibit the effects of theophylline on lipolysis or cyclic AMP accumulation. The stimulation of respiration by added fatty acids was also unaffected by ouabain. Ouabain increased glucose conversion to carbon dioxide and decreased lactate accumulation.

serotonin; ouabain; dibutyryl cyclic AMP; adipose tissue metabolism; glucose metabolism; theophylline; hormone action; propranolol

METHODS

Brown fat cells were isolated from the dorsal interscapular brown adipose tissue of 130- to 160-g Sprague-Dawley female rats (Charles River CD strain) fed laboratory chow ad libitum (7). Cells were isolated by digestion of the minced brown fat-pads with crude bacterial collagenase (Worthington) at a concentration of 1 mg/ml in phosphate buffer containing 4% albumin and 2.8 mM glucose. The buffer was prepared fresh daily, and the pH was adjusted to 7.4 with NaOH after addition of defatted bovine fraction V albumin powder (lot G35607 was used for all studies except those on cyclic AMP accumulation in which lot 36,912 was used) from Armour Pharmaceutical Company. Defatted albumin was prepared by the procedure of Guillory and Racker (11). The phosphate buffer contained NaCl, 128 mM; CaCl2, 1.4 mM; MgSO4, 1.4 mM; KCl, 3.2 mM; and Na2HPO4, 10 mM.

For studies involving uniformly labeled glucose, the free cells were washed 3 times with 4% defatted albumin buffer containing glucose, and then 5 ml of cells was added to each siliconized Warburg single sidearm flask containing 2.5 ml of 4% defatted albumin buffer with 2.8 mM glucose and approximately 0.078 μc of glucose-U-14C/ml. Carbon dioxide was absorbed on rolled filter papers, in the center wells of the flasks, containing 0.2 ml of 10% KOH. The filter papers were removed at the end of the incubation and counted in 10 ml of Bray’s solution (3).

Oxygen consumption was measured in a Gilson respirometer at 37 C. The respirometer flasks were gassed with 100% oxygen for 3 min and equilibrated for 30 min prior to the start of the period over which respiration was measured. The basal rate of oxygen consumption was linear over the entire period. In the respirometer studies involving propranolol, theophylline, and ouabain, the drugs were present in the incubation medium prior to the measurement of oxygen consumption. Lipolytic agents were added from the sidearms at the end of the equilibration period.

In the studies in which lipolysis and cyclic AMP accumulation were measured, the cells were incubated for 30
min in 0.5 ml of 4% albumin buffer with 2.8 mm glucose prior to the addition of lipolytic agents. At the end of this time period, lipolytic agents and all drugs were added, except ouabain, which was present from the start of the experiment. In those tubes used for cyclic AMP analysis, after 5 min of further incubation, 50 μl of 50% trichloroacetic acid were added. Lipolysis was measured in tubes incubated for 60 min after the addition of the lipolytic agents. Total cyclic AMP was determined on duplicate aliquots after extraction of the trichloroacetic acid with ether. The protein-binding assay of Gilman (9) was used for analysis of cyclic AMP. The assay was conducted at pH 6 in a total volume of 35 μl with the use of a binding protein purified up to the diethylaminoethyl cellulose step. Instead of collecting the bound cyclic AMP on membrane filters, it was separated from the unbound cyclic AMP by addition of 0.6 ml of Norit A charcoal suspended (60 mg/100 ml) in 20 mM phosphate buffer, pH 6.0, containing 0.5% bovine serum albumin. After a minimum of 5 and a maximum of 10 min, the tubes were centrifuged to pellet the charcoal. An aliquot (0.5 ml) of the supernatant was taken to determine the bound radioactive cyclic AMP. The use of charcoal is similar to the method of Brown et al. (4).

At the end of the incubation period, aliquots of the medium were taken for determination of glycerol (24), lactate (15), and free fatty acid (7). Triglyceride content was determined from the total fatty acid content and is used for convenience to express the amount of cells present in each experiment (7).

The sources of the chemicals were as follows: Salbutamol (AH-3365), α₁-[(l-butylamino)methyl]-4-hydroxy-m-xylene-α₁,α₂-diol, was a gift of Allen and Hanburys, Ltd.; dipropranolol (Inderal) was a gift of Ayerst Laboratories. The other chemicals were obtained from the following: ouabain octahydrate (g-strophanthin), Sigma Chemical Company; N6, 2'-0-dibutyryl cyclic adenosine 3',5'-monophosphate (DB-cAMP), Schwarz/Mann; 5-hydroxytryptamine creatinine sulfate (serotonin), Aldrich Chemical Company; and l-norepinephrine (noradrenaline or arterenol), Calbiochem.

### RESULTS

**Relationship between lipolysis and respiration.** Salbutamol is a catecholamine derivative which is more potent than isoproterenol as a bronchodilator (12) but less active as an activator of lipolysis (6). In brown fat cells Salbutamol increased lipolysis, and its lipolytic action was potentiated by theophylline (Table 1). It has been suggested that Salbutamol might be more potent than epinephrine as a stimulator of respiration in brown adipose tissue (1). However, the data in Table 1 indicate that there was a good correlation between lipolysis and oxygen consumption by brown fat cells in the presence of Salbutamol.

The potentiation of the lipolytic action of catecholamines by theophylline is presumably due to its ability to inhibit the breakdown of cyclic AMP by phosphodiesterase. We found that dibutyryl 3',5'-cyclic AMP had the same effect as theophylline on the sensitivity to catecholamines. A concentration of 0.01 μM norepinephrine had no effect on oxygen consumption or lipolysis in the absence of DB-cAMP, whereas 0.01 μM norepinephrine in presence of dibutyryl 3',5'-AMP, Brown fat cells (13 μmoles triglyceride/flask) were incubated for 2.5 hr in 3 ml of medium, and oxygen consumption was measured during the last 2 hr. Theophylline (0.5 mM) was added at the start of the incubation period, whereas Salbutamol was added 30 min later at the beginning of the period over which respiration was measured.

### TABLE 1. Stimulation of respiration and lipolysis by Salbutamol

<table>
<thead>
<tr>
<th>Additions, μM</th>
<th>Oxygen Consumption, pmol/mg</th>
<th>Glycerol Release, pmol/mg</th>
<th>Fatty Acid Release, pmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.7 ± 0.3</td>
<td>7 ± 2</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>Salbutamol, 0.66</td>
<td>3.9 ± 0.4</td>
<td>44 ± 8</td>
<td>120 ± 35</td>
</tr>
<tr>
<td>Salbutamol, 6.6</td>
<td>6.6 ± 0.7</td>
<td>78 ± 15</td>
<td>175 ± 9</td>
</tr>
<tr>
<td>Theophylline, 500</td>
<td>2.1 ± 0.3</td>
<td>12 ± 7</td>
<td>35 ± 7</td>
</tr>
<tr>
<td>Theophylline + Salbutamol, 0.044</td>
<td>4.2 ± 0.5</td>
<td>44 ± 9</td>
<td>127 ± 30</td>
</tr>
<tr>
<td>Theophylline + Salbutamol, 0.13</td>
<td>5.6 ± 0.6</td>
<td>60 ± 8</td>
<td>183 ± 35</td>
</tr>
<tr>
<td>Theophylline + Salbutamol, 0.66</td>
<td>7.5 ± 0.8</td>
<td>93 ± 14</td>
<td>235 ± 40</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5 paired replications done on separate days. Brown fat cells (30 μmoles triglyceride/flask) were incubated for 2.5 hr in 3 ml of medium, and oxygen consumption was measured during the last 2 hr. Theophylline (0.5 mM) was added at the start of the incubation period, whereas Salbutamol was added 30 min later at the beginning of the period over which respiration was measured.
of the hormones. In the same experiments, we reinvestigated the genic action of catecholamines (13, 16), we reinvestigated the ouabain-sensitive Na^+ K^+-ATPase is involved in the calori-

The results shown in Fig. 4 indicate that there was a stimu-

However, in the presence of DB-cAMP, serotonin (10–100 μM) markedly increased both respiration and lipolysis. The effects of serotonin were inhibited by dl-propranolol. In other studies (not shown) 4 μM dl-propranolol completely blocked the lipolytic action of 0.1 μM norepinephrine or 50 μM serotonin when propranolol was added at the start of the 1-hr incubation period along with the hormones. In the same experiments, 4 μM dl-propranolol inhibited the lipolytic action of serotonin by 62% and of norepinephrine by 49% (average of three paired experiments).

Effects of ouabain on action of lipolytic agents. Ouabain inhibited the lipolytic action of catecholamines on white fat cells (5, 14), but potentiated that of theophylline (5). Previously we found no effect of 0.04 mM ouabain on the stimulation of respiration or lipolysis by theophylline in brown fat cells (19). In view of the hypothesis that stimulation of a ouabain-sensitive Na^+ K^+-ATPase is involved in the calorigenic action of catecholamines (13, 16), we reinvestigated the question using higher concentrations of ouabain. In the studies shown in Fig. 2, cells had been incubated with ouabain (0.1 or 1.0 mM) for 30 min prior to the addition of

TABLE 2. Stimulation of respiration by serotonin in brown fat cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>Increment Due to Serotonin</th>
<th>Basal</th>
<th>10 μM</th>
<th>50 μM</th>
<th>100 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>3.7 ≥ 0.3 ± 0.14</td>
<td>+1.5 ± 0.20</td>
<td>+1.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td></td>
<td>4.5 +0.3 ± 0.11</td>
<td>+0.6 ± 1.2</td>
<td>+0.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>DB-cAMP</td>
<td></td>
<td>16.7 +10.0 ± 3.2</td>
<td>+17.5 ± 6.3</td>
<td>+22.2 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>DB-cAMP +</td>
<td></td>
<td>17.8 +1.9 ± 1.7</td>
<td>+3.6 ± 2.9</td>
<td>+5.4 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td></td>
<td>29.2 12.7 ± 8.5</td>
<td>+52.3 ± 12.6</td>
<td>+64.2 ± 11.0</td>
<td></td>
</tr>
<tr>
<td>DB-cAMP +</td>
<td></td>
<td>44.0 +8.9 ± 6.6</td>
<td>+12.6 ± 9.3</td>
<td>+20.9 ± 11.6</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE for 3 replications. Brown fat cells (15.7 μmoles triglyceride/flask) were incubated for 3 hr in 3 ml of medium. Propranolol (33 μM) was added at the start of the incubation period. After 1 hr serotonin either without or with dibutyryl cyclic 3’,5’-AMP (0.8 mM) was added, and cells were incubated for 2 hr more, during which time oxygen consumption was measured.

The ability of sodium octanoate to stimulate respiration in brown fat cells was unaffected by incubation with 1 mM ouabain for 30 min prior to addition of the free fatty acids (Fig. 3). Ouabain stimulated glucose oxidation and inhibited lactate accumulation in the presence of added octanoate, which indicated that ouabain was active in the experiments shown in Fig. 3.

Lipolytic agents and cyclic AMP accumulation in brown fat cells. The results shown in Fig. 4 indicate that there was a stimulation of glycerol release from and accumulation of cyclic AMP in brown fat cells due to norepinephrine or serotonin. The basal value for cyclic AMP was 2.6 nmoles/mg triglyceride, and increasing this by only 1.3 nmoles resulted in maximal stimulation of lipolysis (Figs. 4

FIG. 2. Effects of ouabain on brown fat metabolism. Brown fat cells (15.0 μmoles triglyceride/flask) were incubated for 2.5 hr. Ouabain (0.1 or 1.0 mM) and theophylline (0.5 mM) were added at start of incubation period. Thirty minutes later, norepinephrine (0.1 μM) was added and oxygen consumption was measured over next 2 hr. Values are means of 3 paired replications. Basal values are represented by circles, those with theophylline (THEO) alone by triangles, norepinephrine (NE) by stars, and norepinephrine plus theophylline (NE + THEO) by open stars.
and 5). The small increase in cyclic AMP accumulation due to 5 \( \mu \)M serotonin was consistently seen, and the range of values for the three experiments shown in Fig. 4 was from 0.25 to 0.37 nmoles cyclic AMP. Norepinephrine at 0.1 \( \mu \)M gave a maximal stimulation of glycerol release, but a further increase in cyclic AMP accumulation resulted from increasing the norepinephrine concentration to 1 \( \mu \)M. In the studies on cyclic AMP accumulation, serotonin alone was able to increase glycerol release and cyclic AMP content (Fig. 4).

The correlation between the cyclic AMP accumulation at 5 minutes and the glycerol release over 60 minutes of incubation was fairly good in the range between 2.5 and 4 nmoles/m mole triglyceride (Fig. 5). Increasing the cyclic AMP content above this point by high concentrations of methylxanthines was not associated with any increase in lipolysis (Fig. 5). It is of interest that less than a 20% increase in total cyclic AMP is associated with a marked increase in lipolysis. Whether the increases in cyclic AMP accumulation seen 5 min after the addition of lipolytic agents accurately reflect the effects of these agents on cyclic AMP values over the entire period of the lipolysis measurements (60 min) remains to be established.

The stimulation of cyclic AMP accumulation by serotonin was also completely blocked by 33 \( \mu \)M propranolol. Basal cyclic AMP content in three experiments was 2.9 in the absence and 4.0 nmoles/m mole triglyceride in the presence of serotonin. Cyclic AMP in the presence of propranolol alone was 2.9, whereas in the presence of propranolol and 50 \( \mu \)M serotonin, it was 2.8 nmoles/m mole triglyceride.

Ouabain inhibited the increase in both cyclic AMP accumulation and lipolysis due to varying concentrations of norepinephrine (Fig. 6). In these studies the cells were incubated with 1 mM ouabain for 30 min prior to addition of catecholamines.

**DISCUSSION**

In the present studies there was a good correlation between the lipolytic activity of the compounds tested and their ability to stimulate respiration. Reed and Fain (18) previously found that there was a close temporal relationship between activation of lipolysis and respiration in brown fat cells. Approximately 25 sec after the addition of epinephrine to brown fat cells, there was a simultaneous increase in both oxygen consumption and lipolysis (18). The addition of exogenous fatty acids or dibutyryl cyclic AMP mimicked the stimulatory action of catecholamines on respiration in brown fat cells (18-20), which was confirmed in the present experiments.
Correlation between total cyclic AMP content of brown fat cells and glycerol release in presence of lipolytic agents. Brown fat cells (9 nmoles triglyceride/flask) were incubated for 30 min prior to addition of lipolytic agents in 0.5 ml of buffer containing 4% albumin and 2.8 mM glucose. Star in circle represents basal value, open circles those for different concentrations of serotonin, filled circles for serotonin in presence of theophylline (0.8 mM), open squares those for norepinephrine, and filled squares for norepinephrine in presence of theophylline (0.8 mM). Cyclic AMP accumulation was measured 5 min after addition of lipolytic agents and glycerol release after 60 min. Values for each point are means of 3 paired experiments.

Serotonin was a weak lipolytic agent, and in the respiration studies an increase in lipolysis was only seen if an amount of cyclic AMP sufficient to exceed the threshold for lipolysis was present. Our results confirm the findings of Yoshimura et al. (25, 26) that in brown adipose tissue serotonin accelerated both lipolysis and respiration in the presence of theophylline or catecholamines.

In the studies in which cyclic AMP accumulation was measured, serotonin increased both lipolysis and cyclic AMP accumulation in the absence of other agents (Fig. 4). These studies were done at a later time, with another lot of albumin and under different experimental conditions from those involving measurements of respiration. There was an appreciable basal lipolysis in the experiments in which cyclic AMP was examined (Figs. 4 and 5), but not in the other studies. These findings suggest that serotonin is such a weak lipolytic agent that no stimulation will be seen unless there is appreciable basal lipolysis or, if this is minimal, in the presence of submaximal concentrations of another lipolytic agent. This variability might explain some of the earlier confusion created by the reports that serotonin increased the activation of phosphorylase (22) but had no lipolytic activity on white adipose tissue (23). The present results clearly indicate that in brown fat cells serotonin increases the accumulation of cyclic AMP, and this increase is related to the increase in lipolysis (Figs. 4 and 5). The effects of serotonin on brown fat cells appear to be secondary to activation of adenylate cyclase. Serotonin was first found to activate adenylate cyclase in particulate preparations from liver flukes in 1960 by Mansour et al. (17).

The effects of adrenergic antagonists are compatible with the hypothesis that the receptor for the lipolytic activity of catecholamines in brown fat is a beta receptor. Previously we found that both the stimulation of cyclic AMP accumulation and of lipolysis by epinephrine in brown fat cells was blocked by propranolol at a concentration of 3 \( \mu M \) (20). Dichloroisoproterenol, a beta-adrenergic antagonist, blocked the activation by catecholamines of the adenylate cyclase activity in particles isolated from brown adipose tissue homogenates (21). The inhibitory effects of propranolol on serotonin action suggest that it is a weak activator of the beta-adrenergic receptor in brown fat cells.

The relative insensitivity of brown fat cell respiration in the presence of theophylline or fatty acids to ouabain under our experimental conditions is of interest. Ouabain did inhibit the stimulation of respiration by catecholamines, but this appeared to be secondary to reduction in lipolysis and cyclic AMP accumulation. A similar inhibitory effect of ouabain has been seen in white fat cells (5, 14). Although ouabain blocked the lipolytic action of catecholamines, it
did not affect that of theophylline or the stimulation of respiration by either theophylline or added fatty acids. Omission of K+ from the buffer used for isolation and incubation of fat cells mimics the effects of ouabain on the lipolytic action of norepinephrine and theophylline (5, 19).

The relative insensitivity of respiration to ouabain under conditions in which glucose oxidation was increased and lactate formation decreased suggests that calorigenesis in brown fat cells does not result from cyclic flux of K+ via a ouabain-sensitive process. Our results also support the concept that the calorigenic action of amines on brown fat cells is secondary to activation of lipolysis through stimulation of adenylate cyclase via a beta-adrenergic receptor.

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