DECREASED INSULIN BINDING TO PORCINE ADIPOCYTES IN VITRO BY BETA-ADRENERGIC AGONISTS

C. Y. Liu and S. E. Mills

Purdue University, West Lafayette, IN 47907

ABSTRACT

The present study was conducted to determine the influence of dibutyryl-cAMP (dbcAMP), epinephrine, ractopamine and clenbuterol on insulin binding to porcine adipocytes. Dibutyryl-cAMP decreased insulin binding to swine adipocytes by 40 and 20% at 1.8 and 25.8 ng insulin/ml, respectively. Ractopamine and clenbuterol directly reduced insulin binding at the low insulin concentration and decreased binding at high insulin concentrations in the presence of adenosine deaminase. Scatchard analysis suggested that the reduction of insulin binding was due to a decrease in receptor number. Epinephrine alone did not influence insulin binding. In the presence of theophylline, epinephrine decreased binding at both low and high insulin concentrations; however, ractopamine plus theophylline decreased binding only at the low insulin concentration. Clenbuterol did not affect insulin binding in the presence of theophylline. Propranolol blocked the inhibitory effect of epinephrine on insulin binding. These beta-adrenergic agonists can inhibit insulin binding and, thus, antagonize insulin action in swine adipocytes.

(Key Words: Pigs, Adipocytes, Insulin, Receptors, c-AMP, Clenbuterol.)


Introduction

One mechanism whereby hormones regulate cellular metabolism is through modulation of the physiological response to other hormones. Adipocyte metabolism is tightly regulated by the opposing actions of the β-adrenergic agonists and insulin. For instance, insulin inhibits the lipolytic response of the β-adrenergic agonists in rodent (Smith et al., 1984) and swine adipose tissue (Mersmann, 1986), whereas the β-adrenergic agonists antagonize insulin-stimulated glucose transport (Pessin et al., 1983) and lipogenesis (Liu et al., 1989). Further, incubation of rat adipocytes with isoproterenol inhibits the binding of insulin to its cell surface receptor (Pessin et al., 1983).

Ractopamine and clenbuterol are two synthetic β-adrenergic agonists that bind with high affinity to the adrenergic receptor in swine adipocytes (Liu and Mills, 1989) and also limit fat accretion when fed to growing pigs (Ricks et al., 1984; Anderson et al., 1988). How insulin binding is affected by ractopamine or clenbuterol has not been determined. Therefore, the objective of these experiments was to determine the effectiveness of β-adrenergic agonists, including ractopamine and clenbuterol, to influence the binding of insulin to swine adipocytes in vitro.

Materials and Methods

Tissue Processing. Subcutaneous adipose tissue from the shoulder was obtained at slaughter from market weight barrows. Pigs were obtained from both the Purdue swine farm (40%) and from a local abattoir (60%). Pigs from Purdue had ad libitum access to feed up to 2 h before slaughter, but the dietary...
status of the other pigs was not established.

Responses to in vitro treatments were similar in both sets of pigs. All pigs were slaughtered between 0800 and 1000. Tissue was placed in 37°C buffered saline (0.15 M NaCl and 1 mM HEPES, pH 7.4) and processed within 15 min. Tissue slices (<5 mm) were prepared from the outer and middle fat layers using a Stadie-Riggs microtome; adipocytes were isolated by collagenase (1 30 U/ml) digestion as described previously (Liu et al., 1989). Medium used for isolation and subsequent incubation was Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1.25 mM CaCl2, 10 mM HEPES, 5 mM glucose and 1% BSA. Cell density was quantified by a modification of the method of Di Girolamo et al. (1971) as described previously (Liu et al., 1989). Final cell suspensions contained approximately 2 to 3 x 10^6 cells/ml.

Insulin Binding. Procedures for insulin binding followed the protocol described by Etherton and Walker (1982). Duplicate .5-ml aliquots of the cell suspension were preincubated for 30 min in 17- x 100-mm polypropylene tubes in a 37°C gyratory water bath in an atmosphere of 5% CO2 in oxygen. Media additions of dbcAMP, adrenergic agonists, adenosine deaminase (20 mU/ml) or theophylline (.4 mM) were as indicated in each figure and table. Following preincubation, tubes were cooled to 30°C for 5 min and insulin binding was initiated by the addition of porcine [125I]insulin (8 ng/ml) and unlabeled porcine insulin (0 to 25 ng/ml). After a 1-h incubation at 30°C, adipocytes were separated from the media by centrifugation of .1-ml aliquots of each cell suspension in triplicate through .1-ml silicone oil (density .963) in polyethylene microtubes for 45 s at 7,000 × g. Bound insulin in the cell layer was counted using a gamma-counter. Specific binding of [125I]insulin was calculated by subtracting nonspecific insulin binding determined by incubating cells with an excess of unlabeled insulin (100 µg/ml). Insulin binding was expressed as picograms of insulin bound/2 × 10^5 cells. At this cell concentration and .8 ng insulin/ml, specific binding averaged 4.2% of the added insulin.

Insulin degradation occurring during the incubation was assessed by quantifying the percentage of insulin not precipitated by trichloroacetic acid (TCA) before and after incubation. Briefly,.1 ml of the cell suspension was mixed with .1 ml cold 10% TCA and centrifuged for 1 min at 7,000 × g. The supernatant was counted as the estimate of degraded insulin. No corrections in insulin binding due to degradation were made.

Data were analyzed by the General Linear Model procedure of SAS (1985) for a block design; each experiment constituted a block. When appropriate, means separation was accomplished by Student-Newman-Keuls test.

Results

Standardization Studies. Preliminary experiments examined insulin binding as a function of time and cell concentration. The equilibrium of insulin binding was reached by 20 to 40 min and was stable through 120 min at 30°C (data not shown). Based on these data, an incubation time of 1 h was chosen. The amount of insulin...
Treatment

Ractopamine
Clenbuterol

Insulin, ng/ml

Control
Ractopamine (10^{-6} M)
Clenbuterol (10^{-6} M)
SE

1.8
25.8

50.4
44.5b
43.9c
1.6

170.0
157.1
150.7

16.8
10.6

3.3

2.2

*Insulin binding is expressed as picograms of insulin bound/2 x 10^6 cells. Values are means for four pigs.

b,cMeans in a column with similar superscripts do not differ (P < .05).

bound was related linearly (r^2 = .9) to adipocyte concentrations between .5 to 3 x 10^5 cells/ml (data not shown). This linear relationship was used to normalize data when adipocyte concentrations differed. Insulin degradation averaged 12.1 ± 2.8% (n = 7).

Effects of dbcAMP and β-Adrenergic Agonists. The effects of dbcAMP on insulin binding at low (1.8 ng/ml) and high (25.8 ng/ml) insulin concentrations are shown in Figure 1. At concentrations of 2 to 10 mM, dbcAMP decreased insulin binding by 40% at 1.8 ng insulin/ml (P < .01) and by 20% at 25.8 ng insulin/ml (P < .09, Figure 1). Incubation of adipocytes with ractopamine or clenbuterol (10^{-6} M) decreased insulin binding by 13 and 10% at 1.8 and 25.8 ng insulin/ml, respectively, although only the decrease at low insulin concentration was significant (P < .05, Table 1).

The combination of adenosine deaminase plus ractopamine or clenbuterol tended to decrease insulin binding at all insulin concentrations compared to control cells without adenosine deaminase (Figure 2, left panel). Differences reached significance (P < .05) at 1.8, 10.8 and 25.8 ng insulin/ml for ractopamine plus ADA and 25.8 ng insulin/ml for clenbuterol plus ADA. Adenosine deaminase alone tended to decrease insulin binding (data not shown), but its effect was not significant (P > .1). Scatchard analysis revealed nearly parallel plots with different intercepts, suggesting that the total number of insulin receptors was decreased by added compounds (Figure 2, right panel).

Theophylline alone did not influence insulin binding at low or high insulin concentration but it allowed the inhibitory effects of epinephrine to be expressed at both low and high concentrations of insulin (Table 2). Ractopamine plus theophylline decreased insulin binding at the low insulin concentration. However, no inhibition of binding was observed for ractopamine plus theophylline at the high insulin concentration or for clenbuterol plus theophylline at either insulin concentration. A lower concentration of epinephrine (10^{-7} M) in the presence of theophylline decreased insulin binding 16% at 1.8 ng insulin/ml. Propranolol (10^{-6} M) prevented this epinephrine-induced decrease (data not shown).

Because the decrease of insulin binding by beta-adrenergic agonists (Tables 1 and 2) was smaller than that induced by dbcAMP (Figure 1), experiments were repeated with a longer preincubation period. Ractopamine plus adenosine deaminase had similar effects on insulin binding whether the preincubation period was 30 or 120 min (Table 3). With both preincubation times, insulin binding tended to be less in the presence of ractopamine, although differences were not significant (P > .05). Further experiments were conducted to differentiate direct β-adrenergic responses from potential secondary responses due to increased mobilization of fatty acids. Incubation of adipocytes with .1 or .5 mM palmitate had no effect on insulin binding at 1.8 ng insulin/ml (Table 4).

Discussion

Previous work has shown that the number of insulin receptors on rodent adipocytes is decreased by agents that increase the intracel-
lular concentration of cAMP. Incubation of adipocytes with isoproterenol (Pessin et al., 1983; Lonnroth et al., 1987), catecholamines (Lonnroth and Smith, 1983; Pessin et al., 1983), cAMP analogs (Kirsch et al., 1983b; Lonnroth and Smith, 1983; Pessin et al., 1983; Lonnroth et al., 1987) or phosphodiesterase inhibitors (Kirsch et al., 1983b; Pessin et al., 1983) decreased insulin binding by 15 to 50%. Results of the present study show that cAMP-mediated inhibition of insulin binding also is a characteristic of swine adipocytes. Decreased binding of 20 to 40% observed in the presence of dbcAMP is in close agreement with previous results with rodent adipocytes.

To the extent that β-adrenergic agonists elevate intracellular cAMP concentrations, such agonists would be expected to decrease insulin binding similarly. In general, responses in vitro to the β-adrenergic agonists were less than those observed for dbcAMP. Epinephrine decreased insulin binding 20%, but only if the response was enhanced with theophylline. Theophylline acts at multiple sites on the adipocyte to potentiate agonist-induced increases in cAMP concentration (Hu et al., 1987), lipolysis (Hu et al., 1987; Liu et al., 1989) and antilipogenesis (Liu et al., 1989). Because propranolol was able to block the epinephrine response, our data are in agreement with others indicating that epinephrine inhibits insulin binding via a β-adrenergic receptor-mediated increase in adenylate cyclase and cAMP. Decreased insulin binding likely is not a secondary response to increased rates of triglyceride hydrolysis because addi-

<table>
<thead>
<tr>
<th>Insulin, ng/ml</th>
<th>Time, min</th>
<th>Control</th>
<th>RACT + ADA</th>
<th>SE</th>
<th>p&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>.8</td>
<td>30</td>
<td>259.5</td>
<td>28.2</td>
<td>1.1</td>
<td>.48</td>
</tr>
<tr>
<td>25.8</td>
<td>30</td>
<td>350.9</td>
<td>307.2</td>
<td>10.0</td>
<td>.06</td>
</tr>
<tr>
<td>.8</td>
<td>120</td>
<td>24.2</td>
<td>20.1</td>
<td>1.2</td>
<td>.11</td>
</tr>
<tr>
<td>25.8</td>
<td>120</td>
<td>350.6</td>
<td>293.4</td>
<td>19.6</td>
<td>.14</td>
</tr>
</tbody>
</table>

<sup>a</sup>Insulin binding is expressed as picograms of insulin bound/2 × 10⁵ cells. Values are means for four pigs with pooled SE.

<sup>b</sup>Ractopamine (RACT) was present at 10⁻⁶ M and adenosine deaminase (ADA) at 20 mU/ml.

<sup>c</sup>Probability of difference between control and treatment.
TABLE 4. EFFECT OF PALMITATE ON INSULIN BINDING* TO SWINE ADIPOCYTES

<table>
<thead>
<tr>
<th>Palmitateb, mM</th>
<th>0</th>
<th>.1</th>
<th>.5</th>
<th>SE</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>85.3</td>
<td>82.8</td>
<td>88.0</td>
<td>2.8</td>
<td>.47</td>
<td></td>
</tr>
</tbody>
</table>

*Insulin was present at 1.8 ng/ml and binding is expressed as picograms of insulin bound/2 x 10^6 cells. Values are means for four pigs.

A palmitate-BSA (1%) complex was added with suspended cells to a final volume of .5 ml. The molar ratio of palmitate: BSA was .7 at .1 mM and 3.5 at .5 mM.

Probability of difference among palmitate treatments.

The concentrations of ractopamine and clenbuterol used in the present study had been shown to give maximal effects on lipolysis and lipogenesis in swine adipocytes (Liu et al., 1989). Ractopamine and clenbuterol alone decreased insulin binding approximately 10% (Table 1). Whether these effects were mediated by cAMP is questionable because responses were not enhanced with adenosine deaminase or theophylline, and clenbuterol-induced changes in cAMP have not been observed in swine adipocytes (Hu et al., 1987). Ractopamine and clenbuterol bind to the swine adipocyte β-adrenergic receptor with high affinity, but they have a reduced capacity for adenylate cyclase activation relative to epinephrine (Liu and Mills, 1989). Under appropriate conditions in vitro, both agonists stimulate lipolysis and inhibit lipogenesis (Liu et al., 1989; Mills and Liu, 1990), indicating that adenylate cyclase activation occurs. However, we have not observed any effects of either ractopamine or clenbuterol on swine adipocyte metabolism in the absence of theophylline or adenosine deaminase. Whereas insulin binding may be sensitive to small changes in cAMP, we cannot rule out the possibility that secondary actions, independent of adenylate cyclase, result from the coupling of agonist to its receptor. Overall, the influence of ractopamine and clenbuterol on insulin binding to swine adipocytes was small. Furthermore, results appear to be less consistent, especially for clenbuterol, if the media contained adenosine deaminase or theophylline. Although it is tempting to speculate that the presence of adenosine may be necessary for full expression of the agonist response, the present data have not addressed directly this possibility.

The physiological effect of small changes in insulin receptor number is to render the adipocyte less sensitive to insulin without altering maximal cell response (Kahn, 1978). Rightward shifts in the insulin dose-response curve induced by β-adrenergic agonists have been demonstrated when measuring glucose transport (Kirsch et al., 1983a; Pessin et al., 1983) and fatty acid synthesis (Orcutt et al., 1989). Because chronic feeding of the β-adrenergic agonists may lower circulating concentrations of insulin (Beerman et al., 1987; Eisemann and Huntington, 1988), the net result should be a reduced insulin response, lipogenic capacity and rate of triglyceride accretion. We (unpublished observations) and others (Merkel et al., 1987) have observed decreased lipogenic capacity in adipose tissue from ractopamine-treated pigs.

Implications

The beta-adrenergic agonists ractopamine and clenbuterol inhibit insulin binding and thereby antagonize insulin action of fat cells of swine. This decreases capacity for fat synthesis and rate of fat deposition in swine.

Literature Cited


