Regional specificity of ASP binding in human adipose tissue

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Regional specificity of ASP binding in human adipose tissue. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E815–E821, 1999.—Obesity, in particular omental (OM) adiposity, is associated with diabetes and cardiovascular disease. Thus site-specific regulation of fat storage is important to understand. Acylation-stimulating protein (ASP) is a potent stimulator of glucose transport and triglyceride synthesis in adipocytes. In the present study, we characterized receptor binding of $^{125}$I-labeled ASP to human adipocyte plasma membranes from paired OM and subcutaneous (SC) sites in normal (N) and obese (O) male (M) and female (F) subjects ($n = 24$). Overall, specific binding of $^{125}$I-ASP was in the order of SC > OM and O > N (in SC tissue, particularly in F). Receptor affinity of $^{125}$I-ASP was higher [lower dissociation constant ($K_d$)] in SC than in OM ($63.6 \pm 16.2$ vs. $160.7 \pm 38.6$ nM, $P < 0.02$), especially in F ($37.0 \pm 11.1$ F-N vs. $263 \pm 6.7$ nM F-O) and lower ($K_d$) male OM ($291.8 \pm 116.8$ M-N and $149.4 \pm 56.4$ M-O). The greater binding and higher affinity of $^{125}$I-ASP binding to SC suggests that ASP may be an important factor in maintaining regional adipose tissue mass. Conversely, lower binding and receptor affinity in male OM adipose tissue may contribute to the fatty acid imbalance and metabolic complications associated with this syndrome, by reducing the efficiency of adipose fatty acid trapping by the ASP pathway.


citation

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3T3-L1 cells (10, 38). Detailed studies have shown that ASP stimulates triglyceride synthesis (TGS) in human skin fibroblasts (3, 12). Experiments on primary human SC adipocytes have shown that ASP stimulates free fatty acid esterification and incorporation into triglyceride in adipocytes by increasing the maximum velocity for TGS with no change in substrate delivery (Michaelis-Menten constant (Km)) (46). The effect of ASP was much greater than in fibroblasts. ASP stimulated TGS to a much greater extent in differentiated vs. undifferentiated adipocytes (10). Activation of diacylglycerol acyltransferase (48), a regulatory enzyme in TGS, and stimulation of specific glucose transport (21, 36) are the main routes by which ASP action is accomplished. ASP exerts its actions through activation of the protein kinase C (PKC) second messenger system (4).

Growing evidence strongly supports the presence of a specific high-affinity receptor for ASP. Radiolabeled ASP demonstrated specific and saturable binding to normal human skin fibroblasts (9). ASP was internalized and degraded in a time- and concentration-dependent manner (9). The degree of binding to human skin fibroblasts was directly correlated with the extent of TGS (9). Preliminary observations showed that binding of ASP increases markedly with differentiation from human preadipocytes to adipocytes (29), consistent with the increased response of human adipocytes to ASP stimulation of TGS. Radiolabeled ASP binding to skin fibroblasts from hyperlipoprotein B patients was also reduced in correlation with their reduced TGS capacity despite the high fasting plasma ASP levels (13, 49). Furthermore, under basal conditions, TGS was substantially greater in SC adipocytes than in OM adipocytes. The differences were more pronounced when the cells were stimulated with either insulin or ASP (37). Given the effects of ASP on human adipocytes, it is important to examine the binding characteristics of ASP in regional fat tissue. In this study we have focused on the differences in ASP specific binding and receptor affinity to human SC and OM adipose tissue obtained from obese and normal, male and female subjects.

MATERIALS AND METHODS

Subjects. Subjects used in this study were morbidly obese or nonobese patients undergoing abdominal surgery at the Royal Victoria Hospital in Montreal, QC, Canada. Samples of SC and OM adipose tissue were excised under general anesthesia. The protocol was approved by the ethics committee. Morbid obesity was defined as a body mass index (BMI) >35, in which BMI is calculated as weight (kg)/height (m)^2 on the basis of a Canadian population (40).

Particulate membrane preparation. Particulate fractions containing plasma membranes for receptor binding assays were prepared immediately after the tissue samples were obtained, as described previously (5). Adipose tissue (5–10 g wet weight) was excised from blood vessels and connective tissue and was washed in ice-cold Tris buffer (15 mM NaCl and 10 mM Tris-HCl, pH 7.4) containing protease inhibitors calpain (26 µM), leupeptin (26 µM), and aprotinin (7.6 µM), all inhibitors from Boehringer Mannheim (Laval, QC, Canada), and phenylmethylsulfonyl fluoride (1 µM). The tissue was minced, and 3 ml of Tris buffer/g minced tissue were added and homogenized in a glass Dounce homogenizer. The homogenate was centrifuged at 2,500 rpm for 15 min at 4°C, and infranate (below the fat layer) was collected. The infranate was recentrifuged at 40,000 rpm (100,000 g) for 60 min to pellet the particulate membrane (PM) fraction. The pellet was resuspended in ice-cold Tris buffer (200 µl) and frozen immediately at −80°C. Aliquots (10 µl) were frozen separately to measure the protein content by the Lowry method (34) and 5'-nucleotidase activity. Directly before use, the samples were diluted in Tris buffer to obtain a final concentration of 3 mg/ml PM fraction protein.

Receptor binding experiments. Homologous competition binding was performed in 96-well multiscreen-HV filter plates (Durapore filter membrane, pore size 0.45 µM, catalog no. MAHY N45, Millipore, Toronto, ON, Canada). Before experiments, the wells were blocked with 2% BSA in binding buffer (in mM: 50 HEPES, 5 MgCl2, 1 CaCl2) for 1 h at room temperature. The blocking solution was removed by filtration through the membrane filters with a vacuum manifold. Plasma ASP was purified, as described previously (4), and radiolabeled by the iodogen method (Pierce Chemical, Rockford, IL). Specific activity averaged 769.1 ± 29.8 counts/min−1·fmol−1. Ten microliters (10 µg) of crude plasma membrane preparation were added per well. Radiolabeled 125I-ASP (1 nM) and increasing concentrations of unlabeled ASP were added for each assay in duplicate. Preliminary experiments indicated that the concentration of radiolabeled ASP used (1 nM) was substantially below the dissociation constant (Kd) that is a requirement for competition binding experiments. All ASP dilutions were prepared in siliconized tubes in binding buffer. The volume in each well was completed to 100 µl with the binding buffer (final PM protein concentration = 0.1 mg/ml) and incubated on ice for 1 h. Preliminary experiments showed that equilibrium binding was achieved by 20 min at 4°C and was maintained for 1 h. Unbound radioactivity was removed by vacuum filtration, the filter was washed four times with 100 µl of ice-cold binding buffer, and filter membranes were dried. Filter-bound radioactivity was determined using a gamma counter. Wells with no particulate fraction added were used to subtract background sticking of 125I-ASP to the filter membranes. Binding curves were evaluated by curve fitting by use of iterative four-parameter logistic function analysis with Sigma Plot computer software (Jandel Scientific, San Rafael, CA) or by Graph Pad Prism. The data fit best to a one-site (not 2-site) binding isotherm. On average, specific binding constituted 66 ± 6.9% of PM fraction binding.

5'-Nucleotidase assay. Consistency of the plasma membrane content of the PM fraction was determined by using 5'-nucleotidase as a marker enzyme for plasma membranes (47). The assay measures the release of inorganic phosphate from AMP. The reaction was carried out in a final volume of 0.17 ml in each well containing 0.1 M Tris·HCl, 0.1 M MgCl2 (pH 8.5), and 0.1 M AMP (Sigma, St. Louis, MO). PM fraction preparations (50 µl, 0.06 mg protein/ml) were added to each well to give a final concentration of 18 µg/ml. The plates were incubated in a covered humid chamber at 37°C for 20 min. The reaction was stopped by the addition of 20 µl of freshly prepared solution of 7% ascorbic acid and 5% ammonium molybdate. After incubation at 45°C for 2 h, the color was read at 650 nm. Sodium phosphate (1 mM Na2HPO4) was used to prepare the standard curve (0.02–50 nmol/well).

Statistics. All values are expressed as means ± SE. Comparisons were made by paired t-test or ANOVA, as indicated, with significance set at P < 0.05.
RESULTS

The focus of the present study was to determine binding of ASP to SC and OM adipose tissue obtained directly from abdominal operations. To avoid disruption of binding activity, fat tissue was treated with protease inhibitors immediately upon excision of the tissue from the subjects and during plasma membrane preparation; thus local environmental influences that may affect ASP binding to adipose tissue could be studied directly. Moreover, influences from tissue culture conditions such as duration, medium change, and collagenase treatment that may alter the binding characteristics were avoided. Both SC and OM adipose tissue fragments were obtained from 24 patients undergoing abdominal operations. The average age of the patients was 40 ± 1.6 yr, with a range of 23–51 yr. Patient characteristics of each group are summarized in Table 1. Patients were not taking any medications known to affect lipid metabolism. All females were premenopausal. No significant difference was seen among the age groups. Morbidly obese patients were defined as having a BMI > 35 kg/m², and all were undergoing gastric bypass operations. Nonobese females were undergoing abdominal hysterectomy. Nonobese males were undergoing abdominal operations that included bladder removal, colon resection, omentectomy, and umbilical hernia. SC and OM tissues were obtained simultaneously from each patient during each operation.

Competition binding experiments were performed at a constant concentration of radiolabeled 125I-ASP and increasing concentrations of unlabeled ASP by use of the isolated PM fractions. Results were analyzed mathematically by iterative four-parameter logistic function analysis. Specific binding was calculated as the difference between total binding and nonspecific binding and constituted 66 ± 6.9% of total binding to PM fractions. The average 5'-nucleotidase activity for all OM and SC PM fractions for all 24 subjects was 6.19 ± 0.6 U/mg PM, and all values of specific binding were normalized to this activity. No differences were seen in 5'-nucleotidase activity of SC vs. OM or nonobese vs. obese. Overall, the average specific binding of ASP to SC adipose tissue was 141.1 ± 17.5 fmol/mg PM and was significantly higher (2-fold) than in OM tissue, which was 56.4 ± 8.8 fmol/mg PM (P < 0.0001 by paired t-test). The groups were then divided into morbidly obese (O) and nonobese (N). In general, the results indicate that adipose tissue from O patients (n = 12, BMI = 47.1 ± 4.8 kg/m²) had higher specific binding of ASP than tissue from N patients (n = 12, BMI = 28.2 ± 4.6 kg/m²) (O, SC: 177.9 ± 20.2 and OM: 68.1 ± 12.5 vs. N, SC: 104.2 ± 24.2 and OM: 44.7 ± 11.4 fmol/mg PM, P < 0.05 by ANOVA), with a significant difference between O and N in SC (P < 0.04). As well, ASP binding to SC adipose tissue was always significantly greater than ASP binding to OM adipose tissue in these same groups (P < 0.0001). When females (n = 13) were compared with males (n = 11), no significant effect of gender on specific binding in SC or OM was found.

Figure 1 illustrates the individual values for all of the women for ASP binding to SC and OM adipose tissue depots. Comparison between the different groups by two-way repeated-measures ANOVA showed that adipose tissue (SC and OM) from O females (n = 6; Fig. 1,
right) had a significantly higher ASP specific binding than that from N females (n = 7; Fig. 1, left) (P < 0.04). Overall, SC demonstrated significantly greater binding than OM in both O and N females (P < 0.001). The individual data for ASP specific binding for the men are shown in Fig. 2. Although ASP binding to SC was always greater than OM for both N (Fig. 2, left) and O males (Fig. 2, right) (P < 0.0003), there were no significant differences between O males (n = 6) and N males (n = 5). When all subjects (both male and female) were taken together, there was a positive correlation between ASP binding in SC and OM adipose tissue such that those subjects that had the highest level of ASP binding to SC also had the highest binding to OM, and vice versa (Fig. 3, r = 0.701, P < 0.0002).

The affinity of ASP binding (K_d) to the PM receptor preparations was also determined. K_d values were calculated from competition binding curves by computer-assisted analysis as the concentration at which 50% competition is achieved. The average K_d value for the OM depot (160.7 ± 38.6 nM) was significantly higher than that for the SC adipose depot (63.6 ± 16.2 nM, P < 0.02). There was a significant shift of the competition curve to the right in the preparations from OM tissue compared with SC tissue. This shift represents higher K_d values, which indicate lower affinity of ASP to OM adipose tissue receptors. The data were then separated into male vs. female, as shown in Fig. 4. Interestingly, the affinity of ASP to SC adipose tissue in females was significantly higher (lower K_d values) (32.1 ± 6.9 nM) than in male SC adipose tissue (100.8 ± 30.9 nM, P < 0.04). Further subdivision into O and N for both male and female adipose tissue with respect to affinity is given in Table 2. In the females, all adipose tissue,
Table 2. ASP binding affinity

<table>
<thead>
<tr>
<th>Gender</th>
<th>Tissue</th>
<th>Nonobese</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females SC</td>
<td>37.0±11.1</td>
<td>26.3±6.7</td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>70.0±21.4</td>
<td>168.6±80.7</td>
<td></td>
</tr>
<tr>
<td>Males  SC</td>
<td>67.7±17</td>
<td>128.4±52.3</td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>291.6±116.8</td>
<td>149.4±56.4</td>
<td></td>
</tr>
<tr>
<td>All subjects SC vs. OM</td>
<td>P&lt;0.01</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Acylation-stimulating protein (ASP) binding affinity values are means ± SE expressed as a dissociation constant (K_d, in nM) for females (n = 7 nonobese, n = 6 obese) and males (n = 5 nonobese, n = 6 obese). NS, not significant.

except that from O OM depots, demonstrated high-affinity binding. By contrast, the reverse was true of all male adipose tissues, which had substantially lower affinities (higher K_d values), whereas only the K_d for ASP binding to N SC adipose demonstrated high-affinity binding.

DISCUSSION

The ASP pathway appears to be a major determinant of the rate of TGS in adipocytes and, therefore, the efficiency of energy storage. Overall the present results demonstrate that there are clear differences in both specific ASP binding and receptor affinity within the tissues analyzed. The major difference was between SC adipose tissue and OM adipose tissue, where SC adipose tissue had a greater number of ASP specific binding sites as well as a higher affinity receptor, suggesting a more efficient ASP response. These results are consistent with numerous studies that have shown that SC tissue is more efficient in triglyceride storage than OM tissue; this is reflected by the larger size of SC cells (20), by greater TGS rates (37), by increased lipoprotein lipase (20), and by lower lipolytic rates in response to catecholamines compared with OM tissue (32). These results are also consistent with a greater lipogenic response to ASP in SC adipocytes vs. OM adipocytes (37).

Although the major difference in ASP specific binding was between SC and OM tissue, there were also differences, although less marked, between O and N. Results show a trend toward higher specific binding in O compared with N subjects, the trend being more pronounced in the females than in the males. However, there did not appear to be any consistent difference between males and females for ASP specific binding. These differences between SC and OM tissue with regard to specific binding may be explained by a greater ASP receptor number in the SC adipose tissue. This tissue was also found to have a higher density of insulin receptors compared with OM adipose tissue (8). Many factors may be affecting the level of receptor expression in OM adipocytes causing limited ASP binding. OM adipocytes have been shown to respond more intensely to lipid-mobilizing hormones such as catecholamines (2, 25, 32); such hormones may play a role in downregulation of ASP receptor expression in OM adipocytes. On the other hand, hormones such as insulin might also be responsible for upregulation of ASP receptors in SC tissue.

The results indicate clearly the presence of a high-affinity receptor to ASP on adipocyte plasma membranes. This correlates with our previous findings in adipocytes (29) and human fibroblasts (49), and the affinity (nM range) is consistent with the physiological levels of ASP in humans (also nM range) (14). In fact, we have recently demonstrated in human arteriovenous studies that ASP is produced directly by SC adipose tissue and that this production increases postprandially (41).

What was most striking, however, was that the receptor affinity varied markedly depending on adipose tissue site, sex of the subject, and obesity index. Thus the receptor had a higher affinity for ASP in SC vs. OM adipose tissue, had a higher affinity in females compared with males, and in general demonstrated lower affinity in adipose tissue from all O compared with all N subjects. These differences in receptor affinity might be explained through a variety of molecular mechanisms. Vikman et al. (45) proposed that site-specific differences (SC vs. OM) in adipose tissue metabolism may be due to different degrees of phosphorylation of receptors or G proteins. For example, it has been shown that serine/threonine phosphorylation of the insulin receptor may alter receptor affinity (17). Altered insulin receptor affinity has been proposed as an explanation for the greater insulin activity in SC compared with OM adipocytes (8). Insulin receptor affinity is higher in SC than in OM (8). Glucose and gastric inhibitory polypeptide have been shown to increase insulin receptor affinity in both human (8) and rat (23, 43) adipocytes, possibly by slowing the rate of insulin dissociation. Abdominal SC tissue demonstrates higher β- and α2-adrenergic receptor affinities than gluteal SC adipocytes (6). Similar mechanisms may be operating for the ASP receptor. We have shown that ASP stimulation of TGS is dependent on activation of a second messenger pathway, the PKC pathway (4). Activation of the PKC pathway has been shown to be induced through activation of tyrosine phosphorylation, phospholipase C, and phospholipase A2 via G protein-mediated action (39). ASP interaction with its receptor may be acting through any of these pathways. Clearly, once an ASP receptor is identified, elucidation of this pathway in adipocytes will be important to determine in detail.

Differences in ASP receptor affinities between males and premenopausal females might be explained by a sex hormone effect. There are certainly many examples of differences in adipose tissue metabolism between males and females at a cellular level (7, 20, 32, 33, 35). In general, lipogenesis appears to be more efficient in females, whereas lipolysis-related events are more pronounced in males. Specifically, basal lipolysis, catecholamine-stimulated lipolysis, and β1-, β2-, and β3-adrenergic receptors (all stimulators of lipolysis) are present at higher levels in adipocytes from male subjects. By contrast, TGS, lipoprotein lipase enzyme levels, and α2-adrenergic receptors (prolipoxygenase) are all more elevated in female adipocytes. These differ-
ences in adipose tissue metabolism may be mediated directly or indirectly through sex hormone effects. Evidence demonstrating differences in lipolytic activities between premenopausal women compared to postmenopausal women has also been presented (22). These differences could be reversed by hormone therapy (22). Thus, although the presence of sex hormone receptors on adipocytes remains controversial, adipocytes do mediate sex hormone conversion (31), and the present data suggest that there are specific sex-related and site-specific differences in the metabolism of adipose tissue that may mediate, directly or indirectly, changes in the ASP plasma membrane receptor level.

Finally, SC tissue from O females had the greatest receptor affinity for ASP, suggesting that, physiologically, this tissue would be the most responsive to ASP. Certainly, as mentioned above, SC adipose tissue has been shown to be more efficient at TGS than OM adipose tissue and to respond more to ASP than OM tissue (37). What is striking is that in vitro studies also demonstrate that adipocytes derived from obese women remain equally responsive to ASP (46) in contrast to their decreased responsiveness to insulin (15). This may be due to an upregulated receptor expression in morbidly obese females compared with normal females, and, in conjunction with the fact that their circulating levels of ASP are substantially elevated (11), these may be factors that result in increased glucose transport and TGS and may contribute to their obesity. This is especially important because female obesity is primarily SC obesity.

There are, of course, numerous possibilities for genetic and environmental regulation of fat patterning from regulation of hormone secretion (endocrine or autocrine) to regulation of cellular receptors. For example, testosterone inhibits triglyceride assimilation in intra-abdominal (OM and mesenteric) depots and increases this lipid deposition into SC tissue in men (35). Insulin receptors, α2-adenoreceptors, and adenosine receptors (anti-lipolytic/prolipogenesis) are most active in SC fat cells. In addition, agouti protein (27, 30) and angiotensinogen (16, 28, 42) were capable of upregulating enzymes involved in de novo fatty acid synthesis. However, although TGS and storage were increased, the effect was modest (20%) (28). Finally, adipose tissue also secretes both TNF and leptin, which have both paracrine (adipose tissue) and endocrine (brain) effects (26).

In conclusion, the data point to a physiological role of ASP in human adipose tissue in which ASP receptor affinity values are physiologically relevant to ASP concentrations in human plasma (14), such that SC tissue would appear to be more capable of responding to lower concentrations of ASP. There are specific regional, gender, and body size-related differences in binding site numbers and affinity for ASP, which, as well as circulating ASP levels, will influence the impact that ASP has on whole body adipose tissue fat storage. Certainly isolation of the ASP receptor will be a key factor in elucidating the precise mechanism of ASP response. Enhanced ASP binding to SC tissue can lead to an increase in triglyceride storage. Impaired ASP binding in OM adipose tissue, however, can contribute to decreased efficiency of triglyceride storage and increase circulating fatty acid fluxes, leading to metabolic imbalance and disorders that are commonly associated with OM obesity, such as diabetes and cardiovascular disease (18). Our evidence strongly supports the presence of a specific high-affinity receptor for ASP. This is clearly pronounced in the differential binding of ASP to different adipose tissue depots. The results, therefore, suggest an important function for ASP, in which ASP binding and subsequent response may be a significant factor in determining regional differences in fat distribution, a function that would provide further insight to understanding the pathophysiology of obesity.

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