Acute injection of ASP in the third ventricle inhibits food intake and locomotor activity in rats

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ASP promotes TG synthesis in WAT through interaction with its cell surface receptor C5L2. Activation and phosphorylation of C5L2 leads to increased fatty acid uptake, increased glucose transport, and stimulation of diacylglycerol acyltransferase activity (9, 10, 19, 20). ASP is produced through cleavage of the precursor protein complement C3 via factor B and adipin (factor D) interaction generating C3a, which is rapidly desarginated to produce ASP (also known as C3adesArg) (6, 25). C3-knockout mice (C3−/−; also known as C3KO) are deficient in ASP. The striking phenotype of these ASP-deficient (ASPdef) C3KO mice is that, despite being leaner, they have a significant increase in food intake, which is counterbalanced by increased energy expenditure, which is at least partly mediated through elevated muscle fatty acid uptake and oxidation (55). We recently showed that ASPdef C3KO mice on a high-fat diet have preferential utilization of fat as energy substrate (40). In addition, mice lacking the ASP receptor C5L2 (C5L2KO) demonstrated a phenotype similar to ASPdef mice with delayed postprandial TG clearance, increased dietary food intake, and increased muscle fatty acid oxidation (36). However, the mechanism of altered food intake remains unidentified.

The protective potential of ASP deficiency against obesity and involvement of the leptin pathway were examined in lep ob/ob C3−/− double-knockout (2KO), mice which are both ASP and leptin deficient. Food intake in 2KO mice was 9.1% higher compared with lep ob/ob mice, mice that are well known for their marked hyperphagia, and twofold higher than wild-type mice in this study (54). Nonetheless, because of increased energy expenditure in the 2KO mice relative to the lep ob/ob mice, the 2KO mice had a reduction in body weight, leading to improved insulin sensitivity and partial protection from obesity. These results suggest that, even in the absence of leptin, the presence/absence of ASP contributes to alterations in food intake and energy expenditure.

Short-term acute changes subsequent to peripheral (intrapitoneal) ASP administration have been evaluated in a number of mice studies. In lean wild-type, 2KO C3−/− lep ob/ob, obese lep ob/ob, and obese db/db mice, bolus administration of ASP at the beginning of a fat load enhanced postprandial TG, nonesterified fatty acid, and glucose clearance (29–31, 42, 54, 54). Furthermore, in ASPdef C3KO mice, a bolus ASP injection enhanced postprandial TG and nonesterified fatty acid clearance and returned the increased muscle oxidation and decreased adipose tissue fatty acid storage back toward the wild-type phenotype (29, 31). Leptin and insulin, both peripheral hormones affecting nutrient partitioning and adipocyte metabolism, are also known to have central influences on feeding behavior, raising the question of whether the ASP/
CSL2 pathway may be implicated in the central control of energy metabolism and control of food intake.

C3adesArg (ASP) is present in cerebrospinal fluid at concentrations ~15 times lower than plasma (26). However, mRNA for the precursor proteins required for ASP production (C3, B, and adipin/ factor D) are also expressed in brain cells (2). Whether central ASP is produced locally or is derived peripherally is unknown. The presence of CSL2, the ASP receptor, in the brain has been demonstrated (24), and indeed, CSL2 was first identified as an orphan receptor using a brain cDNA library (24). Initial studies evaluating intracerebroventricular (icv) administration of the effects of ASP or C3a (both of which bind the CSL2 receptor) on food intake provide limited data and are contradictory with increases (42), decreases (32–34), and no changes (42, 45) in food intake reported. None evaluated energy expenditure, weight gain, or locomotor activity. The aim of the present study was to examine the effects of icv injection of varying ASP concentrations on acute and extended food intake in rats. Second, we examined the energy expenditure response to central injection of ASP using multiple parameters. Last, we initiated analysis of potential mechanisms through which ASP induces these centrally mediated responses.

METHODS

Animals. Male Wistar rats (Charles River Laboratories, St. Constant, QC, Canada), weighing ~200 g at the beginning of the experiments, were individually housed in stainless-steel cages at 23 ± 1°C under a 12:12-h light-dark cycle (lights on at 0600). The rats had ad libitum access to water and a standard chow diet in powder form (Charles River Rodent Diet no. 5075: 8 kcal% protein and 4.5 kcal% fat; Ralston Products, Woodstock, ON, Canada). The animals were cared for and handled in compliance with the Canadian Guide for the Care and Use of Laboratory Animals, and all experimental procedures received prior approval of the Laval University Animal Care Committee.

Surgery. Following a few days of acclimatization, the rats were permanently implanted with stainless-steel guide cannulas (Plastics One, Roanoke, VA), as described previously (18). Rats were implanted with a 22-gauge single-guide cannula aimed at the third ventricle using the following stereotactic coordinates: 2.3 mm posterior to the bregma on the midline and 8.5 mm ventral to the brain surface (37). The guide cannulas were secured with screws and cranioplast cement (Dentsply International, York, PA). To prevent clogging and to reduce the potential for brain infection, sterile obturators were inserted into the guide cannulas. Cannula placement was also histologically verified a posteriori in every rat. During the recovery period (12 days), every rat was handled twice a day for 3 min to familiarize them with human presence and to simulate the injection procedure.

Intracerebroventricular injection of ASP and artificial cerebrospinal fluid. Briefly, the cap was removed and a 28-gauge injector (Plastics One) inserted into the guide cannula. Once the injector was installed, the rat was held lightly so as to reduce the stress factor during the 30-s injection, which was controlled by an infusion pump (1,065 pmol) and aCSF were done in the same manner as described above. Microinfusion of ASP did not drink 15 ml in the 15 min following the injection was washed, followed by elution with acidified buffer (0.625 N HCl in buffer, pH 2.0). For removal of rHis-tag, rHis-ASP solution was carefully adjusted to pH 8.0, and enterokinase (New England Biolabs, Beverly, MA) was added (0.063 U/ml), vortexed, and centrifuged (3,000 g, 5 min, 4°C), and the supernatant was decanted three times. All supernatants were pooled and HCl precipitated, which results in precipitation of 75% of total protein, with little loss of rASP, consistent with plasma ASP purification methods (19). rHis-ASP was purified with binding to Ni2+-sepharose column (GE Healthcare, Baie d’Urfe, QC, Canada). Following loading of rHis-ASP the column was washed, followed by elution with acidified buffer (0.625 N HCl in buffer, pH 2.0). For removal of rHis-tag, rHis-ASP solution was carefully adjusted to pH 8.0, and enterokinase (New England Biolabs, Pickering, ON, Canada) was added (0.063 μg/ml) and incubated overnight at room temperature (18–22°C) with gentle shaking. For HPLC purification, rHis-ASP or rASP was purified using the same method as for plasma ASP (1). Briefly, ASP was separated on a semipreparative Vydac Protein C4 column with a gradient from 0.1% trifluoroacetic acid to 80% acetonitrile in 0.1% trifluoroacetic acid. The final ASP product recovered from HPLC purification has a purity >98% based on mass spectrometry analysis and was diluted in aCSF to obtain the desired concentration.

Conditioned taste aversion. Male Wistar rats (n = 26, 300–400 g) were kept in individual cages under a 12:12-h light-dark regime. The protocol for cannula implantation in the third ventricle was as described above. Five days after the surgery, all rats received an injection of angiotensin II to assess the cannula placement. Rats that did not drink ≥15 ml in the 15 min following the injection were removed from the experiment.

Saccharin (0.15% wt/vol) was used as the conditioned stimulus and lithium chloride (LiCl; 0.60 M, 1% body weight, administered ip) as the malaise-inducing agent as described (3). Microinfusion of ASP (1,065 pmol) and aCSF were done in the same manner as described above and followed by an intraperitoneal (ip) injection of saline (control) or LiCl.

One week before the onset of the experiment, all rats were handled daily to minimize stress reactions to manipulation. For 6 consecutive days (days 1–6), rats were gradually placed on water deprivation, receiving access to water for 60 min daily until intake stabilized. Rats were then kept under this regimen of restricted access to water throughout the whole experiment. Water consumption and body weight were measured every day. The following day, the conditioning period between each injection. For in situ hybridization, rats were euthanized 90 min after the last injection, with one half provided with food after the injection (ad libitum) and one half remaining fasting (no food).

Two solutions were injected: artificial cerebrospinal fluid (aCSF) and ASP. The aCSF injected was purchased from Harvard Apparatus (Holliston, MA). This solution was chosen because it closely matches the electrolyte concentrations of CSF. It is prepared from high-purity water and analytical grade reagents and is microfiltered and sterile.

Recombinant human ASP was prepared and purified, following the protocol described previously by Cui et al. (9). The portion of the human C3 gene representing ASP (C3adesArg) was cloned into the NcoI/EcoRI restriction sites of pET32a + (Novagen, Madison, WI) and religated to obtain pET_his_ASP vector containing the His-tagged ASP sequence. The cell line used to produce recombinant ASP (rASP) is Origami B (DE3) (Novagen). Frozen OriB-pET-his-ASP scrapes were inoculated and grown overnight at 37°C with shaking (220 rpm) in bacterial medium (25 g of LB Broth; EMD Biosciences, Madison, WI) supplemented with 10 μg/ml kanamycin (Invitrogen, Burlington, ON, Canada) and 100 μg/ml carbenicillin (Invitrogen). Cultures were diluted in bacterial medium (1.5 ml), incubated for 5–8 h (37°C, 220 rpm) until OD600 of 0.6–0.8 was reached, and then supplemented with isopropyl β-D-thiogalactopyranoside (1 mM final concentration; Qiagen, Mississauga, ON, Canada) and incubated overnight (25°C, 220 rpm). Cells were collected and could be frozen until purification of rASP. For rHis-ASP purification, cells were lysed with 10 ml of Bugbuster (EMD Biosciences), 10 mg of lysozyme (Sigma, St. Louis, MO), and 10 μl of benzonase nuclease (20 U/ml; EMD Biosciences), vortexed, and centrifuged (3,000 g, 5 min, 4°C), and the supernatant was decanted three times. All supernatants were pooled and HCl precipitated, which results in precipitation of 75% of total protein, with little loss of rASP, consistent with plasma ASP purification methods (19). rHis-ASP was purified with binding to Ni2+-sepharose column (GE Healthcare, Baie d’Urfe, QC, Canada). Following loading of rHis-ASP the column was washed, followed by elution with acidified buffer (0.625 N HCl in buffer, pH 2.0). For removal of rHis-tag, rHis-ASP solution was carefully adjusted to pH 8.0, and enterokinase (New England Biolabs, Pickering, ON, Canada) was added (0.063 μg/ml) and incubated overnight at room temperature (18–22°C) with gentle shaking. For HPLC purification, rHis-ASP or rASP was purified using the same method as for plasma ASP (1). Briefly, ASP was separated on a semipreparative Vydac Protein C4 column with a gradient from 0.1% trifluoroacetic acid to 80% acetonitrile in 0.1% trifluoroacetic acid. The final ASP product recovered from HPLC purification has a purity >98% based on mass spectrometry analysis and was diluted in aCSF to obtain the desired concentration.

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Protocol was initiated with conditioning sessions on day 7 and one test session on day 10.

During the conditioning session (day 7), all rats were presented with saccharin instead of water for 30 min, after which they were immediately injected icv (ASP or aCSF) and ip with saline ± supplements (ASP-saline, n = 9; aCSF-saline, n = 8; or LiCl as aCSF-LiCl, n = 8/group). Over the next 2 days (days 8 and 9), rats received their daily access to water for 60 min. For the test session (day 10), all rats were presented with saccharin instead of water for 30 min, and water intake was compared in treatment vs. control (day 10) to assess the induced aversion.

Energy expenditure and voluntary motor activity. Oxygen consumption (VO₂), CO₂ production (VO₂CO₂) and respiratory quotient (RQ) were evaluated over 48 h in an open-circuit system with an O₂ (S-3A1; Applied Electrochemistry, Naperville, IL) and a CO₂ analyzer (CD-3A; Applied Electrochemistry). Data are presented as ml·kg⁻⁰·⁷⁵·min⁻¹. Locomotor activity was measured with the AccuScan Digiscan Activity Monitor (AccuScan Instruments, Columbus, OH) with the aid of the VersaMax software (version 1.30; AccuScan Instruments). Rats were placed individually in acrylic chambers for a 48-h adaptation period, and movement was measured for 48 h after injection. Cages contained an array of beams (every 2.5 cm) in all three dimensions (16 μ-axis, 16 μ-axis front-back, and 16 μ-axis vertical). Physical activity was determined by breaks in photo beams and converted into distance from the horizontal beam (cm/24-h period), and total activity was obtained from all of the beams (arbitrary units/day).

Blood plasma analyses. During the final phase of the experiment, plasma samples were collected by cardiac puncture at the time of euthanization. Glucose was measured using the Trinder glucose kit (Sigma, St. Louis, MO). Adiponectin, leptin, and insulin were measured using RIA kits (Linco, St. Charles, MO).

C5L2 mRNA analysis. A subset of rats (nonfasted) were euthanized for C5L2 brain mRNA localization. Brains were collected and separated into five parts, cortex, cerebellum, hypothalamus, hippocampus, and hindbrain, immediately frozen in liquid nitrogen, and then stored at −80°C. Total RNA was isolated using the Qiazol method (Qiagen, Mississauga, ON, Canada), and reverse transcription of 0.25 μg was performed with the RT² first-strand kit according to the manufacturer's instructions (SA Bioscience, Frederick, MD). cDNA (1 μl of 20 μl total) was amplified by real-time PCR using the primer sets for rat C5L2 (Gpr77, no. PPM34422A) (SA Bioscience). Reaction conditions for C5L2 were 40 cycles at 95°C for 10 s and 60°C for 30 s, as recommended for use with RT² fast SYBR green quantitative PCR master mix (SA Bioscience). Primers for the housekeeping gene acidic ribosomal phosphoprotein P0 (ARBP; position 511: TAAA-GACTGGAGACAAAGGTG; position 802: GTGTAGTCAGTCTC-CACA) were used according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Results are expressed as ΔΔCt ratio between target gene C5L2 and housekeeping gene ARBP expression.

In situ hybridization for neuropeptide Y and proopiomelanocortin. Hypothalamic mRNA levels of neuropeptide Y (NPY) and proopiomelanocortin (POMC) were measured by in situ hybridization essentially as described previously (13, 39). Overnight, hypothalamic sections were mounted onto poly-l-lysine-coated slides, dehydrated in ethanol, fixed in paraformaldehyde, digested with proteinase K (10 μg/ml), acetylated with 0.25% acetic anhydride, and dehydrated in ethanol gradient. Sections were incubated overnight with antisense ³⁵S-labeled cRNA probe (10⁵ cpm/ml) for NPY or POMC at 60°C. Slides were rinsed with sodium chloride-sodium citrate solution, digested with RNase-A, washed in descending concentrations of sodium chloride-sodium citrate solution, and dehydrated in ethanol gradient. Slides were dehydrated in toluene, dipped in NTB2 nuclear emulsion (Eastman Kodak, Rochester, NY), and exposed for 7 days before being developed. Slides were examined by dark-field microscopy using an Olympus BX51 microscope (Olympus America, Melville, NY). Images were acquired with an Evolution Q5i camera and analyzed with ImagePro plus version 5.0.1.11 (MediaCybernetics, Silver Spring, MD). The system was calibrated for each set of analyses to prevent saturation of the integrated signal. Mean pixel densities were obtained by taking measurements of three sections of the arcuate nucleus from both hemispheres and subtracting background readings taken from areas immediately surrounding the region analyzed.

Statistical analysis. Results are presented as means ± SE. Groups were compared with two-way ANOVA, ANOVA (with Newman-Keuls or Dunnett’s post hoc analysis as appropriate), or Student’s t-test as appropriate. Statistical significance was set as P < 0.05.

RESULTS

Anorexic effects of acute central injection of ASP. As shown in Fig. 1, there was an acute decrease in cumulative food intake...
Intracerebroventricular injection of ASP diminishes food intake at each time interval

Table 1. Intracerebroventricular injection of ASP diminishes food intake at each time interval

<table>
<thead>
<tr>
<th>Dose, pmol</th>
<th>0–30 min</th>
<th>30–60 min</th>
<th>60–120 min</th>
<th>120–240 min</th>
<th>4–16 h</th>
<th>16–24 h</th>
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<tr>
<td>CTRL (0)</td>
<td>3.86 ± 1.05</td>
<td>0.91 ± 0.87</td>
<td>1.65 ± 1.14</td>
<td>4.53 ± 1.85</td>
<td>21.74 ± 3.13</td>
<td>4.57 ± 4.89</td>
</tr>
<tr>
<td>0.3</td>
<td>4.53 ± 0.97</td>
<td>1.08 ± 1.33</td>
<td>1.90 ± 1.40</td>
<td>4.61 ± 1.95</td>
<td>16.70 ± 3.45</td>
<td>2.12 ± 1.51</td>
</tr>
<tr>
<td>3.75</td>
<td>3.11 ± 1.31</td>
<td>1.64 ± 0.99</td>
<td>1.40 ± 1.01</td>
<td>3.55 ± 2.94</td>
<td>14.65 ± 8.22</td>
<td>4.11 ± 1.96</td>
</tr>
<tr>
<td>18.75</td>
<td>3.74 ± 1.26</td>
<td>1.20 ± 1.02</td>
<td>1.16 ± 0.84</td>
<td>5.08 ± 2.09</td>
<td>17.76 ± 3.36</td>
<td>4.31 ± 0.71</td>
</tr>
<tr>
<td>525</td>
<td>2.28 ± 0.91*</td>
<td>1.70 ± 1.50</td>
<td>1.70 ± 1.07</td>
<td>3.37 ± 1.20</td>
<td>16.25 ± 3.31</td>
<td>5.00 ± 2.04</td>
</tr>
<tr>
<td>1,065</td>
<td>1.65 ± 0.90**</td>
<td>0.60 ± 0.65</td>
<td>2.50 ± 0.61</td>
<td>2.08 ± 1.50</td>
<td>16.25 ± 2.55</td>
<td>5.42 ± 1.34</td>
</tr>
</tbody>
</table>

Values are means ± SE. ASP, acylation-stimulating protein; FI, food intake; CTRL, control. FI at each time interval is presented individually. Statistical changes were analyzed by ANOVA, with posttest for linear trend.

Following icv injection of ASP, this effect on food intake was seen as early as 30 min (58% decrease) and 1 h (45% decrease) at the maximal dose (Fig. 1A). Interestingly, this reduction in food intake was not just an acute response, since it was observed over a 24-h period (Fig. 1), and when evaluated over each time interval separately (rather than cumulatively) there were significant concentration-dependent decreases at each time interval (ANOVA, from $P = 0.013$ to $P < 0.0001$; Table 1). Furthermore, in the groups of rats given the two highest doses of ASP, significant changes in cumulative food intake extended ≥48 h, with a significant decrease (18 and 23%) measured during the second 24-h period (Fig. 1B).

Intracerebroventricular injection of ASP alters weight gain. Following ASP injection in the third ventricle, body weight gain was evaluated. As seen in Fig. 2A, there was a dose-dependent gradual decrease in weight gain in the groups of rats, with those administered the highest ASP dose actually having lost weight following the acute injection (ANOVA, $P = 0.007$). In these two groups, along with their control littermates, observation was continued over a second 24-h period. As shown in Fig. 2B, long-lasting effects of the ASP injection on delaying weight gain are demonstrated. Effectively, even 48 h after a single injection occurred, the ASP-injected rats remained more resistant to weight gain vs. the aCSF-injected rats.

Changes in energy expenditure and RQ levels following a central injection of ASP. Energy expenditure was measured using several parameters, including $O_2$ consumption and RQ, using indirect calorimetry in rats for 48 h after icv injection of ASP at the maximal dose compared with aCSF controls. As shown in Fig. 3A, following icv injection, $O_2$ consumption was lower in rats injected with ASP compared with control CSF-injected rats. This decrease was most marked during the first 12 h after injection, which occurred during the dark phase, when rats are known to initiate eating. During the second 24-h phase (24–48 h postinjection), there was no difference in overall $O_2$ consumption (Fig. 3C). Analysis of RQ is indicative of the balance in energy substrate used (glucose vs. fatty acid) by the rats in the calorimetric chamber. Rats receiving a central injection of ASP had a strong preference for fatty acid oxidation, as indicated by the decrease in RQ compared with CSF-injected control rats (Fig. 3D). In fact, a rapid decrease in RQ following ASP injection is noted especially in the dark phase, a period during which the RQ levels usually change (as seen in the aCSF control rats). Repeated-measures ANOVA analysis of RQ levels over the total 24-h period demonstrates a consistent difference in energy substrate usage between the ASP and control rats, as presented in area under the curve analysis ($P < 0.05$; Fig. 3F). Furthermore, changes in RQ also occurred over the second 24-h period (24–48 h) after ASP injection ($P < 0.05$; Fig. 3F).

Because changes in energy expenditure and RQ in ASP-injected rats might be consequent to their decreased food intake, we evaluated $O_2$ consumption and RQ in rats that were pair-fed the same level of food intake seen in the ASP-injected rats. As shown in Fig. 3, B and E, pair-fed rats demonstrate significant decreases in $O_2$ and RQ ($P < 0.01$). These patterns reflect the same changes as those seen in the ASP-injected rats over the first 24 h (Fig. 3, A and D), and direct comparison indicates that there is no significant difference.

![Fig. 2. Intracerebroventricular (icv) injection of ASP hinders weight gain.](http://ajpendo.physiology.org/Downloadedfrom http://ajpendo.physiology.org)
Intracerebroventricular ASP injection enhances sedentary behavior. Physical activity was monitored during the time the rats were in the calorimetric chambers. This activity was separated into two parts: cumulative distance and total activity. Cumulative distance represents movement only within the x-axis direction, whereas total activity accounts for movement within all three axes. Rats centrally injected with ASP spend less time moving than their control counterparts (aCSF injection). Essentially, over a 24-h period after the injection, ASP-injected rats walked significantly less (Fig. 4A) and overall were more physically inactive compared with control aCSF-injected rats (Fig. 4B). The activity level of the pair-fed rats was also evaluated and demonstrated a decreased distance movement as well as decreased overall physical activity comparable with the ASP-injected rats (Fig. 4A and B). Interestingly, in the ASP-injected rats, this robust effect of the icv ASP injection on total distance was maintained over the 48-h period, as shown in the second 24-h period (Fig. 4C). The total physical activity of the rats returned to normal over the second 24-h period (Fig. 4D).

**ASP is not a malaise-inducing agent.** Saccharin-water consumption was evaluated during the conditioning session (not shown) and the test session (Fig. 5), with significant effects ($P < 0.001$) only in the test session. Whereas aCSF-saline, ASP-saline, and aCSF-LiCl rats had a similar saccharin-water consumption during the conditioning session, aCSF-LiCl rats displayed a significant taste aversion since they consumed less saccharin-water than aCSF-saline and ASP-saline during the test sessions ($P < 0.05$ for LiCl vs. both aCSF-saline and ASP-saline). However, there was no effect of ASP vs. aCSF, indicating no malaise or taste aversion.

**ASP injection enhances expression of the anorexigenic neuropeptide POMC.** To evaluate potential mechanisms of ASP action on central control of food intake, expression of NPY and POMC were evaluated across different brain sections. Rats were injected with the highest dose of ASP used (1,065 pmol) 90 min prior to euthanization. To differentiate the effect of ASP from the effect of refeeding, the rats were separated into two groups, one with ad libitum (AL) access to food following treatment and the other pair-fed (PF).

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**(P = not significant, ASP injected vs. pair-fed for both $O_2$ and RQ); however, there is a longer-lasting effect of ASP on RQ (Fig. 3F).**

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**Fig. 3.** Lower oxygen consumption and respiratory quotient (RQ) levels following an ASP injection. A–C: oxygen consumption was assessed for 48 h following the injection. Rats injected with ASP (A, $P < 0.05$; A) or pair-fed rats (B, $P < 0.01$; B) showed a lower oxygen consumption compared with CTRL aCSF rats (C: A and B) for the first but not for the last 24 h (C). D–F: energy substrate usage was measured for 48 h. ASP-injected rats (A, $P < 0.001$; D) and their pair-fed counterparts (B, $P < 0.001$; E) showed a marked decrease in RQ levels compared with aCSF-injected rats (C: D and E). F: area under the curve (AUC) analysis of the RQ curves for the 48 h following the injection. $V_{O_2}$, carbon dioxide production ($V_{CO_2}$), and RQ ($V_{CO_2}/V_{O_2}$) were measured over 48 h. Results are expressed as means ± SE; $n = 6–12$ rats/group. Significance was determined by 2-way ANOVA (A, B, D, and E) or ANOVA (C and F), where *$P < 0.05$ and **$P < 0.01$.**
the injection (as in the experiments above) and one with continued fasting [no food (NF) access]. Using in situ hybridization, analysis of NPY mRNA indicated that there was no change in the arcuate nucleus when the groups were examined altogether (control vs. ASP; Fig. 6A). When the data were analyzed with fed and fasted rats separately (maintained with NF or AL after the injection; Fig. 6C), the effect of refeeding was clear in both the control and ASP-injected groups, \( P < 0.05 \), but there was still no ASP effect. By contrast, results of POMC in situ hybridization indicate that the effect of central injection of ASP on food intake was promoted via the action of the neuropeptide POMC. As shown in Fig. 6B, a significant increase in mRNA levels of anorexigenic neuropeptide POMC in the arcuate nucleus was detected in ASP-injected rats compared with their control aCSF counterparts (44%, \( P < 0.05 \)). Furthermore, although the trend was evident in the AL rats, there was a greater difference (\( P < 0.01 \)) in the rats that were maintained fasting (Fig. 6D).

Effects of acute ASP icv injection on plasma parameters. Acute effects of ASP on plasma parameters in the ASP-injected rats under both conditions were also evaluated. As shown in Table 2, no significant differences were seen in plasma levels of insulin, leptin, or adiponectin between CSF and ASP in either NF or ad AL-fed animals, although the expected postprandial increase in insulin was noted. Also, central injection of ASP did not affect glucose plasma level (Table 2). Therefore, the differences in POMC expression and physiological parameters (food intake, weight gain, energy expenditure, and physical activity) are not likely related to acute changes in circulating hormones.

Presence of C5L2 in the rat brain. A subset of rats was used to localize C5L2 mRNA expression in the brain. Brains were harvested and cut in the cortex, hypothalamus, hippocampus, cerebellum, and hindbrain and evaluated by real-time PCR for C5L2 expression with evaluation of the spleen as positive control. As shown in Fig. 7, C5L2 mRNA is expressed at comparable levels in three of the five brain regions (cortex, hypothalamus, and cerebellum), with higher expression in the hindbrain and hippocampus areas.

DISCUSSION AND CONCLUSION

In the present study, we observed that an acute icv injection of ASP has effects on both energy intake and expenditure, substrate preference, physical activity, and weight gain by inhibiting acute and short-term feeding habits in rats. Also, our results showed that the most robust changes resulted from the injection of the highest amount of ASP. These effects were obtained at physiologically relevant concentrations of ASP, and not pharmacological doses, based on human CSF concentrations that are ~15-fold lower than plasma ASP (rat CSF concentrations are unknown). Interestingly, these results were not associated with any significant ASP-induced changes in leptin, insulin, or adiponectin plasma levels, all hormones that are known to influence energy metabolism, suggesting a process directly related to signaling by ASP, where the mechanism may involve POMC activation. It should be noted that although the ASP injection strongly reduced the feeding response, it did...
not induce malaise, since there was no development of a conditioned-taste aversion response, although an aversive dose of LiCl did, as expected.

The aim of the present study was to evaluate comprehensively a potential ASP effect on food intake and related parameters and to determine a mechanism by which the ASP-C5L2 pathway modulates food consumption. Previous initial studies evaluating icv administration of ASP or C3a (both of which bind the C5L2 receptor) effects on food intake provide limited data and are contradictory, with increased (42), decreased (32–34), and no changes (42, 45) in food intake reported. Our initial study demonstrated that icv administration of ASP (5 μg = 560 pmol) produced a small increase in food intake, with no effect over 24 h. At a higher dose (25 μg = 2,800 pmol), there was no effect at all (41). Unfortunately, no other parameters were evaluated, in contrast to the present study, which is much more robust. We can only speculate that the disparities may be explained in part by the use of ASP derived from plasma, which has been shown to be less bioactive than our current recombinant ASP (9). On the other hand, our results are consistent with those of Ohinata and colleagues (32–34), which demonstrated that centrally administered C3a (which binds both C5L2 and C3aR) dose dependently (10 –100 pmol/mouse) decreased food intake in mice.

This study complements our previous studies, which demonstrated that a deficiency of the ASP-C5L2 pathway leads to increased food intake and energy expenditure in ASP-(C3KO) and C5L2-deficient (C5L2KO) mice (29, 31, 36, 40, 55). Specifically, previous studies on the ASP-C5L2 pathway demonstrated that C3KO mice were hyperphagic (29, 31). Similar results were recently obtained in C5L2KO mice, which are ASP receptor-deficient mice (36). We also observed the influence of the ASP-C5L2 pathway on food

Table 2. Blood plasma values

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NF</th>
<th>AL</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, ng/ml</td>
<td>1.14 ± 0.07</td>
<td>0.78 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>0.78 ± 0.08</td>
<td>0.08 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>9.29 ± 1.19</td>
<td>7.23 ± 1.40</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>7.23 ± 1.40</td>
<td>1.40 ± 1.60</td>
<td>NS</td>
</tr>
<tr>
<td>Adiponectin, μg/ml</td>
<td>4.91 ± 0.46</td>
<td>4.01 ± 0.45</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4.01 ± 0.45</td>
<td>0.45 ± 0.56</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>207 ± 11</td>
<td>179 ± 10</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>179 ± 10</td>
<td>10 ± 2</td>
<td>NS</td>
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Values are means ± SE. AL, ad libitum; NF, no food; aCSF, artificial cerebrospinal fluid; NS, not significant. Note that there is no significant difference between AL and NF except for insulin (P < 0.001).

Fig. 6. ASP icv injection influences neuropeptide expression in the arcuate nucleus, as assessed by in situ hybridization. Following ASP injection, rats were provided free access to food [ad libitum (AL)] or maintained in the fasting state [no food (NF)]. A and C: the mRNA expression of neuropeptide Y (NPY) was not affected by acute central administration of ASP but was significantly lower in the group with AL access to food. B and D: the anorexigenic neuropeptide proopiomelanocortin (POMC) mRNA expression showed an increase in ASP- (black bar) compared with aCSF-injected rats (open bar). E: visualization by microscopy of POMC in situ hybridization; AL and NF groups with ASP and aCSF CTRL injection. Results are expressed as means ± SE; n = 8 rats/group. Significance was determined by 1-way ANOVA or t-test, where *P < 0.05 and **P < 0.01. ns, Not significant.
consumption in double-knockout ASP and leptin-deficient mice (C3−/−lep ob/ob). Surprisingly, the lack of ASP in ob/ob mice further aggravated the hyperphagia of the ob/ob mice (54).

In summary, our previous studies support the hypothesis that mice lacking ASP (C3KO) or C5L2 (C5L2KO) signaling displayed increased energy intake via a non-leptin-mediated mechanism.

In addition to reduction in food intake, decreased energy expenditure and locomotor activity were also noted. Since food intake is a strong regulator of energy metabolism (4, 5, 51), we used aCSF-injected, pair-fed control littersates to potentially dissociate the effects of ASP on energy expenditure with those of food intake. Results demonstrated a comparable decrease in oxygen consumption and similar changes in RQ in the ASP-injected vs. aCSF pair-fed group, suggesting that the effects of centrally administered ASP on energy expenditure could simply be a consequence of the decrease in food intake. However, a recent study on chronic (3 wk) subcutaneous delivery of ASP or antibody to ASP using minipumps in mice demonstrated changes in energy expenditure (decreased and increased, respectively) without any substantial change in food intake (35). Furthermore, changes in muscle and adipose tissue function could be directly linked to an ASP mechanism. This suggests that ASP-mediated food intake and energy expenditure changes are not necessarily mediated via the same mechanism and that peripheral vs. central or short-term vs. long-term effects may be dissimilar.

Locomotor activity levels are another component of energy expenditure that is directly affected by energy intake. Both ASP-injected rats and their pair-fed counterparts showed a decrease in total distance and total activity in the 24-h period following the injection. However, ASP administration was more potent as a long-term inhibitor of voluntary movement than food restriction, as shown by the extended effect of ASP. We speculate that this might be due to the relatively high presence of C5L2 in multiple key areas of the brain, some of which control food search and locomotor activity (23), as suggested by our RT-PCR analysis of the brain. It is also noteworthy to mention that C5L2 is found to be expressed in motoneurons (52), and this is an area that needs to be explored in future studies.

The ivc effects of ASP are similar, in part, to those of leptin (15, 44) and insulin (50), where acute administration inhibits food intake in the first 24 h, mediated through stimulation of POMC gene expression in the arcuate nucleus, acting through the central melanocortin system (44, 47, 56). Why, in the case of ASP, this does not lead to increased energy expenditure, as with leptin and insulin, will require examination of downstream activation parameters.

In recent years, many peripheral signals have been demonstrated to contribute to feeding behavior and body weight regulation, and it should be recognized that both short-term and long-term food intake and energy balance (storage and energy expenditure) are regulated through distinct but interacting mechanisms. Some short-term signals (such as nutrients and gastrointestinal hormones) act primarily as determinants of satiety. The list comprises members of the gastrointestinal hormone family glucagon-like peptide-1 (27, 49), oxyntomodulin (11, 12), pancreatic polypeptide, and others (16), and these have a markedly different function than long-term regulators, such as insulin (50) and the adipokine leptin (7, 14), that are related to both body adipose stores and energy consumption over a more prolonged period of time.

Our observations presented here demonstrate that ASP can now be added to this list of centrally active hormones. Whether the ASP is generated peripherally or centrally, and is primarily a short-term or a long-term regulator, remains to be determined. In humans, plasma ASP has been shown to change acutely, with increased intra-adipose tissue produced postprandially, albeit without changing general circulating levels in the short term (21, 43, 48). Chronically increased circulating plasma ASP is associated with obesity, metabolic syndrome, insulin resistance, type 2 diabetes, cardiovascular disease, and polycystic ovary disease (8). Whereas ASP correlates with adiposity, increased ASP is noted in metabolic disturbances even without obesity (53, 57).

New data is emerging, suggesting an active role of tanycytes, cells lining the third ventricle, in controlling the movement of blood-borne molecules to the CSF and also from the CSF to the arcuate nucleus (28, 38). It is tempting to speculate that chronically increased plasma ASP may lead to increased central ASP (as with insulin and leptin), and if so, this could induce a similar state of ASP resistance, as is demonstrated with insulin resistance and leptin resistance. On the other hand, the brain has the capacity to produce ASP directly; however, when, where, and why this might occur is unknown. In summary, these results suggest that ASP may have central in addition to peripheral effects.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


