Evaluation of the lack of anorectic effect of intracerebroventricular insulin in rats

Jessen L, Clegg DJ, Bouman SD. Evaluation of the lack of anorectic effect of intracerebroventricular insulin in rats. Am J Physiol Regul Integr Comp Physiol 298: R43–R50, 2010. First published October 28, 2009; doi:10.1152/ajpregu.90736.2008.—Insulin detemir is a novel human insulin analog that does not show the usual propensity for weight gain in diabetic patients. We speculated that this beneficial effect could be due to insulin detemir exerting stronger anorectic effects within the brain than other insulins. To study the central effects of regular human insulin and insulin detemir on food intake, the present study was undertaken. We used acute intracerebroventricular insulin injections to compare food intake and body weight in rats fed ad libitum. Contrary to previously published data, we found that neither regular human insulin (8 or 32 mU) nor insulin detemir (1,290 pmol) reduced food intake in this model. Melanotan-II was also injected intracerebroventricularly as a positive control, and significantly reduced food intake and body weight, suggesting that our intracerebroventricular model is able to show anorectic effects. A series of experiments was therefore conducted in which different set-ups were tested to investigate which factors would be required to produce the reported anorectic effect of intracerebroventricular insulin. Although we varied rat strain, stereotactic coordinates, formulations of insulin and vehicle, dose, volume, and time of injection, the anorectic effect of intracerebroventricular insulin could not be replicated. Therefore, we suggest that acute intracerebroventricularly injected insulin does not robustly inhibit food intake in rats. Based on our results, the acute intracerebroventricular injection procedure may not be a preferred method for studying the central anorectic effects of insulin in rats. Instead, administrations over time or locally in hypothalamic nuclei might be recommended.

Insulin therapy; food intake; body weight; brain insulin; insulin detemir

BODY WEIGHT GAIN is a common problem in diabetic patients treated with insulin (8, 25). Insulin-associated weight gain can be substantial and is often of clinical concern, especially in patients with type 2 diabetes. These patients are usually overweight at diagnosis, and further weight gain is undesirable because it increases diabetic morbidity and mortality (25, 29).

Insulin detemir (Levemir, Novo Nordisk, Denmark) is a novel long-acting human insulin analog; it is acylated with a fatty acid that enables reversible albumin binding in interstitial fluid and circulation. The binding of insulin detemir to albumin causes the delayed time-action profile (10). Several clinical studies have shown that insulin detemir compared with NPH (Neutral Protamine Hagedorn) insulin leads to less weight gain at equivalent levels of glycemic control in both type 1 and type 2 diabetes (12). This finding is interesting because it suggests some intriguing putative mechanisms whereby weight gain with insulin therapy might be limited. Thus elucidation of the mechanism underlying the beneficial weight effect of insulin detemir might provide valuable insights into the ways insulin influences weight both normally and in insulin-treated diabetics.

It has been speculated that the altered effect of insulin detemir on body weight could be mediated via actions in the brain (11, 24). Endogenous insulin is believed to act as a central negative feedback signal in the long-term regulation of food intake and body weight (22). Consistent with this hypothesis, insulin is secreted in plasma in direct proportion to body fat and is transported from plasma in the brain where it acts on insulin receptors in the hypothalamus and other brain areas (28). It has been shown that chronic infusion of exogenous insulin directly in the hypothalamic tissue or the cerebral ventricles reduces food intake and body weight in animals (4, 13, 16, 27). In addition, some studies have shown that acute injection of insulin in the third cerebral ventricle also may reduce food intake and body weight in rats (1, 5, 7). Keeping in mind that insulin detemir therapy does not show the usual propensity for weight gain, we speculated that insulin detemir could exert more pronounced anorectic effects in the brain than other insulins. Insulin detemir could perhaps more potently induce hypothalamic insulin signaling, or it could for instance, because of its acylated nature, enter the brain at a higher rate than other insulins, which has also been speculated by others (11, 24). Evidence in support of this theory is lacking. To date, there is no literature on the mechanisms by which insulin detemir may affect food intake and body weight. However, some studies in mice and humans have suggested that insulin detemir might reach or affect the brain to a greater extent than regular human insulin (11, 24). Therefore, our main interest was to investigate whether insulin detemir may serve as a stronger central regulator of food intake and body weight. For this purpose, we decided to use the acute intracerebroventricular insulin injection paradigm because this model had been used in previously published brain insulin studies and is relatively easy to perform.

To this end, we had to establish the intracerebroventricular insulin model described in the literature, with acute central administration of insulin suppressing food intake and body weight in rats (1), with the perspective of using this model to study insulin detemir. In study I, regular human insulin and insulin detemir were injected in the third cerebral ventricle to investigate their effects on food intake and body weight in rats fed ad libitum. In this study, contrary to the previously published data, acute intracerebroventricularly injected regular human insulin did not induce an anorectic effect. Therefore, a series of subsequent experiments (study II) was performed to...
explore the experimental conditions that might influence intracerebroventricularly injected insulin’s actions in the brain to regulate food intake and body weight.

**MATERIALS AND METHODS**

**Study I**

The purpose of study I was to establish the model of intracerebroventricularly injected regular human insulin reducing food intake and body weight in rats, and use this model to study the effects of insulin detemir.

**Animals.** Male Long Evans rats obtained from Taconic (Germantown, NY) were housed individually in a climate controlled room under a 12:12-h light-dark cycle (lights on from 2200 to 1000) in the Novo Nordisk animal facilities (Maaloev, Denmark). Standard pelleted chow (Altromin 1324, Brogaarden, Denmark) and drinking water were available ad libitum. All procedures were approved by the Danish Animal Experiments Inspectorate, Ministry of Justice, Denmark.

**Surgery.** Under isoflurane/N2O anesthesia, all rats were stereotactically implanted with a 22-gauge stainless steel guide cannula (C313G, Plastics 1; Bilaney Consultants, Düsseldorf, Germany) aimed at the third ventricle. The guide cannula was lowered at a 10° angle (lateral to medial) in the brain; the stereotactic coordinates were 1.0 mm posterior to bregma, 1.5 mm lateral to the sagittal suture, and 7.5 mm ventral from the skull surface (20). The rats received analgesic (2 mg carprofen sc; Rimadyl vet 50 mg/ml; Orion Pharma, Nivaa, Denmark) and antibiotic (sulfadifen 40 mg sc, trimethoprim 8 mg sc, Tribriessen vet 24%; Schering-Plough, Ballerup, Denmark) on the day of surgery and the two following days. The recovery period was at least 1 wk. During this period, the rats were handled each day and also habituated to the injection procedure by removing and inserting their dummy cannula (obturater). Rats were only used for experiments after they had regained their preoperative body weight.

**Verification of brain cannula placement.** After the study, cannula placement was verified by histological examination of the brains. Under anesthesia (isoflurane/N2O), each rat received an intracerebroventricular injection of 3 μl black ink (Drawing Ink A; Pelikan, Hannover, Germany). Later (5 min), the rats were killed; brains were removed and frozen. A microtome was used to section the brains (20 μm) coronally. Following contrast staining with thionin, the brain slices were examined under a microscope. Rats that did not have ink placements was verified by histological examination of the brains. Under anesthesia (isoflurane/N2O), each rat received an intracerebroventricular injection of 3 μl black ink (Drawing Ink A; Pelikan, Hannover, Germany). Later (5 min), the rats were killed; brains were removed and frozen. A microtome was used to section the brains (20 μm) coronally. Following contrast staining with thionin, the brain slices were examined under a microscope. Rats that did not have ink

**Experimental protocol.** At the beginning of study I, rats weighed 316 ± 5 (SE) g. Four experiments were performed. In experiments IA and IB, human insulin (100 IU/ml Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was diluted in vehicle and injected intracerebroventricularly in doses of 8 or 32 mU in 3 μl (for human insulin, 1 mU corresponds to 6 pmol). In experiment IC, melanotan-II (MT-II) (in-house synthesis; Novo Nordisk, Maaloev, Denmark) dissolved in vehicle was injected intracerebroventricularly in a dose of 294 pmol in 3 μl. MT-II is a synthetic melanocortin receptor agonist known to inhibit food intake; it was used as positive control (23). Finally, in experiment ID, an intracerebroventricular injection of 1,290 pmol insulin detemir (in-house production; Novo Nordisk) diluted in vehicle (3 μl) was given. The insulin detemir dose was chosen because of the observed dosage difference in rats between insulin detemir and human insulin after peripheral administration (9). In each experiment, control rats received an intracerebroventricular injection of vehicle (3 μl). Vehicle consisted of a weak sodium phosphate buffer (2 mM) and NaCl (140 mM); pH was 7.4–7.7. At least 1 wk was allowed between the experiments.

Before each experiment, food intake during a baseline period (i.e., noninjection days) was measured over three consecutive days. Rats were randomly assigned to drug and control groups matched with respect to body weight and baseline food intake. In all four experiments, the experimental protocol of Air et al. (1) was used. On experimental days, food was removed at 0900, and the rats received an intracerebroventricular injection of drug or vehicle. The injection was given 1 h before lights off because food intake in rats mainly takes place during the dark phase. The injection was performed by holding each rat and replacing the obturator by an injector cannula (C313L, Plastics 1; Bilaney Consultants, Düsseldorf, Germany) that extended 1 mm beyond the guide cannula. The total volume (3 μl) was administered in the third ventricle in intervals of 1 μl/10 s. After the injection, the injector cannula was left in place for an additional 30 s to allow the injected solution to move away from the cannula tip. The rats were then returned to their cages. Food was returned at the onset of dark (at 1000) and intake was measured after 4, 6, and 24 h.

After the experiments, the concentration of the two insulin solutions was confirmed using high-performance liquid chromatography (HPLC). This analysis was done to verify that the rats had received the intended insulin dosages. The insulin was identified by HPLC to be intact and thereby biologically active insulin.

**Data analysis.** Data are presented as means ± SE. Food intake differences between groups (vehicle vs. regular human insulin, insulin detemir, or MT-II) were analyzed using Student’s two-tailed, unpaired t-test. Food intake differences between baseline values and vehicle, human insulin, insulin detemir, or MT-II were done using Student’s two-tailed, unpaired t-test. Percent change (over 24 h) in body weight during baseline and after administration of vehicle or drug was calculated, and differences between groups (vehicle vs. human insulin, insulin detemir, or MT-II) were evaluated using Student’s two-tailed, unpaired t-test. Percent change (over 24 h) in body weight between baseline values and vehicle, human insulin, insulin detemir, or MT-II were analyzed using Student’s two-tailed, unpaired t-test. All analyses were performed using GraphPad Prism, version 5.00, 2007 (GraphPad Software, San Diego, CA). P < 0.05 was considered statistically significant.

**Study II.**

Because study I did not show reduced food intake after intracerebroventricular injection of regular human insulin, the purpose of study II was to investigate experimental conditions previously demonstrated for intracerebroventricularly injected insulin to induce an anorectic effect.

**Animals.** Study II included experiments at Novo Nordisk in Maaloev, Denmark, with Sprague Dawley rats (Charles River, Sulzfeld, Germany), as well as experiments at the University of Cincinnati, Cincinnati, OH, with Long Evans rats (Harlan, Indianapolis, IN). The procedures were approved by the Danish Animal Experiments Inspectorate, Ministry of Justice, Denmark or the Internal Animal Care and Use Committee at the University of Cincinnati. For the experiments at Novo Nordisk (study IIa), the animal housing conditions were the same as in study I, with standard pelleted chow (Altromin 1324; 53% carbohydrates, 19% protein, 5% fat) and tap water ad libitum. The rats used in the experiments at the University of Cincinnati (study IIb) were housed individually and were also maintained in a climate-controlled room on a 12:12-h light-dark cycle, where lights were on from 0400 to 1600. The rats received standard pelleted chow (Harlan Teklad 7012; 54% carbohydrates, 20% protein, 6% fat) and tap water ad libitum.

**Surgery.** Two different sets of stereotactic coordinates were used. The rats in studies IIa 1–5 underwent stereotactic surgery as described in study I; the guide cannula was positioned in the third cerebral ventricle at an angle. The rats in studies IIb 1–4 were implanted with guide cannulas that were inserted vertically in the third cerebral ventricle. This procedure demanded that a 9- to 12-mm² skull window was outlined using a drill and removed with forceps. The sagittal sinus was displaced laterally as the guide cannula was lowered vertically in the brain. The stereotactic coordinates were as follows: 2.2 mm posterior to bregma; 7.5 mm ventral to dura; and on the midline (1).
After surgery, all rats were housed individually. The recovery period was at least 1 wk. During this period, the rats were handled each day and also habituated to the injection procedure by removing and inserting their dummy cannula (obturator). Rats were only used for experiments after they had regained their preoperative body weight.

**Verification of brain cannula placement.** Before the experiments, cannula placement was verified by performing the ANG II test. During the light phase, all rats were injected intracerebroventricularly with ANG II (10 ng/rat; Sigma-Aldrich) and observed for a positive dipogenic response. Rats that failed to drink at least 5 ml of water within 60 min were excluded from the data analyses. In studies IIA–I, cannula placement was additionally verified by histological examination of the brains. This was done as described in study I.

**Experimental protocol.** In study II, we performed a series of experiments searching for the optimal condition that would enable the anorectic effect of intracerebroventricularly administered insulin. To this end, we investigated the following conditions: rat strain, stereotactic coordinates, formulation of insulin and vehicle, insulin dose, injection volume, and time of intracerebroventricular injection. In total, nine experiments were performed. Before each experiment, rats were randomly divided into groups. At least 1 wk was allowed between the experiments. The different procedures used in the nine experiments are described in Table 1.

Briefly, study IIA followed the experimental protocol of study I with the following three adaptations: as vehicle we used artificial cerebrospinal fluid [aCSF, composed of a weak sodium phosphate buffer (2 mM NaCl, 3.1 KCl, 1.3 CaCl2, and 1.2 MgCl2; pH 7.4)]; the injection volume was 3, 5, or 6 μl; and regular human insulin was injected in doses of 4, 8, 13, 16, or 32 mU.

In study IIB, the following two different insulin solutions and corresponding vehicles were tested: 1) human insulin (Humulin Regular 100 IU/ml; Eli Lilly, Indianapolis, IN) diluted with saline; and 2) chemically pure human insulin (in-house production; Novo Nordisk) dissolved in vehicle [composed of a weak sodium phosphate buffer (2 mM) and NaCl (100 mM); pH 7.7]. The insulin dosage was 10 mU in 1 μl. On the experimental day, rats were weighed and injected intracerebroventricularly with insulin or vehicle 6 h before lights off. At the same time, their food was removed, to avoid accidental meals until the start of the dark period. Food was returned at the onset of dark (i.e., at 1600), and food intake was measured after 4 and 24 h. This was the standard experimental set-up at the University of Cincinnati laboratory, where these experimental conditions for acute intracerebroventricular insulin previously had been able to inhibit food intake.

**Assessment of insulin loss in the injection system.** Like most proteins, insulin has a tendency to adhere to surfaces. Thus, to verify that the rats received the intended insulin dosages, the injection system was validated. The intracerebroventricular injection system consisted of a 50-μl Hamilton glass microsyringe connected by Tygon tubing to an injector cannula (C313L, Plastics 1; Bilaney Consultants, Düsseldorf, Germany). The microsyringe was prefilled with sterile water; the tubing and injector cannula were filled with a known volume of insulin solution (separated from the sterile water by a small air bubble). The insulin solution was prepared by diluting 100 IU/ml Actrapid (Novo Nordisk) with aCSF to a final concentration of 16 μM; the concentration of intact insulin was confirmed using HPLC. This concentration was chosen because it was identical to the lowest insulin concentration used in study I. The tubing was then allowed to soak in the insulin solution for 2 min. Thereafter, the insulin solution was injected in a glass vial containing a known volume of buffer solution (5 mM sodium phosphate buffer, 100 mM NaCl, 0.007% polysorbate 20, pH 7.7); this buffer solution prevented insulin from adhering to the glass surfaces of the vial. The intact insulin concentration in the glass vial was measured using HPLC, and the loss of insulin in the injection system was then calculated. This method of validation mimicked the injection procedure used in both study I and study II.

**Data analysis.** Data are presented as means ± SE. Within each of the nine experiments, food intake differences between groups (vehicle vs. insulin) were analyzed using Student’s two-tailed, unpaired t-test. To further study variation size between and within treatments, the data from the nine experiments were then combined (pooled), and identified as the following seven data sets: one data set with all baseline data; three vehicle data sets (saline, buffer, and aCSF); and three insulin data sets [low dose (<10 μM), medium dose (10–20 μM), and high dose (>20 μM)]. Percent change (over 24 h) in body weight during baseline and after administration of vehicle or insulin was calculated and expressed in accordance with the above-mentioned seven groups. Analyses on the pooled data (comparisons between the means of the baseline data set, the vehicle data sets, and the insulin data sets) were done using one-way ANOVA followed by Tukey’s posttest. All analyses were performed using GraphPad Prism, version 5.00, 2007 (Graph Pad Software). *P < 0.05 was considered statistically significant.

Table 1. Experimental designs of study II

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Study IIA</th>
<th>Study IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test facility</td>
<td>Male Sprague Dawley (two cohorts, n = 26)</td>
<td>Male Long Evans (three cohorts, n = 71)</td>
</tr>
<tr>
<td>Site of icv. injection, mm</td>
<td>Posterior 1.0, ventral 8.5, lateral 1.5, angle 10°</td>
<td>Posterior 2.2, ventral 8.5, on the midline</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>440 ± 5</td>
<td>354 ± 3</td>
</tr>
<tr>
<td>Light-dark cycle, h</td>
<td>2200–1000</td>
<td>0400–1600</td>
</tr>
<tr>
<td>Time of injection and removal of food</td>
<td>1 h before dark period</td>
<td>6 h before dark period</td>
</tr>
<tr>
<td>Insulin and vehicle solutions</td>
<td>Human insulin (Actrapid) dilute in aCSF</td>
<td>Two different insulins: 1) Human insulin (Humulin Regular) dilute in saline 2) Human insulin in phosphate buffer</td>
</tr>
<tr>
<td>Injection volume, μl</td>
<td>3, 5, or 6</td>
<td>1</td>
</tr>
<tr>
<td>Experiments</td>
<td>IIA-1) Cohort 1, two groups: 4 μl insulin (in 5 μl); vehicle IIA-2) Cohort 1, two groups: 8 μl insulin (in 5 μl); vehicle IIA-3) Cohort 2, two groups: 13 μl insulin (in 6 μl); vehicle IIA-4) Cohort 2, two groups: 16 μl insulin (in 6 μl); vehicle IIA-5) Cohort 2, two groups: 32 μl insulin (in 6 μl); vehicle</td>
<td>IIB-1) Cohort 1, two groups: 10 μl insulin A; vehicle A IIB-2) Cohort 1, two groups: 10 μl insulin B; vehicle B IIB-3) Cohort 2, two groups: 10 μl insulin A; vehicle A IIB-4) Cohort 3, four groups: 10 μl insulin A; 10 μl insulin B; vehicle A; vehicle B</td>
</tr>
<tr>
<td>Verification of guide cannula placement</td>
<td>ANG II test</td>
<td>ANG II test</td>
</tr>
</tbody>
</table>

aCSF, artificial cerebrospinal fluid. *100 IU/ml Actrapid (Novo Nordisk, Bagsvaerd, Denmark). †100 IU/ml Humulin Regular (Eli Lilly & Company, Indianapolis, IN). ‡Chemically pure human insulin (in-house production, Novo Nordisk). The actual concentration of these solutions was confirmed using HPLC.
RESULTS

Assessment of Insulin Loss in the Injection System

The injection system was validated to verify that the rats received the intended insulin doses. The injection system was filled with 16.18 μM insulin. The insulin concentration of the solution flowing from the injection system was 14.94 μM. Therefore, the loss of insulin was only 1.24 μM, i.e., for this insulin concentration 7.7%.

Study I

The purpose of study I was to establish the acute intracerebroventricular insulin injection model by reproducing the central inhibitory effects of insulin on food intake and body weight in rats fed ad libitum (as reported in the literature) and to use this model to study insulin detemir. The reference compound MT-II was used as positive control. Two rats were excluded from the data analyses because of incorrect brain cannula placement.

The effects on food intake and body weight are depicted in Figs. 1 and 2. There were no significant differences in 4, 6, or 24 h food intake between rats intracerebroventricularly injected with 8 or 32 mU regular human insulin and the rats injected with vehicle (Fig. 1, A and B). In addition, no differences in body weight changes were observed between the insulin- and vehicle-treated rats (Fig. 2). Food intake and body weight changes in vehicle- or insulin-treated rats did not differ significantly from baseline values either (Fig. 1, A and B). Thus regular insulin did not have an anorectic effect.

As depicted in Fig. 1C, intracerebroventricular administration of MT-II (294 pmol) significantly reduced food intake at 4 (P = 0.0015) and 6 (P = 0.0003) h compared with vehicle. The anorectic effect persisted and remained significant after 24 (P = 0.0001) h. Consequently, the rats treated with MT-II also significantly lost body weight (P = 0.0021) during the 24 h after injection compared with the vehicle-treated rats (Fig. 2). MT-II significantly reduced food intake compared with baseline values as well, at 4 (P = 0.0001), 6 (P = 0.0001), and 24 (P = 0.0001) h (Fig. 1C). Likewise, whereas normal untreated rats always gain weight, MT-II caused a significant reduction in body weight changes relative to baseline values (P = 0.0001) (Fig. 2).

There were no significant differences in 4, 6, or 24 h food intake between rats intracerebroventricularly injected with 1,290 pmol insulin detemir and rats injected with vehicle (Fig. 1D). Likewise, no differences in body weight changes were observed between groups (Fig. 2). Insulin detemir did not cause a significant reduction in food intake and body weight compared with baseline values either. Therefore, insulin detemir did not induce an anorectic effect in this model.
be observed that intracerebroventricular injection per se had an anorectic effect (Fig. 3A), since both rats injected with buffer vehicle and rats injected with the medium dose insulin (10–20 mU) significantly reduced their food intake compared with baseline (P = 0.0001). Likewise, during baseline, rats normally gain body weight, as we also observed in our study (Fig. 3B), but intracerebroventricular injection caused a significant loss of body weight, independent of the solution administered (insulin or vehicle) (P = 0.0001). There were no significant food intake differences between the vehicle and insulin groups (Fig. 3A). The 24 h body weight changes differed significantly between the saline group and the insulin medium dose (10–20 mU) group. There were no significant differences in 24 h body weight changes between any of the other vehicle and insulin groups. To summarize, in the experiments in study II, acute intracerebroventricular injections of insulin did not inhibit food intake to a greater extent than vehicle solutions.

DISCUSSION

In study I, we aimed at reproducing the data in the literature reporting that insulin injected in the third cerebral ventricle inhibits food intake in rats fed ad libitum (1). In our hands, however, intracerebroventricular injection of neither regular human insulin nor insulin detemir resulted in an anorectic effect (Figs. 1 and 2). The reason for this discrepancy is unclear.

To validate whether our setup indeed is able to show food intake effects, MT-II was used as a reference compound. MT-II is a synthetic melanocortin receptor agonist known to inhibit food intake in rats after intracerebroventricular administration (23). MT-II indeed strongly induced a significant anorectic effect (Figs. 1C and 2), confirming the reliability of our model studying food intake after intracerebroventricular injections. Study I followed the experimental protocol of Air et al. (1); in that paper, 4 and 24 h food intake in rats were suppressed by injecting insulin intracerebroventricularly in doses of 4 and 8 mU. In our study, injection of insulin in the same dose range (8 mU) did not affect 4, 6, or 24 h food intake in the rats (Fig. 1). Even injection of a four times higher insulin dose (32 mU) did not reveal an anorectic effect (Fig. 1). A potentially greater insulin loss in our injection system cannot explain this difference. We estimated the loss of insulin to only 7.7%, which means that the rats in our study most likely received the intended insulin dosages. In addition, the concentrations of the two insulin solutions we used for the intracerebroventricular injections were verified by HPLC after the experiments. Injection of insulin detemir did not affect food intake, but, because

Study II

The objective of study II was to attempt to replicate the experimental paradigms previously published indicating that acutely injected intracerebroventricular insulin reduces food intake and body weight. This might also clarify why insulin injected intracerebroventricularly did not reduce food intake in study I. Therefore, we varied different experimental factors such as rat strain, stereotactic coordinates, formulation of insulin and vehicle, insulin dose, injection volume, and time of intracerebroventricular injection. Ten rats were excluded from the data analyses because of incorrect brain cannula placement. Initially, the data of each of the nine experiments were analyzed separately (Supplemental Figs. 1 and 2; supplemental data for this article are available online at the American Journal of Physiology: Regulatory, Integrative and Comparative Physiology website). This did not enable us to conclude which experimental factors were of importance for the anorectic effect of insulin, because insulin did not reliably reduce food intake and body weight in the rats. Only in one of the nine experiments did food intake and body weight of insulin-treated rats decrease significantly compared with vehicle-treated rats. This effect was observed after intracerebroventricular injection of 10 mU insulin in Long Evans rats (experiment IIB-1). However, replication of the experiment (experiment IIB-3) did not reveal any anorectic effect. Because some increased variation within groups after injection with vehicle or insulin was suspected, we decided to pool all data and analyze whether there were overall changes in variation that could mask any anorectic effect of insulin. Table 2 shows the pooled groups’ averages and variation coefficients, suggesting that variation within all groups indeed increased after intracerebroventricular injection, regardless whether this was with vehicle or with insulin. Figure 3 shows the 24 h food intake and the 24 h body weight changes of the pooled groups after intracerebroventricular injection of different vehicle and insulin solutions. It can be observed that intracerebroventricular injection per se had an effect (Figs. 1A and 2), confirming the reliability of our model studying food intake after intracerebroventricular injections.
LACK OF ANORECTIC EFFECT OF INTRACEREBROVENTRICULARLY INJECTED INSULIN

of the lack of effect of regular insulin, no conclusions can be made comparing insulin detemir with regular insulin.

To understand why regular human insulin injected intracerebroventricularly did not suppress food intake in study I, a series of experiments (study II) was conducted in which different experimental paradigms were tested to investigate which factors are required to produce the anorectic effect of insulin. Although we varied rat strain, stereotactic coordinates, the time of intracerebroventricular injection, insulin doses, injection volumes, and insulin and vehicle formulations, insulin did not induce robust anorectic effects across the experimental paradigms tested. Food intake and body weight decreased significantly after insulin administration in only one of the nine experiments in study II, and this effect could not be reproduced. Furthermore, the pooled data only revealed a significant difference in body weight changes between the saline vehicle group and the insulin medium dose (10–20 mU) group (Fig. 3B). There were no significant food intake differences between any of vehicle and insulin groups (Fig. 3A).

Because none of the experimental procedures employed in either laboratory resulted in reproducible anorectic effects of insulin, it is unlikely that any of the differences between the various methods (including animal characteristics such as rat strain, age, body weight and insulin sensitivity, food type, and fasting period) can explain the lacking anorectic effect of insulin. Our main conclusion is therefore that intracerebroventricularly injected insulin with neither of the different experimental methods employed induces a robust anorectic signal in rats.

In both studies I and II, intracerebroventricular injection per se might have affected the rats, even though they were habituated to the experimental conditions. This was reflected by reductions in food intake relative to baseline and/or loss of body weight (<1–3% weight loss, while rats during baseline periods gain weight). These injection effects were independent of whether insulin or vehicle solutions were administered. In line with our observations, both vehicle- and insulin-treated rats in the study of Air et al. (1) lost body weight (≈2–5%) after intracerebroventricular administration. Despite potential injection stress, that study still found an anorectic effect of insulin. Consequently, injection stress is unlikely to have abolished an anorectic effect of insulin in our study. In this context, it should be emphasized that the rats in study I seemed to be least affected by the injection procedure (food intake and body weight were not significantly reduced compared with baseline values); nevertheless, insulin had no effect. We also observed increased variation within both vehicle and insulin groups after intracerebroventricular injection, but this can only have masked very subtle potential effects of insulin. Furthermore, MT-II injected intracerebroventricularly induced a clear anorectic effect in our study, despite that intracerebroventricular injection per se may affect rats.

The major finding in our study is that regular human insulin injected in the third cerebral ventricle does not reliably inhibit food intake in rats fed ad libitum. Because experimental procedures and injection stress cannot explain the lack of insulin’s anorectic effect, it may be related to the route of insulin administration. Perhaps the intracerebroventricular route of administration is an inefficient way of insulin delivery to target sites within the brain (17). In keeping with this, no conclusive evidence has shown that the inputs to the insulin-mediated central regulation of food intake actually arise from insulin present in CSF. The inputs could arise from insulin transported across the epithelium of the blood-brain barrier in the brain interstitial fluid and/or from insulin transported in blood-brain microvessels through circumventricular organs that are devoid of a blood-brain barrier (14). If the latter hypothesis is true, insulin administered in the third cerebral ventricle could have difficulty in penetrating the brain parenchyma, thereby giving a reduced or no anorectic effect. This is supported by a study of McGowan et al. (15); these authors showed that chronic infusion of insulin in the third cerebral ventricle was less effective in reducing food intake and body

Fig. 3. Twenty-four-hour food intake (A) and body weight changes (B) during baseline and after ivc injection of vehicle solutions [△, saline, buffer, or artificial cerebrospinal fluid (aCSF)] or insulin solutions [●, low dose (<10 mU), medium dose (10–20 mU), or high dose (>20 mU)]. P < 0.05 vs. saline (one-way ANOVA, Tukey’s posttest) (#) and vs. baseline values (one-way ANOVA, Tukey’s posttest) (*)

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weight in rats than infusion of the same dose directly in the hypothalamic tissue. In addition, van Dijk et al. (26) reduced food intake in rats with intrahypothalamic injection of insulin in a dose almost a thousand times below the intracerebroventricular doses used in the study of Air et al. (1). It could also be that insulin, when it is administered by one single bolus intracerebroventricular injection, does not remain in the target location long enough to equilibrate its transport in the brain tissue and elicit an anorectic effect (17).

Because the original studies (1, 7) cannot easily be reproduced, this could lead one to question the putative role of insulin as a central regulator of food intake. However, the intracerebroventricular route of insulin administration and the insulin dosages cannot be considered physiological. Therefore, our data suggesting a lack of an anorectic effect of intracerebroventricular insulin does not eliminate the possibility that endogenous insulin plays a physiological role in the inhibition of food intake. Evidence to support this role for circulating insulin is found in a study conducted in insulin-receptor knockout mice showing that mice with brain-specific disruption of the insulin-receptor gene experienced hyperphagia and body weight gain (3). Furthermore, chronic intracerebroventricular infusion (7 days) of an antisense oligodeoxynucleotide designed to blunt the expression of insulin-receptor protein in the hypothalamus was accompanied by hyperphagia and increased fat mass in rats (18). Interestingly, it was chronic changes in insulin action within the brain that led to the observations reported in those two studies. This might imply that endogenous insulin predominantly regulates body weight on the long term. In support of this, multiple studies have shown that chronic infusion of insulin directly in the brain reduces food intake and body weight in animals (4, 13, 16, 27). In addition, repeated intracerebroventricular injections (over 72 h, 7 injections were given) of insulin in starved rats have been shown to affect the expression of hypothalamic neuropeptides (mRNA neuropeptide Y, mRNA proopiomelanocortin) involved in the regulation of food intake and body weight (2, 21). In contrast to the chronic studies, only few studies have suggested that acute intracerebroventricular administration of insulin has similar effects (1, 5, 7), and these results are controversial because of conflicting results in the literature (6, 19).

The present study was undertaken because we speculated whether insulin detemir could exert more pronounced anorectic effects within the brain than other insulins. An enhanced central insulin action might explain why insulin detemir results in less body weight gain in diabetic patients. Because our findings show that acutely intracerebroventricularly injected human insulin does not consistently reduce food intake and body weight in ad libitum-fed rats, no conclusions can be made as to whether insulin detemir affects food intake to the same or a greater extent than human insulin in rats. However, further research should be done to elucidate the association between insulin detemir and reduced body weight gain. A better understanding of the mechanism underlying the beneficial weight effect of insulin detemir might promote a new concept for insulin therapy in diabetes.

**Perspectives and Significance**

Our results show that regular insulin acutely injected in the third cerebral ventricle does not reliably inhibit food intake in rats fed ad libitum. We furthermore evaluated experimental factors that could influence central insulin’s anorectic effect in this model. Based on this finding that previous studies are difficult to reproduce, the acute intracerebroventricular injection procedure may not be a preferred method for studying the central anorectic effect of insulin in rats fed ad libitum. Instead, chronic administrations in the third ventricle or injections directly in the target areas could be preferred, since such methods may not suffer from the aforementioned potential confounding factors of handling stress or pharmacokinetic issues, and furthermore may be closer to the physiological role of insulin as long-term regulator of body weight.

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**DISCLOSURES**

No conflicts of interest are declared by the authors.

**REFERENCES**


