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Influence of high-fat feeding, diet-induced obesity, and hyperamylinemia on the sensitivity to acute amylin

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Abstract: Obesity results in the increased secretion of various hormones controlling food intake and body weight, such as leptin, and insulin; increased circulating levels of pancreatic amylin have also been described in obese humans and rodents. Because leptin-resistance is present in diet-induced obese (DIO) rats, and because hyperleptinemia seems necessary for the full development of leptin resistance, we tested whether amylin sensitivity is inversely correlated with adiposity, such that DIO reduces the anorectic action of acute amylin. We also determined if hyperamylinemia leads to a change in amylin sensitivity. In the first experiment, rats were chronically exposed to a high fat (HF; 60% fat) diet or fed standard chow for control. The anorectic response to amylin was tested on several occasions over a 14 week observation period. HF feeding led to the expected increase in body adiposity; the response to an acute amylin injection (5-50 g/kg s.c.) was unaltered for 10 weeks of HF feeding. Even after 12 weeks on a HF diet, which clearly caused obesity, acute administration of amylin (5 g/kg, s.c.) was still able to suppress food intake, although the suppression was not statistically significant. Further experiments using additional doses of amylin will be necessary to demonstrate possible amylin resistance after HF feeding or in DIO rats. In the second experiment, we tested more specifically whether hyperamylinemia that may result from HF feeding and subsequent obesity, reduces the sensitivity of the amylin signaling system. To avoid confounding factors, we chronically infused lean chow fed rats with amylin (5 or 10 g/kg/day s.c.) to elevate their plasma amylin concentration to levels observed in obese rats (30-40 pM). In the absence of obesity, hyperamylinemia did not lead to a reduced sensitivity to acute amylin (5-20 g/kg s.c.) injections; acute amylin reduced eating similarly in all groups of rats. Overall, we concluded that direct diet effects by short term exposure to HF appear to be of little importance for amylin sensitivity; further, long-term maintenance on a HF diet and the resulting obesity only slightly attenuated the anorectic response to acute amylin. Because we observed no marked changes in amylin sensitivity in lean, chow fed rats with induced hyperamylinemia, amylin receptor downregulation in chronic hyperamylinemia does not seem to occur.

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Influence of high-fat feeding, diet-induced obesity, and hyperamylinemia on the sensitivity to acute amylin

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Keywords: amylin; satiation; adiposity; high-fat; food restriction; hyperamylinemia

Key statements:

1 Long-term maintenance on a HF diet only slightly attenuates acute amylin action.

2 Attenuation of amylin action seems more related to obesity than HF exposure.

3 Acute amylin sensitivity is not reduced by chronic hyperamylinemia.

ABSTRACT

Obesity results in the increased secretion of various hormones controlling food intake and body weight, such as leptin, and insulin; increased circulating levels of pancreatic amylin have also been described in obese humans and rodents. Because leptin-resistance is e.g. present in diet-induced obese (DIO) rats, and because hyperleptinemia seems necessary for the full development of leptin resistance, we tested whether amylin sensitivity is inversely correlated with adiposity, such that DIO reduces the anorectic action of acute amylin. We also determined if hyperamylinemia leads to a change in amylin sensitivity. In the first experiment, rats were chronically exposed to a high fat (HF; 60% fat) diet or fed standard chow for control. The anorectic response to amylin was tested on several occasions over a 14 week observation period. HF feeding led to the expected increase in body adiposity; the response to an acute amylin injection (5 – 50 µg/kg s.c.) was unaltered for 10 weeks of HF feeding. Even after 12 weeks on a HF diet which clearly caused obesity, acute administration of amylin (5µg/kg, s.c.) was still able to suppress food intake, although the suppression was not statistically significant. Further experiments using additional doses of amylin will be necessary to demonstrate possible amylin resistance after HF feeding or in DIO rats. In the second experiment, we tested more specifically whether hyperamylinemia that may e.g. result from HF feeding and subsequent obesity, reduces the sensitivity of the amylin signaling system. To avoid confounding factors, we chronically infused lean chow fed rats with amylin (5 or 10 µg/kg/day s.c.) to elevate their plasma amylin concentration to levels observed in obese rats (30 – 40 pM). In the absence of obesity, hyperamylinemia did not lead to a reduced sensitivity to acute amylin (5 – 20 µg/kg s.c.) injections; acute amylin reduced eating similarly in all groups of rats. Overall, we concluded that direct diet effects by short term exposure to HF appear to be of little importance for amylin sensitivity; further, long-term maintenance on a HF diet and the resulting obesity only slightly attenuated the anorectic response to acute amylin. Because we observed no marked changes in amylin sensitivity in lean, chow fed rats with induced hyperamylinemia, amylin receptor downregulation in chronic hyperamylinemia does not seem to occur.

1. INTRODUCTION

Obesity typically results in increased secretion of various hormones controlling food intake and body weight, such as leptin, and insulin [1, 2]; elevated circulating levels of these adiposity signals in obesity eventually results in the decreased peripheral and central sensitivity to leptin and insulin [3, 4], respectively, which only further potentiates the obese state. Leptin-resistance, for example, is present in diet-induced obese (DIO) mice and rats, and is thought to result from several factors including defective leptin receptor signaling and decreased leptin transport across the blood brain barrier [Reviewed in 5, 6]. It was also shown recently that hyperleptinemia is required for the full development of leptin resistance [7]. Obesity can also affect the sensitivity to satiation hormones, which control meal size, such as glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK) [8, 9].

Increased circulating levels of amylin have also been described in obese humans and rodents ("tonic" increase) [10-12]. Amylin is a pancreatic hormone which buffers glucose flux during a meal by decreasing food intake, gastric emptying, and glucagon secretion. Thus, in response to nutrient ingestion, circulating amylin concentrations rise rapidly within minutes after meal onset ("phasic" increase), peak within 60 min, and return to baseline by 120 min [13]. When administered exogenously (peripherally or centrally), acute amylin dose-dependently decreases food intake, causing a decrease in meal size though having no effect on the intermeal interval [14, 15]. Furthermore, the decrease in meal size is not a result of an aversive or toxic effect of amylin [14, 16].

Some rodent models of obesity (e.g. *ob/ob* and MC4Rko mice, *fa/fa* rats) require higher doses of the amylin receptor agonist, salmon calcitonin (sCT) to reduce eating [17], suggesting that obesity may attenuate amylin sensitivity. Previous pilot work has also indicated that high circulating amylin levels may reduce the ability of amylin to slow gastric emptying [18]. Furthermore, clinical tests report that higher doses of the amylin analog, pramlintide, are necessary to promote weight loss in type 2 versus type 1 diabetics, suggesting that amylin deficiency, as found in type 1 diabetics, may perhaps increase the efficacy of exogenous amylin [19].

Based on these data, we tested whether amylin sensitivity is inversely correlated with adiposity, such that diet-induced obesity reduces the anorectic action of acute amylin. We also determined if factors that cause obesity, such as consumption of a high-fat diet, or that are associated with obesity, such as hyperamylinemia, can lead to a change in amylin sensitivity, independently of obesity.

2. MATERIALS AND METHODS

2.1 Experimental animals

Sprague-Dawley rats (initial body weight 240-300g; Harlan NM Horst, the Netherlands) were used for all experiments; some animals served as a model for diet-induced obesity (DIO). The animals were individually housed in hanging, stainless steel wire-mesh cages and were maintained in a temperature-controlled environment (21± 2°C), on a 12/12h light-dark cycle. Water and food were accessible ad libitum, unless otherwise indicated. All rats were habituated to the housing conditions for at least one week prior to the start of an experiment. During habituation, rats were handled daily, and they were also allowed 30 min access to a common, enriched environment to play. All experiments were approved by the Veterinary Office of the Canton of Zürich, Switzerland.

2.2 Experimental Diets

During the acclimatization phase of each experiment and during the entire duration of the second experiment, rats were allowed ad libitum access to standard pelleted chow (Diet 3430, Provimi Kliba AG, Kaiseraugst, Switzerland; energy content: 13 kJ/g, protein (w/w): 21%, carbohydrates: 39.8%, fat: 5%), except where noted. In experiment one, DIO was induced in one group of rats by providing ad libitum access to a pelleted high fat diet (60% energy from fat; Diet 2127, Provimi Kliba AG, Kaiseraugst, Switzerland; energy content: 22 kJ/g, protein (w/w): 26%, carbohydrates: 1%, fat: 38.0%). Standard chow was also fed to control animals in this experiment; the third group of rats in experiment one also received chow but was restricted to 80% of ad libitum intake.

2.3 Amylin

Amylin (Bachem AG; Bubendorf, Switzerland) was dissolved in 0.9% NaCl (Fresenius Kabi AG, Stans, Switzerland) in various concentrations, depending on the experiment (see below).

2.4 Osmotic minipump implantation

Amylin was chronically administered peripherally by subcutaneous osmotic minipumps with a mean pumping rate of 1.0µl per hour for seven days (alzet®, Model 2001, Durect Corporation, Cupertino USA). On the morning of implantation, the minipumps were filled

under sterile conditions with saline or amylin (total volume = 238µl/minipump, in doses described below). Rats were initially anesthetized by inhalation of 5% isoflurane (IsoFlo®, Provet AG Lyssach, Switzerland), then maintained on 2-3% isoflurane and placed on a heating pad to maintain body temperature during surgery. At the site of implantation, rats were shaved and the skin was disinfected with Betadine® (Provet AG, Lyssach, Switzerland). Under sterile conditions, a small incision was made between the scapulae and the minipump was subcutaneously implanted. The wound was closed with interrupted cutaneous sutures.

2.5 Blood Sampling

Prior to blood sampling, rats were food deprived for the last 6 hrs of the dark phase. Immediately after being briefly anesthetized by inhalation of 5% isoflurane, rats were placed in a supine position and the tongue was extended from the mouth using a cotton-tipped applicator. One of the sublingual veins was punctured with a 20G needle, and blood was collected in a 500µl serum tube (Microvette®, SARSTEDT, Nümbrecht, Germany) and mixed with 5µl of a Protease Inhibitor Cocktail (P2714, Sigma, Missouri, USA). The blood remained at room temperature for at least 30 minutes and then centrifuged for 10 minutes at 2500g. Serum was then transferred to clean tubes and stored at - 20°C until use.

2.6 Hormone Measurements

Circulating levels of serum amylin, insulin and leptin were measured in duplicate by fluorescence immunoassay using the Rat Endocrine Panel Milliplex MAP kit from Millipore (Millipore Corporation, Billerica, MA, U.S.A.). Sensitivity of the assay was 0.32 ng/mL for insulin, 6.2 pM for amylin, and 0.1 ng/mL for leptin. Intraassay CVs were less than 10%. Data were analyzed by Bio-Plex Manager™ software versions 4.0 and 5.0 (Bio-Rad Laboratories, Inc.)

2.8 Body Composition Analysis

Body adiposity was determined postmortem by computerized tomography (CT) using the La Theta LCT-100 scanner (Aloka, Tokyo, Japan). The X-ray source tube voltage was set at 50 kV with a constant 1 mA current. The frozen carcasses were placed supine in the holders with an inner diameter of 120mm. Abdominal scans were done between

vertebrae L1 and L6. Aloka software was used to estimate the volumes of adipose tissue, bone, air, and remaining tissue, using differences in X-ray density. Intra-abdominal (IAAT) and subcutaneous adipose tissue (SAT) were distinguished based on the detection of the abdominal muscle layers; in some cases, this automated classification required manual image-by-image correction. Whole-body IAAT and SAT were estimated using previously validated calculations [20, 21]. Total adiposity was calculated as the sum of whole-body IAAT and SAT mass.

2.9 Description of Experiments One and Two

2.9.1 Experiment One: Do diet and body composition influence the anorectic effect of amylin?

The aim of the first experiment was to assess if the acute anorectic response to amylin is altered in rats chronically fed a high fat diet or food restricted, thus achieving a state of DIO or reduced body weight by chronic underfeeding.

Following habituation, rats were divided into three randomized groups (7-8 rats/group). The control group was fed ad libitum with standard chow diet. A second group was fed ad libitum with the 60% HF diet to achieve DIO. A third group was maintained on chow, but restricted to 80% of the ad libitum intake of the chow-fed controls for 11 weeks; the total daily allotment of food was provided at dark onset. Starting at week 12, restricted rats were switched to the HF diet ad libitum for the remainder of the experiment.

Over the course of the 14-week experiment, six feeding trials were performed, each testing the acute anorectic response to different doses of amylin at various points of HF feeding or food restriction. The timing and amylin dosing for the trials were as follows (also shown in Fig. 1A): Trial 1: week 2, 5μg/kg; Trial 2: week 3, 20μg/kg; Trial 3: week 6, 20μg/kg; Trial 4: week 7, 50μg/kg; Trial 5: week 10, 50μg/kg; Trial 6: week 12, 5μg/kg. In all trials food was removed from the cages one hour before dark onset.

In trials 1, 2, and 6, amylin or saline were administered subcutaneously immediately before dark onset, food was returned immediately after treatment, and energy intake was measured at 60 and 120min post-injection. After the completion of trials 1 and 2, we presumed that strong hunger, habituation and conditioning to dark-onset feeding prevented amylin-induced anorexia in the chronically restricted rats in these trials.

Because the goal of the study was to test if amylin sensitivity changes in lean respectively underweight rats, and not in hungry rats, in trials 3, 4, and 5, all rats were allowed to pre-feed for the first hour of the dark phase, following which amylin or saline was subcutaneously administered and energy intake measured at 60 and 120min. In trials 3 and 4, rats were given ad libitum access to their respective diet during the 60min pre-feeding phase. In trial 5, the amount of the food which was given to all rats in the pre-feeding phase was matched to the approximate caloric load that the chow control group had consumed in trials 3 and 4 (50 kJ).

For all trials, food intake is depicted as energy intake (in kJ) to allow the comparison between standard chow and HF intake. All feeding trials were performed in a crossover manner within diet groups so that each rat received both saline and amylin treatments.

Sublingual blood samples for hormone measurements were collected during weeks 9 and 14. Following blood sampling in week 14, rats were euthanized via an overdose of pentobarbital (300mg/kg i.p.), and frozen prior to performance of CT scans to determine body composition.

*2.9.2 Experiment Two***:** *Does chronic elevation of peripheral amylin levels induce a change in amylin sensitivity?*

To investigate if elevated basal levels of circulating amylin change the sensitivity to the acute anorectic action of amylin in non-obese rats, circulating basal amylin was clamped to different levels using osmotic minipumps. Two amylin doses (5 and 10µg/kg/day) were chosen based on pilot studies, on circulating levels of DIO rats observed in Experiment 1, and on published work [16, 22], and were compared to saline infused controls.

For the duration of the experiment, rats were allowed ad libitum access to standard pelleted chow. The modified counterbalanced experiment was performed over three weeks, during which every animal received each combination of minipump infusate (saline, 5 or 10µg/kg/day amylin, s.c.), mimicking different levels of “tonic” amylin, and three acute injection (saline, 5 or 20µg/kg amylin, s.c.), mimicking the “phasic” meal induced release of amylin. To allow plasma amylin levels to equilibrate to the target concentration, the first of three feeding trials was administered approximately 72h following minipump implantation (corresponding to day 3 in Fig. 2A); trials 2 and 3 were

performed on days 5 and 7, respectively. For all feeding trials, saline or amylin was administered to non-fasted rats immediately before dark onset. Energy intake was measured 30, 60 and 120min following the treatment.

On day 7 of each week, two hours following the completion of the third feeding trial and before the minipumps were exchanged, sublingual blood samples were taken to assess basal circulating amylin levels.

2.10 Statistical Analysis

All data are expressed as mean ± SEM. Experiments performed in a crossover manner were analyzed using paired t-test, when appropriate. When more than two groups were compared, data were analyzed using one-way ANOVA, with Bonferroni's post-hoc test used to determine differences between individual groups. A p-value <0.05 was considered to be statistically significant. Statistical analyses were performed using GraphPad Prism (version 5.0, San Diego, CA, USA).

3. RESULTS

3.1 Experiment One

3.1.1 Development of DIO

Baseline body weight was similar across groups (240-260g), with no significant differences at the start of the experiment. Figure 1A shows the average body weight of the three groups for the duration of the study. At the beginning of week 12 (day 77), the restricted rats were switched to the HF diet, which was then offered ad libitum for the remainder of the experiment.

Comparison of the average body weights of the chow control group and the HF fed animals, demonstrates a demarcation between obese and non-obese rats, with a significant difference starting at week 9 and remaining significantly different for the duration of the observation period. Compared to the ad libitum chow and HF fed rats, the mean body weight of the restricted rats was significantly lower during the entire experiment, even at the end of the 3-week HF ad libitum refeeding period (weeks 12-14).

3.1.2 Amylin sensitivity in DIO and food-restricted rats

Trial 1

Figure 1B shows the effect of acute peripheral amylin in rats maintained on the different feeding regimens for two weeks. The rats were injected in a crossover manner with either saline or a dose of 5µg/kg amylin with 2 days in between trials. With the exception of the restricted group, where amylin only caused a slight decrease in eating, amylin-treated rats showed a significant reduction in energy intake, as compared with the saline controls, at 60 and 120 min after injection. At this time point, the body weight between ad libitum chow and HF rats did not differ.

Trial 2

Rats were tested again one week later (week 3) for their response to a higher dose of amylin (20µg/kg). The effects on eating were similar to trial 1 in that amylin significantly reduced eating in chow and high-fat rats but not in restricted rats; the latter group again only showed a slight tendency to eat less after amylin (Fig. 1C). Body weight was significantly lower in restricted than in the chow or HF rats, but did not differ between the latter two groups.

Trials 3, 4, and 5

Because one aim of the experiment was to test if amylin efficacy is modified by decreased body weight, and because the string drive to eat in the food-restricted group may have prevented amylin from decreasing food intake significantly within the 2h observation period in trials 1 and 2, the experimental design was changed. In trials 3 through 5, all animals were allowed to eat for one hour prior to the amylin injection, i.e. during the first hour following dark onset. In trials 3 and 4, all rats were provided with 132 kJ of food during this hour, which corresponds to approximately 20% of average daily ad libitum intake. During the 1h prefeeding phase, the food-restricted group ate the entire 132 kJ before saline or amylin treatment, while the chow controls and HF-fed rats only ate on average 57 and 53 kJ, respectively. In trial 3, 20µg/kg of amylin still did not suppress food intake in the food-restricted rats at 60 or 120 min after administration (data not shown). With the same design and when the amylin dose was increased in trial 4 (50µg/kg), the decrease in food intake induced by amylin in the food-restricted group was significant (Fig. 1D). In trial 5 (Fig. 1E), in which all groups were pre-fed with the same amount of food (50 kJ), and despite giving the relatively high dose of 50µg/kg amylin, the amylin effect disappeared, i.e., no significant decrease in food intake was observed in the food-restricted group. In each of these trials, the chow- and HF-fed groups continued to show a similar and strong anorectic response to acute amylin.

Trial 6

For the final trial, the restricted animals were refed ad libitum with the HF diet; the chow and HF ad libitum fed groups of rats were fed as before. Given on days 3 and 4 following the switch to HF diet, a low dose of amylin (5µg/kg) significantly reduced food intake in the chow-fed group only (Fig. 1F). Interestingly, the anorectic effect of amylin in both high fat groups was not significant even though the absolute reduction in eating appeared to be similar across groups; when tested a few days later, the same dose of amylin again did not reduce eating significantly in the HF ad libitum group of rats (results not shown). It is important to note that the average body weight was significantly different between all groups at this time point, i.e. body weight in the HF group was significantly higher than in the chow group.

3.1.2 Effects of HF and food-restriction on hormone levels and body composition

Table 1 shows basal concentrations of amylin, leptin, and insulin in fasted rats during weeks 9 and 14. In week 9, rats fed HF demonstrated significantly elevated levels of amylin and leptin, when compared to chow-fed or food-restricted rats. Chow-fed and restricted rats did not differ even though restricted rats weighed significantly less. Furthermore, insulin levels were significantly higher in HF- and chow-fed rats compared to restricted rats. In week 14 (note that the previously restricted rats had been switched to HF diet 3 weeks earlier), the differences in amylin and insulin levels were no longer detectable, despite a significant difference in body weight between formerly restricted and chow-fed or HF-fed rats. At this time point, circulating leptin was still higher in the HF group compared to the chow controls, but there was no significant difference in leptin levels between the HF group, which had been maintained on HF diet for 14 weeks, and the previously restricted group, which had only been on HF for 3 weeks.

Average body composition of the three groups at the time of sacrifice (week 14), as determined by CT scan, is also shown in Table 1. There was no significant difference in total body fat mass between the HF and the previously restricted group, but both groups had significantly higher total body fat than the chow-fed controls. Similarly, no difference in the subcutaneous fat mass was detected between the high fat and previously restricted group, but both groups had significantly higher levels compared to the chow-fed group. Comparison of total intra-abdominal fat mass revealed significantly higher amounts in HF-fed rats compared to the other two groups, and the previously restricted rats also had significantly more intra-abdominal adipose tissue compared to chow-fed controls.

3.2 Experiment Two

3.2.1 Effects of chronic amylin infusion on body weight and basal hormone levels

Figure 2A shows the average percentage of body weight gain under the influence of two doses of chronic amylin treatment (5 and 10µg/kg/day) compared to control animals receiving saline. Body weight was set to 100% at the beginning of each of the three infusion weeks, and all data were combined across the three weeks and the three infusion groups. Although there was no significant difference among the three groups, control animals gained slightly more weight than the amylin treated rats. Hence, there was a tendency that body weight gain was inversely correlated to the amylin dose. We observed a similar tendency for decreased weekly food intake following 7-day amylin

infusion, but again no significant differences were detected between saline and amylin-infused groups at the low doses used here (data not shown).

Figure 2B shows the levels of basal circulating amylin as measured at the end of each of the three infusion weeks in ad libitum fed rats. We observed significantly higher circulating amylin values in rats that received chronic amylin at a dose of 5 or 10 µg/kg/day. For insulin and leptin, there was a tendency for amylin infusion to result in lower insulin and leptin levels, though no significant differences were observed (Fig. 2C and D).

3.2.2 Influence of elevated amylin levels on sensitivity to acute amylin

Figure 3 shows the effect of acute peripheral amylin on energy intake in rats with differing basal amylin concentrations but similar body weight. We observed no substantial difference in the anorectic effect of acute amylin administration dependent on the basal amylin levels. Across all groups, the higher the dose of the acute amylin treatment, the larger the acute anorectic effect of amylin.

More precisely, at all time points and in all infusion groups energy intake was significantly lower following an acute injection of 20µg/kg amylin than in the saline-injected control group. In rats with the 5µg/kg/day minipumps, we also saw a significant decrease in energy intake after 5µg/kg at 30 and 60min post-treatment. The main difference post acute amylin treatment was observed after two hours, where 5µg/kg amylin significantly decreased food intake in the control group, but not in the 5µg/kg/day nor the 10µg/kg/day infusion groups.

DISCUSSION

The aims of this study were to investigate the influence of body weight, exposure to a HF diet, and hyperamylinemia on the sensitivity to acute amylin injections in rats. The results of our studies suggest three main points. First, the suppression of food intake by a variety of amylin doses was comparable between rats fed chow or HF diet for up to 10 weeks. Second, maintenance on a HF diet for longer duration and resulting obesity, only slightly attenuated the anorectic response to acute amylin. Finally, we observed no marked changes in amylin sensitivity in lean, chow fed rats with induced hyperamylinemia.

5.1 Amylin-induced suppression of energy intake is decreased after 11-week maintenance on high fat diet

To test the hypothesis that amylin sensitivity is inversely correlated with body weight or adiposity, we investigated if the effect of acute amylin to reduce eating is altered in DIO or food-restricted rats. These obese and lean rodent models were validated by measurement of the adiposity signals amylin, leptin and insulin 9 and 14 weeks after exposure to the HF diet, and by directly assessing body composition when terminating the study in week 14, using CT. It is known that elevated fasting leptin and insulin levels reflect increased adipose mass [1, 2], at least under relatively weight stable conditions [21]. Furthermore, Pieber and colleagues showed that similar to leptin and insulin, the basal circulating level of amylin also correlates positively with adipose mass when measured across individuals [10]. Consistent with these reports, we observed that after 9 weeks on the HF diet, rats exhibited significant increases in circulating fasting amylin and leptin levels, compared to the chow-fed rats. Unexpectedly, we did not observe an effect of the HF diet-induced weight gain on fasting insulin levels. By the end of the experiment in week 14, the HF group still showed higher leptin levels than the chow control group, and terminal body composition analysis showed significant increases in total adipose tissue, intra-abdominal adipose tissue, and subcutaneous adipose tissue in HF fed rats compared to chow fed rats. Even though it would have been preferable to assess the development of obesity and associated adiposity signals at more time points, most of our data are in general agreement with the expected changes and corroborate the successful induction of DIO.

Rats restricted to 80% of the control food intake had significantly lower body weight than the other two groups during the entire experiment. This difference also remained significant during the refeeding phase, i.e. when the previously restricted rats were fed HF diet ad libitum for 3 weeks following the 11 week period of food restriction. These findings are consistent with previous studies showing that following prolonged food restriction, rats maintained a body weight that was lower than the pre-restriction baseline, even after ad libitum refeeding for as long as four months [23]. In addition to lower body weight, at 9 weeks the food restricted group had lower leptin, insulin, and amylin levels compared to HF fed rats, and lower insulin levels than the chow controls. The three week HF refeeding phase in the previously restricted rats had dramatic effects on circulating hormones (see also [21]) and body composition. Interestingly, by week 14, there was no difference between the circulating leptin levels of the previously restricted group and the HF group, despite the marked difference in body weight (HF: 533.5 ± 8.4g vs. [previously] Restr: 455.3 ± 10.5g). There were also no differences in total nor subcutaneous fat mass between the previously-restricted rats and the rats maintained on HF for the entire 14 weeks, which is consistent with the leptin values of the two groups observed at the termination of the study. Only the intra-abdominal adipose fat mass of the previously-restricted group remained lower compared to the HF fed rats. Thus, following 3 weeks of HF refeeding, the previously restricted rats nearly reached the same level of total body fat mass as observed in the rats fed the HF diet ad libitum for 14 weeks. Furthermore, the increase in body weight during the 3 weeks of ad libitum HF feeding appears to be largely due to an accrual of body fat and not lean mass. Once again, our results are reminiscent of those reported by Brownlow and colleagues, who observed that following a 4-month refeeding period, rats that had been food restricted to 50% for two months had a greater body fat percentage than ad libitum fed control rats [23], and who suggested that following food restriction for an extended period, rats develop efficient fat-depositing mechanisms which lead to rapid weight gain once food is provided ad libitum. Unfortunately, because body composition was not assessed between the food restriction and HF refeeding phases, it is not clear if the previously-restricted rats gained more subcutaneous adipose tissue than intra-abdominal adipose tissue during refeeding, or if they had disproportionately lost mass from these depots during food restriction. Reports have demonstrated that intra-abdominal fat depots are more responsive than subcutaneous adipose tissue during periods of caloric restriction [24, 25]. This supports the notion that food restricted rats did not necessarily gain more

subcutaneous adipose tissue during refeeding, but actually had lost a higher proportion of intra-abdominal adipose tissue during the prior restriction phase.

Validation of the obese and lean phenotypes, even though only performed at specific time points and not continuously throughout the development of obesity, assists us in drawing conclusions from the acute amylin feeding trials. In the first 10 weeks of the experiment, we observed significant and similar decreases in food intake following amylin injection in rats on the chow control diet and in rats receiving the HF diet ad libitum. In a study of shorter duration, Covasa and Ritter had observed a similar effect of exogenous amylin in rats fed a HF (approx. 75% by energy) diet for three weeks; there was no difference in the acute anorectic effect of amylin between animals adapted to either a low or high fat diet [8]. Interestingly, using the same paradigm, there was a significant attenuation of the CCK effect in Sprague-Dawley rats after maintenance on the HF diet. While the highest dose of CCK tested (1µg/kg) produced a reduction of food intake that did not differ between low- and high-fat fed rats, lower doses of CCK, while still effective in HF-fed rats, were significantly less powerful at reducing food intake, compared to low fat-fed rats.

After 11 weeks on the HF diet, rats showed a slightly decreased response to a low dose of amylin, which was no longer significant, whereas the chow control group still ate significantly less after amylin treatment. This result suggests that long-term maintenance on the high fat diet may eventually attenuate the anorectic effect of acute amylin. Clearly, experiments using multiple doses of amylin and performed under different experimental conditions will be necessary to demonstrate possible amylin resistance.

The change in sensitivity could either be the result of the diet itself, or the result of the increase in body weight, body fat mass, or circulating hormones or metabolites that resulted from the HF intake or ensuing obesity. Because we did not see any decrease in amylin sensitivity after HF feeding for the first 10 weeks, it seems unlikely that the change in amylin sensitivity would be due solely to the fat content of the diet; otherwise, a decrease in amylin sensitivity would very likely be anticipated earlier in the study, such as is e.g. the case for insulin [26] or leptin [27]. It is more probable that a change in amylin sensitivity would be based on the resulting change in body weight or body composition or, possibly, hormone or metabolite levels that differ between obese and leaner rats. This idea is supported by the fact that the point in time when the anorectic

effect of amylin was no longer significant in the HF group corresponded with a highly significant difference in body weight between the HF and chow fed groups. The change in amylin sensitivity also followed elevated leptin and amylin levels observed in week 9. Still, the exact mechanism by which DIO may reduce sensitivity to amylin remains unclear. While elevated levels of circulating amylin do not appear to be a main contributor to this effect (discussed in section 5.2), at this time, we can only speculate that elevated leptin levels, or a potential increase in plasma metabolites (e.g., free fatty acids or triglycerides, which were not measured in this study), might be involved in attenuated amylin sensitivity following DIO [28]. Further experiments, performed under a variety of dietary and metabolic conditions, will be necessary to demonstrate possible amylin resistance.

Unlike the HF fed rats, food-restricted rats only responded to an acute amylin injection when the animals were pre-fed for one hour with 50% of their daily amount of food and when they received a comparably high dose of amylin (50µg/kg). Because the anorectic effect was only observed after pre-feeding, we believe that the drive to eat after prolonged food restriction may simply have been too strong, and likely masked the anorectic properties of amylin. The phenomenon was still apparent in the last acute amylin trial, i.e. 3 to 4 days after allowing the (previously) restricted rats ad libitum access to HF food. Overall, we therefore believe that decreased body weight, at least under these test conditions of habituation to dark onset presentation of food and acute amylin injections, does not appear to reveal increased sensitivity to amylin.

Unfortunately, it was not possible in the frame of this study to test other satiating hormones, like CCK, under these chronically restricted conditions. Thus, we cannot conclude that our observation was specific to amylin or that it was part of a more generalized phenomenon. It is well-established, however, that food deprivation produces a number of neural and physiological responses aimed at stimulating eating and correcting for the impinging energy deficit; these responses possibly contributed to our findings. Plasma insulin is one of the signals that is reduced in food-deprived rats, which was also exhibited in this study following 9 weeks of food-restriction, to allow the mobilization of stored energy from adipose tissue and to promote gluconeogenesis [29]. Data demonstrate that amylin and insulin interact to suppress food intake in rats, suggesting that the presence of very low insulin levels below a certain range may have prevented the acute anorectic response of amylin [30]. Additionally, the concentration of

the hunger signal ghrelin is known to increase after fasting and it is also known that a loss in body weight leads to an increase in circulating ghrelin [31, 32]. The upregulation of these and other mechanisms in food restricted rats may explain why we did not observe an anorectic effect of acute amylin in the chronically restricted animals, at least with amylin doses that reduce eating in non-restricted animals.

Another contribution to changes in amylin sensitivity in obese and lean rats could be the interaction between amylin and leptin. It is known that a co-infusion of amylin and leptin reduces body weight and adiposity synergistically in DIO prone rats [22]. Additionally, it has been shown that peripheral administration of amylin restores leptin sensitivity in obese rats and humans [33]. These findings suggest that an interaction exists between exogenous amylin and the prevailing circulating leptin values. It is possible that exogenous amylin only shows its anorectic effect in a certain range of circulating leptin levels. Although speculative, this may explain why we observed a reduced sensitivity to the anorectic activities of acute amylin in both obese rats, with high circulating leptin levels, and in restricted rats, with very low circulating leptin levels [28]. The fact that obese Zucker rats, which lack functional leptin receptors, show decreased responsiveness to CCK [34], supports the notion that the absence of normal leptin signalling can modify the effectiveness of satiating signals, such as CCK and perhaps also amylin.

5.2 Hyperamylinemia alone does not cause amylin insensitivity

In experiment one, we observed a slight decrease in amylin sensitivity after long-term exposure of rats to a HF diet, which resulted in increased body weight, fat mass, hyperleptinemia and hyperamylinemia. Because it remained unclear which of these factors may primarily contribute to decreased amylin sensitivity after prolonged high fat consumption, we investigated whether hyperamylinemia alone, independent of other obesity-related factors, causes a decrease in the sensitivity to acute amylin administration. In addition to becoming obese, hyperinsulinemic and hyperleptinemic DIO rats fed a high energy diet also show a decrease in insulin and leptin sensitivity, respectively [35, 36]. It was also recently shown that hyperleptinemia is necessary for the full development of leptin resistance [7]. Because obesity is also associated with hyperamylinemia, we therefore tested whether elevated levels of circulating amylin might contribute to decreased amylin sensitivity.

By chronically infusing amylin at 5 or 10µg/kg/day for seven days, we achieved average basal circulating amylin levels of approximately 30 to 40 pM, which were significantly higher than the baseline concentration in the lean rats in this experiment (16.5 $\pm$ 2.7 pM), and which were in the range of obese rats in the fed state (Boyle et al, unpublished observations). In contrast to our hypothesis, we observed no marked difference in amylin sensitivity among the groups with different background circulating amylin levels. Thus, regardless of the level of circulating amylin, acute peripheral amylin decreased food intake in a dose-dependent manner, and this effect was similar under all conditions. These results suggest that hyperamylinemia alone, without the impact of obesity or obesity-associated parameters, does not cause a marked decrease in (acute) amylin sensitivity. It is important to note, however, that two hours after acute amylin treatment with a dose of 5µg/kg, the anorectic effect of acute amylin was no longer detected in the animals with the moderate (~30pM) and high (~40pM) circulating baseline amylin levels, though the effect was present in the control rats receiving saline infusion. This suggests the presence of a subtle reduction in amylin sensitivity in rats receiving chronic amylin infusion, and that after two hours the concentration of the acutely administered amylin was likely too low to induce a further decrease in food intake in animals that already have elevated baseline circulating amylin. Therefore, it is possible that by administering a lower dose of acute exogenous amylin, a decrease in amylin sensitivity might have been uncovered at earlier time points.

Amylin activation of calcitonin receptors (CT-R) in the area postrema (AP) is considered the main mechanism mediating amylin's anorectic effects [37-39]. The present results suggest that it is unlikely that the expression of CT-Rs in the AP is downregulated following 7-day infusion of physiological doses of amylin. The results also indicate that this degree of hyperamylinemia has little effect on the amylin signaling pathways which control feeding behavior [28]. Based on these data, one could also speculate that decreased sensitivity occurs independently of amylin receptor dysfunction in the AP. We have collected some preliminary evidence to support this idea, because only little difference was observed between amylin-induced cFos expression in the AP of lean and obese rats, however, additional studies are needed [28].

Previous studies had shown that chronic subcutaneous or third ventricular infusion of amylin produced a transient, dose-dependent decrease in daily food intake and a decrease in body weight gain [40, 41]. Mack and colleagues showed that amylin

(10µg/kg/day) infused subcutaneously for four weeks decreased food intake and body weight in rats fed a HF diet for five weeks [16]. Consistent with these findings, our rats receiving exogenous amylin showed a tendency to gain less body weight over the one week infusion period, and the effect seemed to be dose-dependent. However, probably because of the low doses of administered amylin and the short duration of the study, the difference across the groups for the effect on body weight gain or baseline food intake did not reach significance. In a similar study, the minimal effective dose for suppressing daily food intake was ~32.3µg/kg/day (7pmol/kg/min) which produced plasma amylin levels of approximately 80 pM [42], i.e. much higher than the physiological levels observed in DIO rats and much higher than the concentrations in our experiment. Though our elevated levels of amylin, which were still in the physiological range, did not produce dramatic changes in body weight and food intake, our results do provide further evidence that amylin may play a role in the long-term control of energy balance, similar to well known adiposity signals, like insulin and leptin [43].

In our experiments, it was important to consider what effect chronic hyperamylinemia might have on circulating levels of insulin or leptin. It had been previously reported that insulin secretion is increased by the administration of an amylin antagonist in isolated rat islets [44], suggesting that an interaction exists between amylin and insulin secretion. This idea was further supported by findings showing that insulin secretion in the rat pancreas was suppressed by high doses of amylin [45]. Additionally, Gebre-Medhin and colleagues observed that amylin deficient mice showed increased plasma insulin responses following glucose administration, whereas baseline insulin and glucose levels were normal [46]. Together, these data demonstrate that high amylin levels may feedback to suppress insulin secretion. Our data are also consistent with these reports. Though physiological doses of hyperamylinemia did not significantly suppress circulating insulin in this study, we observed that the higher amylin dose had a tendency to lower insulin concentration. Furthermore, because of the known interaction between amylin and leptin [22, 33], we determined whether an increase of amylin, independent of obesity, alters circulating leptin levels. Again we observed that as circulating amylin levels increased, there was a trend toward a decrease in leptin levels. Whether this was a direct effect of the hyperamylinemia cannot be addressed; it is quite possible that the slight lowering of leptin levels was a result of slightly decreased weight gain induced by amylin infusion, rather than a direct effect of hyperamylinemia on the leptin concentration.

5.3 Summary

Our study tested the ability of acute amylin to suppress food intake and its modulation by the metabolic status in the rat. While up to 10 weeks of HF feeding did not seem to reduce amylin's acute anorectic action in rats, extended ad libitum exposure of rats to the HF diet and ensuing obesity may result in a reduced amylin effect; even though there still appeared to be a reduction in food intake, the effect was no longer significant. However, experiments with additional doses of amylin will be necessary to demonstrate possible amylin resistance. We also found that rats that were chronically food restricted to 80% of their ad libitum intake appeared to be resistant to a short term reduction in eating under our experimental conditions and induced by acute amylin injection; the drive to eat at the time when the daily food ration was presented was most likely too strong and hence overcame the satiating signal generated by the (single) amylin injection. Interestingly, even during the initial phase of catch up growth when the previously restricted rats were allowed ad libitum access to HF food, acute amylin did not significantly reduce eating in these rats. Food restricted rats did react to amylin, though, if prefed prior to injection. Defining the exact mechanism underlying these phenomena will require follow up studies. Finally, the last experiment indicated that even in the presence of hyperamylinemia in a concentration range typical for obese rats, acute amylin was able to reduce short-term food intake. Hence, chronic elevation of the circulating amylin concentration does not seem to result in downregulation of the amylin signaling system. Overall, our studies provide new information in terms of amylin's effectiveness to reduce eating under conditions of HF induced DIO or chronic hyperamylinemia.

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FIGURE LEGENDS

FIGURE 1: Effects of high fat feeding and food restriction on body weight development and amylin sensitivity in acute feeding trials.

(A) Mean (± SEM) body weight of rats maintained on standard chow (Chow; white circles), 60% high fat diet (HF; gray triangles) or restricted to 80% intake of the chow ad libitum group (Restr; black squares). At the start of week 12, Restr rats were given ad libitum access to HF diet. Solid arrows indicate feeding trials and the corresponding dose of amylin administered (μg/kg); dashed arrows indicate blood sampling for hormone analysis. Body adiposity was determined by CT scan following sacrifice in week 14. Mean (± SEM) cumulative energy intake 60 and 120 minutes after saline or amylin injection in rats maintained on chow, HF, or 80% food restriction for 2 weeks (B; 5 μg/kg amylin), 3 weeks (C; 20 μg/kg amylin), 7 weeks (D; 50μg/kg amylin), 10 weeks (E, 50μg/kg amylin), and 12 weeks (F, 5μg/kg). Symbols in A denote significant differences between the three diet groups; + chow vs restricted, °chow vs high fat, * high fat vs restricted; Symbols in B-F denote significant differences between saline- and amylin-treated groups within diet groups; *p<0.05, **p<0.01, ***p<0.001.

FIGURE 2: Effects of 7-day amylin infusion on body weight gain and circulating hormone levels.

(A) Mean (± SEM) percent body weight gain in rats (n=20) implanted with osmotic minipumps infusing NaCl (white circles), 5μg/kg/day amylin (gray triangles) or 10μg/kg/day amylin (black squares) for 7 days. Mean (± SEM) amylin (B), insulin (C), and leptin (D) levels measured on day 7 of NaCl (white bars), 5μg/kg/day amylin (gray bars) or 10μg/kg/day amylin (black bars) infused rats. Symbols denote significant differences between infusion groups; *p<0.05, **p<0.01.

FIGURE 3: Effect of 7-day amylin infusion on amylin sensitivity in acute feeding trials.

Mean (± SEM) cumulative energy intake 30, 60 and 120 minutes after acute saline (white bars) 5μg/kg amylin (gray bars) or 20μg/kg amylin (black bars) injection in rats implanted with a osmotic minipump infusing either saline (A), 5μg/kg/day amylin (B) or 10μg/kg/day amylin (C). Symbols denote significant differences between saline- and amylin-treatment within infusion groups; *p<0.05, **p<0.01, ***p<0.001

TABLE LEGEND

TABLE 1: **Effects of high fat feeding and food restriction (and subsequent HF refeeding) on circulating hormone levels and body adiposity**

Mean ± SEM leptin, insulin and amylin levels measured in rats maintained on chow, high fat diet (HF), or 80% food restriction (Restr) for 9 weeks. Hormones were assessed again after 14 weeks, at which time previously restricted (Prev Restr) rats had been switched to HF-diet presented ad libitum for 3 weeks. After sacrifice in week 14, total (TAT), subcutaneous (SAT), and intra-abdominal adipose tissue (IAAT) were assessed by CT scan. Letters denote significant differences compared to a: Chow, b: HF, and c: Restr or Prev Restr groups, where $P<0.05$.