Cheese-induced body weight gain is not accompanied by an increase of gastric cells producing leptin, ghrelin, gastrin, or pancreastatin in mice.

by ***Camilla Waldum[1], Chun-Mei Zhao[1,2], Helge L. Waldum[1], Jostein Halgunset[2] & Duan Chen[1*]***
[1]Departments of Clinical and Molecular Medicine and [2]Laboratory Medicine, Norwegian University of Science and Technology, Trondheim, Norway.[*]

Summary
The stomach is a source of several circulating peptides/hormones, such as gastrin, pancreastatin, leptin and ghrelin, which are thought to play important roles in the regulation of food intake and body growth. The present study was undertaken in mice in order to examine the effects of diet composition on the body weight gain and the gastric cells that produce these peptides/hormones.
Both young and adult female mice (BALB/cABBom strain) were given a standard pelleted dry diet, with or without cheese *ad libitum*, during a 7 week period. The diet supplement consisted either of carbohydrate-free white cheese containing 27% fat or sweet-tasting but sucrose-free Norwegian "brown cheese" containing 29% fat and 39% carbohydrate, mainly lactose. The total intake of the various types of food and the change in body weight were recorded. At the end of the observation period, blood samples were obtained for determination of gastric hormone levels by radioimmunoassay, and the stomachs were removed for examination of hormone producing cells by immunohistochemistry.
The young mice increased their body weight more than the adult mice. In the groups offered white cheese, both young and adult mice increased their body weight more than the animals kept on the standard diet alone. In contrast, the "brown cheese" supplement led to a relative overweight only in adult mice. Despite the changes in body weight gain, there were no differences with respect to the circulating levels of gastrin, leptin or ghrelin, and to the numbers of cells stained with antibodies to pancreastatin (including ECL cells and G cells), leptin (subpopulation of chief cells) and ghrelin (A-like cells) in all groups.
Body weight gain was increased in both young and adult mice by a white cheese diet supplement, whereas 'brown cheese' produced overweight only in adult mice. The increased body weight gain was not accompanied by an icrease of gastric cells producing leptin, ghrelin, gastrin, or pancreastatin.

Sammendrag
I magesekken produseres en rekke peptider/hormoner, som bidrar til regulering av matinntaket og dermed av vekst og kroppsvekt, slik som gastrin, pancreastatin, leptin og ghrelin. Den foreliggende studien ble gjennomført for å undersøke effekten av ulike diettfaktorer på vektutviklingen hos mus og for å se om diettinduserte endringer i kroppsvekt har sammenheng med funksjonelle forandringer i de hormonproduserende cellene i ventrikkelen.
Grupper av unge og voksne hunnmus (BALB/cABBom) fikk standard tørrfor med eller uten tilskudd over en periode på 7 uker. Diettilskuddet bestod enten av karbohydratfri gulost med 27% fett eller norsk 'brunost', som inneholder 27% fett og 39% karbohydrat, vesentlig i form av laktose. Slik 'brunost' har søtlig smak, men inneholder ikke sukrose. Totalinntaket av ulike næringsmidler ble registrert, og dyrene ble veid regelmessig. Ved avslutning av forsøksperioden ble dyrene avlivet, og det ble tatt prøve av blod til bestemmelse av ventrikkelhormoner med radioimmunologisk teknikk. Magesekken ble tatt ut og preparert for mikroskopi og immunhistokjemisk undersøkelse av hormonproduserende celler.

Correspondence: Professor Duan Chen, Department of Surgery, University Hospital in Trondheim, 7006 Trondheim, Norway. Tel: +47 73 86 94 93; Fax: +47 73 86 94 95; E-mail: Duan.Chen@medisin.ntnu.no

De unge musene økte mer i vekt enn de voksne. I gruppene som fikk tilskudd av gulost, økte både unge og voksne mer i vekt enn tilsvarende gruppe på standarddiett. Derimot var det bare de voksne musene som utviklet relativ overvekt ved tilskudd av 'brunost'. Til tross for klar effekt på vektutviklingen var det ikke påvisbare forskjeller i serumnivå av verken gastrin, leptin eller ghrelin. Immunhistokjemisk undersøkelse med antistoff mot henholdsvis pancreastatin (ECL-celler og G-celler), leptin (en underpopulasjon av hovedceller) og ghrelin (A-lignende celler) viste samme antall positive celler i slimhinnen i samtlige grupper. Konklusion: Tilgang på gulost førte til økt vekt hos både unge og voksne hunnmus, mens 'brunost' resulterte i vektøkning bare hos de voksne dyrene. Økningen i kroppsvekt var ikke ledsaget av noen påvisbar endring i aktiviteten i celler som produserer gastrin, pancreastatin, leptin eller ghrelin.

Introduction

Over the past two decades, obesity has become more prevalent in both children and adults, which may reflect changing patterns of food consumption (Nicklas et al. 2001). The stomach is known to play a key role in food digestion, and moreover, it has been recognized as an endocrine organ, playing a role in the regulation of food intake, body growth and metabolism. Several hormones have recently been shown to participate in a complex interplay to control body weight and tissue composition. In the vertebrate stomach, seven different endocrine cell types have been identified, based on their histochemical and ultrastructural features, i.e. ECL cells (closed type), gastrin (G) cells (open), antral D cells (open), fundic D cells (closed), A-like cells (closed), EC cells (closed) and D_1/P cells (closed) *(Solcia et al. 2000)*. Besides producing gastrin, the stomach is the main source of circulating pancreastatin (chromogranin A-derived peptide) *(Håkanson et al. 1995)* and ghrelin *(Kojima et al. 1999; Dornonvill de la Cour et al. 2001)* and a source of leptin *(Bado et al. 1998)*. Ghrelin is a growth hormone-releasing hormone, recently identified as an acylated 28-amino acid peptide *(Kojima et al. 1999)*. Injection of ghrelin induced adiposity in mice *(Tschop et al. 2000)*. An increased circulating ghrelin level was observed in diet-induced weight loss in subjects *(Cummings et al. 2002)*, while a reduced circulating ghrelin level occurred in human obesity *(Tschop et al. 2001)*. Animal experiments have demonstrated that dietary fat *ad libitum* increases the incidence of obesity *(Salmon & Flatt, 1985; West et al. 1992)* and that a high amount of dietary fat is associated with an increased body fat content *(West & York, 1998)*. Experimental studies have also suggested that sweet-tasting diets promote hyperphagia and obesity in rodents *(Sclafani, 1987)*. The aim of this study was to search for a possible link between diet-induced body weight gain and gastric endocrine function. Thus, we examined the circulating gastric peptides/hormones and their cell populations in the stomach of both young and adult female mice. The animals were provided with a standard mouse/rat pelleted diet with free access to either carbohydrate-free white cheese containing a high percentage of fat (27%) or sweet-tasting, sucrose-free, Norwegian "brown cheese" containing similar levels of fat (29%) but also carbohydrate (39%), the latter mainly lactose.

Materials and Methods

Animals

Thirty-six female mice (BALB/cABBom strain) were purchased from Möllegaard (Skensved, Denmark). Eighteen of the mice were 2 months of age, whereas 18 were adult of age 3 months. The animals in each of the two age groups were randomly allocated to one of three groups, thus defining a total of 6 groups with 6 mice in each. The animals were housed in wood-powder bottom cages of 800 cm^2 floor area and 18 cm interior height, kept at 20°C and 40-45 % humidity, with 12 hours light/dark cycle. One cage contained one experimental group. All groups had unlimited access to standard mouse/rat food pellets with or without supplementary cheese and to tap water (Table 1). The nutritional composition and values of the food pellets and the two kinds of cheese are shown in Table 2. The body weight of each individual mouse and the amounts of food pellets and cheese per cage

were recorded twice per week. After 7 weeks, the mice were killed under deep anesthesia with a subcutaneous injection of 0.02 ml/kg of a solution containing 2.5 mg/ml fluanison, 0.05 mg/ml fentanyl (Janssen Animal Health, Buckinghamshire, UK) and 1.25 mg/ml midazolam (Alpharma AS, Oslo, Norway). The experiment was approved by the Norwegian State Commission for Animal Experimentation.

Table 1: Experimental design

Group (n)	Age (months)	Body weight (g)	Food*	Cheese**
I (6)	2	24.4±0.6	Standard pellets	No
II (6)	2	22.2±0.6	Standard pellets	White cheese
III (6)	2	23.1±0.9	Standard pellets	"Brown cheese"
IV (6)	3	31.9±0.9	Standard pellets	No
V (6)	3	29.7±0.4	Standard pellets	White cheese
VI (6)	3	30.7±0.6	Standard pellets	"Brown cheese"

Means ± SEM (n=6). *, purchased from B&K Universal Ltd., Hull, UK; **, purchased from Tine Norske Meierier. Brown cheese: Gudbrandsdalsost; White cheese: Norvegia. The body weight was at the start of the experiment.

Table 2: Nutritional compositions and values of food pellets and cheese*

Composition	Standard pellets	White cheese	Brown cheese
KCals/100g	390.2	351.0	461.0
Protein/100g	19.7	27.0	11.0
Carbohydrate/100g	50.2	0.0	39.0
Fat/100g	4.1	27.0	29.0

*, values provided by the producers (B&K Universal Ltd., Hull, UK, and Tine Norske Meierier, for the standard pellets and for the cheese, respectively).

Blood

Blood was drawn from the abdominal aorta at sacrifice from freely fed mice. Serum was stored at -80°C prior to measurements by specific radioimmunoassay for gastrin *(Stadil & Rehfeld, 1973)*, ghrelin available kit, Phoenix Europe gMBh, Karlsruhe, Germany) and leptin (available kit, Linco Research Inc., St. Charles, MO, USA.

Stomach

The stomach of each mouse was collected, opened along the major curvature, and rinsed with 0.9 % NaCl. Tissue specimens were taken from the glandular area and fixed in 4% buffered formalin, dehydrated and embedded in paraffin. Four μm thick serial sections were cut perpendicularly to the mucosal surface, and representative sections were stained with haematoxylin and eosin. The thickness of the oxyntic mucosa was measured microscopically. Immunohistochemistry was performed using primary antibodies against the following peptides/hormones: pancreastatin (Euro-Diagnostica, Malmö, Sweden) at a final dilution of 1:1000, leptin (Phoenix Pharmaceuticals, Mountain view, CA, USA) at a final dilution of 1:400, and ghrelin (Phoenix Pharmaceuticals, Mountain view, CA, USA) at a final dilution of 1:2000. Antigen-antibody binding was visualized with the avidin-biotin-peroxidase complex (ABC) method using commercial ABC kits (Vector Laboratories Inc., Burlingame, CA, USA). Immunoreactive cells were counted in at least three sections from each mouse, and the cell density was expressed as number of cells per mm length of mucosa.

Statistical analysis

Results are expressed as means ± SEM unless otherwise stated. Dunnett test was used to evaluate differences between groups. A p value of < 0.05 was considered statistically significant.

Results

Food intake

Although the total food intake determined by weighing food was similar in all groups, the fat intake was very high in both cheese groups. The mice given "brown cheese" had a lower intake of protein and carbohydrate than the controls, whereas the mice in the white cheese groups ingested more protein, but less carbohydrate than the other two groups. Thus, the relative contribution of fat to the energy balance was remarkably similar in the four cheese groups, with ~30% of the ingested energy derived from fat, as opposed to ~10% in the controls (Table 3).

Table 3. Intakes of standard food, cheese, protein, carbohydrate and fat in terms of weight or energy (kCals) per cage of 6 mice during the period of 7 weeks.

Group (n)	Total food intake (g)	Total protein(g)	Total carbohydrate (g)	Total fat (g)	Energy from protein (%)	Energy from carbohydrate (%)	Energy from fat (%)
I (6)	4305.6	848.2	2579.1	176.5	22,2	67,4	10,4
II (6)	4718.2	1055.5	1791.8	588.9	25,3	42,9	31,8
III (6)	4321.2	698.3	2220.8	615.1	16,2	51,6	32,2
IV (6)	4461.6	878.9	2672.5	182.9	22,2	67,4	10,4
V (6)	4706.3	1052.3	1791.8	585.7	25,3	43,1	31,7
VI (6)	4716.6	765.2	2431.2	662.8	16,3	51,9	31,8

Body weight gain

The mice fed additionally with white cheese had an increased weight gain (150% in young mice and 250% in adult mice). In the mice fed with "brown cheese", the adults showed increased weight gain (230%). However, the young mice did not differ significantly from the corresponding controls (Fig. 1).

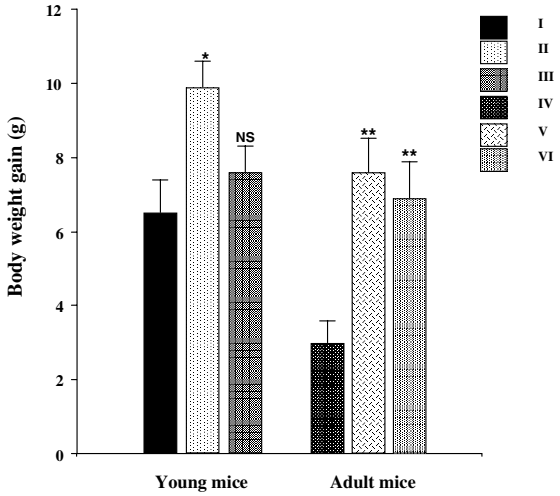

Fig. 1: The gain of the body weight in mice of various groups (I-VI, see Table I for details). Means ± SEM (n=6). *: $p<0.05$; **: $p<0.01$; NS: not significant

Serum levels of gastrin, leptin and ghrelin

The serum gastrin concentration was similar among the various groups. Statistically, there was no significant difference in the serum leptin concentration between control and cheese groups neither in the young nor in the adult mice. However, we could not exclude the possibility that lack of the significance of comparison was due to the weakness of statistical power (by the Dunnet test) or the limited number of animals (6 in each group). The serum ghrelin concentration did not differ from each other within each group of young mice or of adult mice in freely fed state (Table 4).

Table 4: Serum peptides/hormones levels

Group(n)	Gastrin (pM)	Leptin (ng/ml)	Ghrelin (ng/ml)
I (6)	24.5±2.3	6.4±1.3	2.8±0.7
II (6)	24.7±3.2	11.5±2.6	2.6±0.3
III (6)	23.8±3.4	5.8±1.5	2.7±0.7
IV (6)	23.0±1.9	10.4±1.7	4.2±0.3
V (6)	24.7±3.1	14.0±4.4	3.6±0.5
VI (6)	21.8±1.5	16.7±3.2	3.7±0.4

Means ± SEM. No significant difference between the cheese groups (II/III and V/VI) and the corresponding control groups (I and IV).

Hormone-producing cells in the stomach

No gross difference was observed between the various groups. Histologically, there was no diet dependent change in the thickness of the oxyntic mucosa. There were no differences in terms of the cell density in pancreastatin positive cells in the antrum (including G cells) and in the fundus (most were ECL cells), leptin positive cells in the fundus (subpopulation of chief cells), and ghrelin positive cells in the fundus (A-like cells) (Table 5).

Table 5: Histological data of the stomach

Group (n)	Thickness of oxyntic mucosa (mm)	Pancreastatin-positive cells (no.per mm) in antrum	fundus	Leptin-positive cells (no.per mm) in fundus	Ghrelin-positive cells (no.per mm) in fundus
I (6)	0.42±0.03	14.1±1.5	50.6±2.6	36.9±4.9	18.4±2.3
II (6)	0.39±0.03	16.0±2.3	43.9±2.2	46.5±7.2	14.9±1.2
III (6)	0.43±0.04	15.8±2.0	38.2±2.0	45.1±4.7	17.2±1.6
IV (6)	0.43±0.03	20.5±1.8	42.5±1.2	43.4 ±4.1	14.5±0.6
V (6)	0.42±0.02	16.8±1.2	38.4±1.3	50.9±5.4	15.0±1.4
VI (6)	0.39±0.02	14.7±0.9	38.4±1.6	54.4±4.6	14.3±1.2

Means ± SEM. Immunohistochemistry was performed with antibodies to pancreastatin, leptin or ghrelin, respectively, and the number of immunoreactive cells was counted as number per mm length of horizontal mucosa. No significant difference between the cheese groups (II/III and V/VI) and the corresponding control groups (I and IV).

Discussion

In the present study, the groups of mice were given the laboratory standard mouse/rat diet supplemented *ad libitum* with either of two kinds of cheese, both of which contain much more fat than standard pellets. Although neither the young nor the adult mice became hyperphagic when given the opportunity to eat cheese, animals in both age groups showed increases in their fat intake and body weight during the observation period of 7 weeks. It is generally believed that sweet-tasting diets promote hyperphagia and obesity in rodents (*Sclafani, 1987*). On the other hand, it was shown that mice fed with a low-fat, high-sucrose diet were leaner than mice fed with a high-fat, high-sucrose diet, and in the absence of fat, free access to sucrose had no effect on the body weight *(Surwit et al. 1995; Black et al. 1998)*. In the present study, we chose the Norwegian "brown cheese" as a source of fat and carbohydrate because it has a soft solid consistency, and it tastes sweet without containing any sucrose. We found that the mice given "brown cheese" as a diet supplement consumed as much fat as did the mice fed with white cheese. However, the "brown cheese" did not induce hyperphagia, and, although its consumption caused overweight in the adult mice, it did not show this effect in the young, despite an increased energy intake also in this group. A main difference between the mice eating white cheese and those eating "brown cheese" was that the intake of carbohydrate was lower in the former group. However, carbohydrate is more thermogenic than fat, and energy expenditure is higher when a positive energy balance is produced by a diet with a high carbohydrate/fat ratio than if the ratio is low *(Astrup, 2000)*. This suggests that the tendency to develop obesity may depend on some age-specific factor in addition to being a consequence of the food intake (see also *Bachmanov et al. 2001*).

The mechanism of diet-induced obesity is unclear. In the present study, we have focused on the endocrine function of the stomach, which is a source of several circulating peptides/hormones. Pancreastatin (together with histamine), the secretion of which is regulated by a gastrin dependent pathway, is released from the ECL cells in the oxyntic mucosa of the stomach in response to food intake *(Håkanson et al. 1995)*. The physiological significance of pancreastatin is unknown. In the present study, the serum gastrin concentration did not differ between the various groups of mice, and the number of cells that produce pancreastatin (most are ECL cells) was found to be unchanged. The stomach has been reported also to be a source of leptin, which is the product of the ob gene and which provides feedback information on the size of fat stores to central ob receptors that control food intake and body weight homeostasis *(Bado et al. 1998)*. Gastric leptin may also have a local effect, contributing to the control of body weight *(Azuma et al. 2001)*. In the present study, the leptin-immunoreactive cells were found to distribute mainly in the basal part of the oxyntic mucosa, most likely in a sub-population of chief cells. The cell density was unchanged in the overweight mice regardless of their age or diet, although the serum leptin levels seemed to be elevated with age but independent on the diet composition. Recently, the novel hormone ghrelin was found to be produced in the A-like cells in the stomach *(Kojima et al. 1999; Dornonvill de la Cour et al 2001)*. The ghrelin mRNA level in the stomach was increased but the peptide content decreased in response to fasting, and both returned to normal after refeeding *(Toshinai et al. 2001)*. In keeping with this, ghrelin has been shown to be a stomach derived appetite-stimulatory signal *(Asakawa et al. 2001)*, the serum levels of which are high during starvation and lowered in response to feeding both in rodents and humans *(Dornonvill de la Cour et al. 2001; Tschop et al. 2001)*. Studies in rats and mice have shown that peripheral daily administration of ghrelin increased body weight gain *(Tschop et al. 2000)*, and ghrelin has therefore been proposed to play a role in the development of adiposity *(Kojima et al. 1999; Tschop et al. 2000)*. In the present study, we found no responses either in the serum ghrelin levels or in the A-like cell density to high fat or high fat plus carbohydrate diet, either in young or in adult animals. The results of the present study do not provide evidence that gastric ghrelin is involved in high fat diet-induced body weight gain in mice. On the other hand, a smaller body weight gain induced by long-term omeprazole treatment in young rats was also found not to be associated with gastric ghrelin *(Cui et al. 2001)*. However, these "negative" data do not rule out the possibility of ghrelin's role in other experimental settings.

The propensity for diet induced obesity seems to vary among strains of rats and mice. Thus, C57/JB mice are reportedly more sensitive in this respect than the strain we chose. In addition to the age related differences described above, genetic factors may either protect animals from becoming overweight or predispose them to such an outcome when placed in an environment where large quantities of dietary fat are available.

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