Enterostatin - target proteins and intracellular mechanisms. Function in food intake and energy metabolism

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Function in food intake and energy metabolism

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Abstract
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In this thesis, a possible target protein for enterostatin has for the first time been found in a neuronal cell line and further identified in rat brain membranes. The target protein was surprisingly identified as the beta-subunit of the F1F0-ATP synthase. The binding of enterostatin to the beta-subunit was further verified in pure bovine F1-ATPase and was also demonstrated in the insulin producing cell line, INS-1. In INS-1 cells, the targeting of enterostatin resulted in decreased ATP synthesis, enhanced heat production and increased oxygen consumption. Enterostatin was further shown to decrease the insulin secretion in these cells. In addition, in an animal experiment, enterostatin was shown to decrease the feed efficiency in rats eating high fat food. Altogether, this indicates that enterostatin is involved in the regulation of energy metabolism.

The binding of enterostatin to neuronal cells and F1-ATPase was shown to be inhibited by μ-opiate agonists, e.g. beta-casomorphin, but not by a kappa-opiate agonist. In rats, the inhibition of high-fat food intake by intravenous injection of enterostatin in low doses was abolished after simultaneous equimolar administration of beta-casomorphin. However, higher doses of enterostatin instead increased the high-fat food intake, and a synergistic increase on food intake together with beta-casomorphin was demonstrated.

The association between insulin secretion and uncoupling protein-2 (UCP2) expression was compared in INS-1 cells after long-term exposure to oleic acid in combination with forskolin and TNF-alpha, both substances known to influence the insulin secretion. It was concluded that fatty acid-induced increase in UCP2 expression is not always associated with a decrease in insulin secretion.

Key words: enterostatin, appetite, satiety, obesity, fat intake, F1F0-ATP synthase, ATP, energy metabolism, insulin, uncoupling protein, beta-casomorphin, opiate, SK-N-MC, INS-1

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ENTEROSTATIN - TARGET PROTEINS AND INTRACELLULAR MECHANISMS

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Hälsoråd

Luft är en blandning av kväve och syre.
Akta dig noga för kvävet mitt pyre!
Kväve ger krut och riskabelt nitrat
medan syret förbränner vår mat.

Berselia Schele

Ur Alf Henriksson Samlade dikter

CONTENTS

ABSTRACT

Due to the increasing prevalence of obesity worldwide, it is of great importance to understand the mechanisms behind appetite and energy metabolism. Hunger and satiety are the results of complex neural events that involve several neurotransmitters and peptides. Enterostatin is an appetite-regulating peptide produced by cleavage of procolipase, which is released from pancreas in response to fat ingestion. Procolipase is cleaved by trypsin in the intestine to form enterostatin and colipase, an obligatory cofactor for pancreatic lipase during fat digestion. Enterostatin has been shown to specifically decrease fat intake, but has also shown metabolic effects like inhibition of insulin secretion, stimulation of sympathetic activity and increased expression of uncoupling proteins (UCPs). Enterostatin has both central and gastrointestinal sites of action, although the gastrointestinal action is dependent on afferent vagal transmission.

In this thesis, a possible target protein for enterostatin has for the first time been found in a neuronal cell line and further identified in rat brain membranes. The target protein was surprisingly identified as the beta-subunit of the F1F0-ATP synthase. The binding of enterostatin to the beta-subunit was further verified in pure bovine F1-ATPase and was also demonstrated in the insulin producing cell line, INS-1. In INS-1 cells, the targeting of enterostatin resulted in decreased ATP synthesis, enhanced heat production and increased oxygen consumption. Enterostatin was further shown to decrease the insulin secretion in these cells. In addition, in an animal experiment, enterostatin was shown to decrease the feed efficiency in rats eating high fat food. Altogether, this indicates that enterostatin is involved in the regulation of energy metabolism. The binding of enterostatin to neuronal cells and F1-ATPase was shown to be inhibited by μ-opiate agonists, e.g. beta-casomorphin, but not by a kappa-opiate agonist. In rats, the inhibition of high-fat food intake by intravenous injection of enterostatin in low doses was abolished after simultaneous equimolar administration of beta-casomorphin. However, higher doses of enterostatin instead increased the high-fat food intake, and a synergistic increase on food intake together with beta-casomorphin was demonstrated. The association between insulin secretion and uncoupling protein-2 (UCP2) expression was compared in INS-1 cells after long-term exposure to oleic acid in combination with forskolin and TNF-alpha, both substances known to influence the insulin secretion. It was concluded that fatty acid-induced increase in UCP2 expression is not always associated with a decrease in insulin secretion.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. **Berger, K.**, Sörhede Winzell, M. and Erlanson-Albertsson, C. Binding of enterostatin to the human neuroepithelioma cell line SK-N-MC. *Peptides* 19 (9), 1525-1531, 1998.

II. **Berger, K.**, Sivars, U., Sörhede Winzell, M., Johansson, P., Hellman, U., Rippe, C. and Erlanson-Albertsson, C. Mitochondrial ATP synthase – a possible target protein in the regulation of energy metabolism *in vitro* and *in vivo*. *Nutritional Neuroscience* 5 (3), 201-210, 2002.

III. **Berger, K.**, Sörhede Winzell, M., Mei, J. and Erlanson-Albertsson, C. Intravenous enterostatin and β-casomorphin may use the same receptor system in regulating fat intake. Manuscript

IV. **Berger, K.**, Kvist Reimer, M., Gustafsson, M., Ahrén, B. and Erlanson-Albertsson, C. Relation between insulin secretion and fatty acid induced UCP2 expression in INS-1 cells. Submitted manuscript, 2003.

ABBREVIATIONS

ADP	adenosine diphosphate
ApoA	apolipoprotein A
ATP	adenosine triphosphate
ATPase	ATP synthase
BAT	brown adipose tissue
β-CM	β-casomorphin
BBB	blood brain barrier
BMCP1	brain-specific mitochondrial carrier protein 1
BMI	body mass index
BS^3	bis(sulphosuccinimidyl) suberate
cAMP	cyclic adenosin monophosphate
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
CNS	central nerve system
CoA	Coenzym A
EE	energy expenditure
EI	energy intake
Ent	enterostatin
FAD	flavin adenine dinucleotide
$FADH_2$	dihydroflavin adenine dinucleotide
FFA	free fatty acid
G6P	glucose-6-phosphate
GDP	guanosine diphosphate
GI	gastrointestinal
GK	glucokinase
GLUT	glucose transporter
5-HT	5-hydroxytryptamin, serotonin
HDL	high density lipoprotein
HF	high-fat
IC_{50}	concentration for 50 % inhibition
i.c.v.	intracerebroventricular
i.d.	intraduodenal
i.p.	intraperitoneal
i.v.	intravenous
K_d	Dissociation constant
kDa	kilo Dalton
LCFA	long chain fatty acids
LF	low-fat
LH	lateral hypothalamus
MALDI-TOF-MS	matrix-assisted laser desorption ionisation time of flight mass spectrometry
mRNA	messenger ribonucleic acid
NA	noradrenalin
NAD	nicotinamide adenine dinucleotide
NADH	dihydronicotinamide adenine dinucleotide
NPY	neuropeptide Y
NTS	nuceus tractus solitarus
OM	osborne-mendel
PVN	paraventricular nucleus
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate polyacrylamid gelelectrophoresis
SNS	sympathetic nervous system
UCP	uncoupling protein
VMN	ventromedial nucleus
WAT	white adipose tissue
WT	wild-type

GENERAL INTRODUCTION

The ability to assure constant availability of energy despite fluctuations in the energy supply in the environment is of highest importance for survival. Higher organisms have solved this problem by developing the capacity to store excess energy as triglycerides in adipose tissue, from which stored energy could be rapidly released. During prolonged starvation the decrease in energy stores is sensed, leading to decreased energy expenditure. On the contrary, during prolonged nutritional abundance, the voluntary food intake is reduced and energy expenditure increased to avoid excessive energy storage. Despite of the regulation system the prevalence of obesity, for adults as well as for children, has increased to epidemic proportions in both developed and developing countries during the past few decades. According to the World Health Organisation (WHO), overweight and obesity are now so common that they are replacing the more traditional public health problems like undernutrition and infectious diseases as the most significant contributor to ill-health (WHO, 1998). There are several complications associated with obesity, including type 2 diabetes (non-insulin dependent diabetes mellitus), cardiovascular diseases, cancers, gastrointestinal diseases, respiratory dysfunctions and arthrosis (Pi-Sunyer, 1993).

The fundamental causes of the increasing obesity are the sedentary lifestyle and an excess of food with high energy density, resulting in low energy expenditure combined with a high energy intake. Hence, to reduce the obesity, there must be a reduction of total energy intake in combination with an increase in energy expenditure. Even though it immediately seems to be an easy problem to solve, a change in the energy balance could be compensated by adaptations and regulations of regulatory systems. Since it is genetically important to preserve body mass in periods of starvation, there is a strong defense against undernutrition and weight loss while the defense against overweight is much weaker. The treatment of obesity would be facilitated if we had better knowledge about the genetic, cellular and physiological control of the energy regulation and energy balance.

The regulation of appetite and food intake is a complex part in this network. Hunger and satiety are the results of complex neural events that involve actions by, and interactions between, neurotransmitters and peptides released from the nervous system, gastrointestinal tract, adipose tissue, exocrine glands and endocrine glands. In this thesis I have primarily studied one of the components in this regulating system, the peptide enterostatin.

BACKGROUND

Obesity

The prevalence of obesity has developed into a global problem and is not restricted to the more developed countries. With increasing "Westernisation", the frequency of overweight and obesity appears to be rising even in those countries with current food deficiency (fig. 1). In the majority of the European countries, the obesity problems have increased by about 10-40% in the past 10 years (IOTF) and today there are more than 135 million obese Europeans. Sweden is still one of the European countries with lowest proportion of obese citizens, but it is calculated that Sweden will reach the level of USA in 5-10 years (table 1). The World Health Organization (WHO) has since the 1990s decided to develop strategies to prevent the increasing epidemia (WHO, 1998). The WHO has a system for classification of overweight and obesity in adults based on body mass index (BMI) calculated as weight in kg divided by the square of the height in meter (kg/m^2) (table 2). However, BMI is not a perfect way for classification of obesity since the accumulation of body fat, especially abdominal fat, is of importance in the evaluation of obesity and the related diseases. A weakness of the BMI is that high values obtained for muscular individuals with low body fatness give a false indication of overweight. For a more accurate classification according to health risks, BMI should be complemented with waist girth or waist/hip ratio.

Table 1. The increasing prevalence of obesity (BMI≥30) in Sweden (Lissner *et al.*, 2000)

	Men	Women
1980/81	6.6 %	8.8 %
1988/89	7.3 %	9.1 %
1996/97	10.0 %	11.9 %

Most individuals maintain a relatively stable body weight, which is a result of energy intake (EI) and energy expenditure (EE) being in equilibrium. Weight gain is a result of a positive energy balance, EI>EE, where the excess of energy is stored as fat in the adipose tissue. This can be the result from increased energy

intake, especially from energy-dense food like fat (Westerterp-Plantenga, 2001) or decreased energy expenditure, as reduced physical activity (Westerterp, 2001).

Table 2. The WHO definitions (WHO, 1998)

Classification	BMI (kg/m^2)	Associated health risk
Underweight	<18.5	Low (but risk of other clinical problems increased)
Normal range	18.5-24.9	Average
Overweight	25.0-29.9	Mildly increased
Obese	**≥30.0**	
Class I	30.0-34.9	Moderate
Class II	35.0-39.9	Severe
Class III	≥40.0	Very severe

Obesity related genes and environmental changes

What has changed during the last decades causing the dramatic global rise in overweight and obesity? The body weight and body composition are determined by interactions between genetics and environment. The genes have not changed much during this time while the environment is completely different regarding the access to food. In the past, with periods of poor access to food, those who had the capacity to store energy as fat had a survival advantage in times of starvation. Thus, individuals with genetic or other biological predisposition for obesity were in advantage, but now the opposite is true for such individuals. The "thrifty gene hypothesis" postulates that certain populations have mutations that increase the ability for fat storage which in time of famine is positive for survival (Groop and Tuomi, 1997). The life style today, at least in the Western part of the world, is characterized by a supply of relatively inexpensive, palatable and energy-dense food, in combination with low required physical activity. Such a lifestyle promotes high energy intake and low energy

expenditure leading to epidemic obesity (Hill and Peters, 1998). In addition to the changed environment regarding food and its availability the physical activity, an important part in the energy expenditure, used to be at a much higher level (Egger *et al.*, 2001).

The phenotypes of obesity, like BMI and fat mass, are estimated to be inheritable to an extent of at least 40% in average (Comuzzie and Allison, 1998). There are numerous genes being postulated as candidate genes for human obesity, e.g. genes for regulating obesity, energy balance, feeding behaviour, appetite regulation, satiety and adipose differentiation. Some of these proteins or peptides coded by the postulated genes will be described later in this thesis. The most common forms of human obesity are multifactoral and arise from the interaction of multiple genes, environmental factors and behaviour.

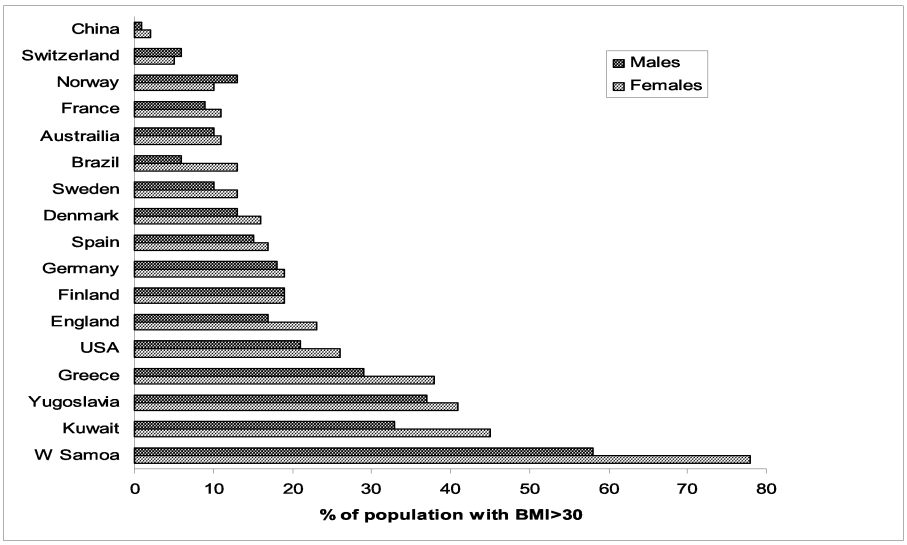

Figure 1. Global prevalence of obesity. Obesity defined as BMI ≥ 30. Surveys conducted between 1988 and 1994 (IOTF).

The metabolic syndrome and type 2 diabetes

Health risks increase progressively as overweight increases. The risk of developing type 2 diabetes is increased six fold for women who are even slightly overweight and up to 90 times greater for the severely obese (IOTF). The symptoms developing as a consequence of obesity have given rise to the concept of the metabolic syndrome. The WHO recently published definitions of the metabolic syndrome. For men it is defined as insulin resistance or presence of impaired glucose tolerance or type 2 diabetes and the presence of at least two of the following criteria (values for men): abdominal obesity (waist/hip ratio > 0.90 or BMI ≥ 30), dyslipidemia (serum triglycerides ≥ 1.70 mM or HDL cholesterol < 0.9 mM), hypertension (≥160/90 mmHg) or microalbuminuria (Alberti and Zimmet, 1998). Most type 2 diabetes patients have the metabolic syndrome before onset of the diabetes. This was shown in a study on men who met the WHO definitions of the metabolic syndrome. These had a nearly ninefold greater likelihood of developing diabetes than healthy men (Laaksonen *et al.*, 2002). Definitions of the metabolic syndrome can thus be used to detect new cases of diabetes prospectively.

Type 2 diabetes, also known as non-insulin dependent diabetes mellitus (NIDDM), is associated with severe insulin resistance in the peripheral tissue, combined with impaired insulin secretion. Before the onset of the type 2 diabetes, insulin resistance may exist for a decade or more. The insulin resistance is initially compensated by increased insulin secretion with resulting maintained normal plasma glucose levels. Eventually, the β-cells may not be able to compensate for the increasing resistance, and the β-cells lose the secretory function (LeRoith, 2002). The result is a loss of secretory function and hyperglycemia. The earliest physiologic indication of β-cell dysfunction is a delay in the first phase of the insulin response to glucose that begins immediately after food intake and ends within 20 minutes. The effect of this first response is to shift hepatic glucose metabolism from a state of production to a state of uptake of glucose. The second peak of insulin secretion begins at about 20 minutes after food intake and lasts for 20-40 minutes. The result is a sharp rise in postprandial glucose, and to dispose of it, a hyperinsulinemic second-phase insulin response (Porte and Kahn, 1995). Although genetically influenced, insulin resistance and β-cell dysfunction are closely associated with obesity and are probably mediated by chronically elevated levels of fatty acids and glucose. Unlike genetic defects, these problems may be temporary and reversible if the metabolic conditions can be corrected. Randle *et al.* (Randle *et al.*, 1965) showed early that increased availability of FFAs inhibited carbohydrate oxidation and glucose uptake in rat muscle cells. The insulin resistance itself

enhances the release of FFAs from adipose tissue, especially from abdominal adipose tissue. Abdominal fat cells quickly break down stored lipids with resulting increased levels of fatty acids in the blood stream (Katzel *et al.*, 1992). The resulting oversupply of FFAs competes with glucose metabolism and the cells further increase their rate of FFA oxidation (LeRoith, 2002) and inhibits the responsiveness of β-cells to glucose stimulation (fig. 5). This might be a consequence of downregulation of the expression of acetyl-CoA carboxylase, an enzyme involved in the regulation of malonyl-CoA, which is a key regulator of FFA oxidation (Brun *et al.*, 1997). A decrease in signalling molecules such as malonyl-CoA has also been a proposed mechanism in the glucotoxicity that desensitizes the β-cells to glucose stimulation, caused by chronic hyperglycemia in combination with hyperlipidemia (Prentki and Corkey, 1996). In addition, glucotoxicity is proposed to give rise to desensitization of the ATP-dependent potassium channels crucial for insulin secretion (Aguilar-Bryan and Bryan, 1999) (fig. 2), and enhance the insulin resistance through downregulation of the glucose transporter system (LeRoith, 2002).

The pancreatic islet of Langerhans

The islets of Langerhans are distributed throughout the pancreas, forming endocrine islets with a diameter from 0.4 to 4 μm, in the exocrine pancreas. The total mass is 1-2% of the entire gland. The islet consists of four major cell types; the centrally located insulin-producing β-cells (60-80% of the islet cell mass) and the surrounding glucagon-producing α-cells, somatostatin-producing δ-cells and pancreatic polypeptide-producing F-cells. Additionally, other regulatory peptides such as islet amyloid polypeptide (IAPP) and neuropeptide Y (NPY) are released from the islets. The secretion of hormones from the islet is stimulated by endocrine, paracrine and nervous factors. The vessels of the islet differ from those of the exocrine pancreas in that they are wider, thinner-walled and more fenestrated and thereby able to facilitate a rich exchange with the endocrine cells (Henderson and Moss, 1985). The pancreatic islet is richly enervated by parasympathetic, sympathetic and sensory nerves (Ahrén, 2000). Insulin secretion is stimulated by parasympathetic nerves or their neurotransmitters and inhibited by the sympathetic nerves and their neurotransmitters. The autonomic nerves seem to be of importance in mediating the cephalic phase of insulin secretion, in the regulation of islet hormone secretion and optimizing islet hormone secretion during metabolic stress like hypoglycaemia (Ahrén, 2000).

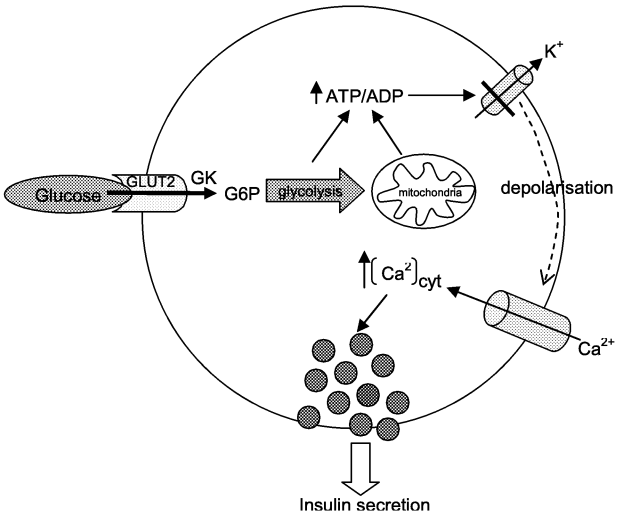

Figure 2. Glucose induced insulin secretion in the pancreatic β-cell. Glucose is transported into the cell by the GLUT2 receptor. Glucose is immediately phosphorylated to glucose-6-phosphate (G6P) by the rate limiting enzyme glucokinase (GK). G6P is metabolized in glycolysis, citric acid cycle, electron transport chain and finally the oxidative phosphorylation of ADP by F_1F_0-ATPase, resulting in the end product ATP. The increased ATP/ADP ratio causes a closure of the ATP-dependent K^+-channels and the following depolarisation of the cell results in an opening of Ca^{2+}-channels and subsequent increase of intracellular Ca^{2+}, which stimulates secretion of insulin from the granule.

β-cell and insulin secretion

The initial step in the glucose-induced insulin secretion is the uptake of glucose to the β-cell by the glucose transporter GLUT2 (fig. 2). When inside the cell, glucose is phosphorylated to glucose-6-phosphate by the high-K_m glucokinase, which is rate limiting for glucose metabolism and viewed as a glucose sensor. Glucose is further metabolized by glycolysis, citric acid cycle, electron transport chain and finally oxidative phosphorylation and ATP production by F_1F_0-ATP synthase. The glucose dependent intracellular increase in ATP/ADP ratio causes closure of ATP-dependent K^+-channels of the plasma membrane leading to

depolarisation of the cell membrane which in turn opens voltage-sensitive Ca^{2+}-channels. The influx of Ca^{2+} increases the normally low intracellular concentration of Ca^{2+} approximately 10-fold which stimulates insulin secretion from storage granule (fig. 2). Not only glucose is able to induce stimulation of insulin secretion although it is the most important secretagogue. Hormones and neurotransmitters as well as amino acids and fatty acids are involved in the regulation of insulin secretion, using different intracellular signalling pathways.
Normal insulin action in muscle and fat cells begins when the postprandial glucose levels rise, the subsequent increase in circulating insulin activates binding of insulin to its cell membrane receptor, whereupon the glucose transporter protein GLUT4, stored in vesicles within the cell, is translocated from the storage compartment to the plasma membrane. This process is reversible such that when circulating insulin levels decline, GLUT4 transporters are removed from the plasma membrane by endocytosis. The complexity of these regulatory processes provides numerous potential targets that may be defective and eventually result in peripheral tissue insulin resistance and possibly diabetes (Watson and Pessin, 2001).

Appetite and satiety

According to Blundell the biopsychological system for expression of appetite is present at three levels: Psychological events (hunger perception, cravings) and behaviour operations (meals); peripheral physiology and metabolic events; and neurotransmitter and metabolic interactions in the brain (Blundell, 1991). Appetite is a synchronous operation of events and processes on all three levels. Neuronal events trigger behaviour which in turn involves a response in the peripheral physiological system which activates the brain and decide the strength of motivation and willingness to feed. The eating behaviour of mammals is characterized by eating periods interspersed with periods of non-eating. When food consumption suppresses hunger and inhibits further eating, two processes are involved; satiation and satiety (Blundell, 1991). Satiation is the process which brings a period of eating to an end, controlling meal size. Satiety is defined as inhibition of hunger and eating as a consequence of the meal, controlling of post-meal interval. Satiety is divided into four mediating processes classified as sensory, cognitive, pre-absorptive and post-absorptive, together referred to as the satiety cascade (fig. 3). Even before the start of a meal, there is a cephalic phase of appetite, when physiological signals are generated by the smell, sight and thought of food (Powley, 1977). The cephalic

phase responses gear up the body to better absorb and use ingested nutrients of food by increased salivation, gastric activity and insulin release (Nederkoorn *et al.*, 2000). During the satiation process, when ingesting the meal, the afferent signals provide the major control over appetite. According to Smith *et al.*, the afferent signals from the mouth provides primarily positive feedback for eating while the signals from the stomach and small intestine primarily act as negative feedback regulators (Smith, 1990). In the early satiety, the sensory processes like smell, taste, texture and visual appearance are factors terminating the meal. The cognitive processes following the sensory processes in the satiety cascade are characterized by the beliefs and psychological feelings about the properties of foods.

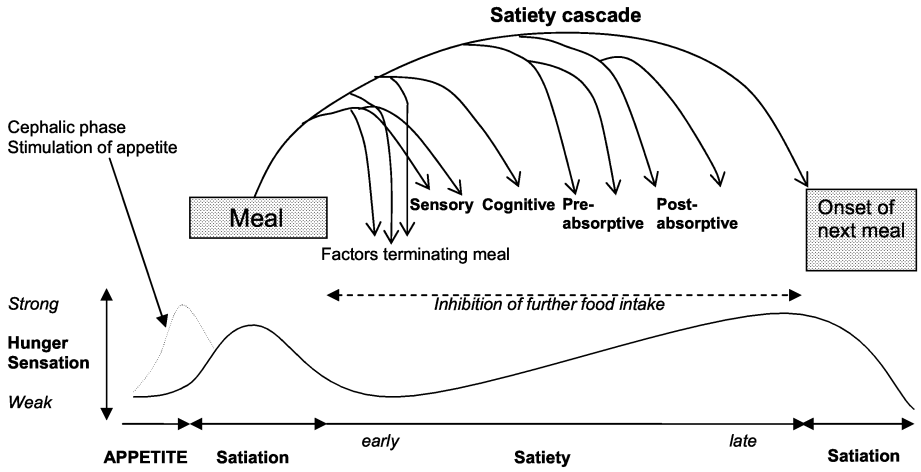

Figure 3. The satiety cascade consists of two phases of appetite, satiation and satiety, which outlines the processes during and following a meal. These are ordered into four broad categories overlapping each other: sensory, cognitive, pre-absorptive and post-absorptive effects. Sensory components refer to the mouth sensory effects of the physical properties of the food consumed, cognitive effects refer to the beliefs about the food, pre-absorptive effects are effects of the food prior to absorption of the nutrients and post-absorptive effects are after the metabolites have reached the blood stream. Figure modified from Blundell and Green *et al.* (Blundell, 1991 11; Green *et al.*, 1997).

When the food reaches the gastrointestinal tract, chemo- and mechanoreceptors monitor activity and send information to the brain, mainly via the vagus nerve. This afferent signals together with the release of hormones (e.g. insulin, enterostatin, CCK) act as satiety signals in the post-ingestive control of appetite (fig. 11). After digestion of the nutrients, i.e. during the post-absorptive phase in late satiety, the metabolites reach the circulation. The idea of the satiety cascade is that the nutrient composition will affect the satiety mediating processes and will therefore exert different effects on satiation and satiety (Kovacs, 2002).

Fat as macronutrient

The ingestible fat consists to 95% of triacylglycerol and the rest is phospholipids, free fatty acids, cholesterol and fat-soluble vitamins (A, D, E and K). The composition of the macronutrient intake differs much between populations, and as a result, the amount of energy from ingested fat diverges between countries. In Greenland, more than 50 % of energy comes from fat (primarily marine), while in Africa it is about 6 %, USA 42 % and Sweden 38%. The Swedish government recommends a decrease in fat intake to 30 % of energy intake (SLV, 2003). A general decrease in fat consumption would most probably result in a general decrease in obesity (Bray and Popkin, 1998). High-fat diets lead to high levels of energy intake, due to passive overconsumption. Foods high in dietary fat have a weak effect on satiation and has a weaker effect on postingestive satiety compared to other macronutrients (Blundell *et al.*, 1996). The overconsumption is dependent upon both the high energy density and high palatability of high-fat food (Blundell and MacDiarmid, 1997). After an increase of dietary fat, the body should normally react to maintain energy balance, either by oxidizing the fat because of the need of energy or by regulation by subsequent reduced fat intake. Several studies have tested if addition of fat to a meal increases fat oxidation or energy expenditure, but according to Bennet *et al.* no such correlation have been established (Bennett *et al.*, 1992). On the other hand, fatty acid oxidation has been suggested to have a role in the maintenance of satiety, since inhibition of fatty acid oxidation has been shown to be associated with enhanced high-fat consumption due to a decrease in the intermeal interval (Langhans and Scharrer, 1987b). This proposed satiety signal of fatty acids seems to be mediated by vagal afferent transmission after hepatic fatty acid oxidation (Langhans and Scharrer, 1987a). Addition of carbohydrates to a meal has been shown to increase the

carbohydrate oxidation (Bray and Popkin, 1998). Bray and Popkin have reviewed results from 28 clinical trials regarding reduced fat consumption (Bray and Popkin, 1998). They showed that a reduction of 10 % in the proportion of energy from fat was associated with an average reduction of bodyweight of 16 g/day, and thus conclude that the relative amount of dietary fat plays a role in the development of obesity.

Fat digestion

In most mammals the pre-duodenal digestion of fat starts in the stomach by gastric lipase, in humans secreted from the chief cells in the fundus region (Moreau *et al.*, 1988b). However, in rodents there is instead a lingual lipase secreted from serous glands present at the posterior part of the tongue, and in polygastric species the pre-duodenal lipase is a pharyngeal lipase released from the glosso-epiglottic area (DeNigris *et al.*, 1988; Embleton and Pouton, 1997; Moreau *et al.*, 1988a). Pre-duodenal lipases are structurally identical and are all active in the acidic environment of the stomach. The fat is partly emulsified in the stomach by physical forces and by dietary phospholipids that absorb to the oil/water interface and envelopes the triglyceride droplets. Thereafter, the pre-duodenal lipase spontaneously initiate the hydrolysis of the emulsion particle, resulting in the release of diglycerides and fatty acids (Carriere *et al.*, 1993). As much as 10-40 % of the fat digestion occurs in the stomach and this gastric lipolysis is further believed to be important for initiating intestinal lipolysis (Hamosh, 1984) (Carriere *et al.*, 1993).

When the chyme reaches the duodenum, it is neutralized by the bicarbonate-rich pancreatic juice and further emulsified by amphiphilic molecules like bile salt, phospholipids and cholesterol present in the bile secreted from the liver via the gall bladder. Biliary compounds interfere with the oil-in-water emulsion and the phospholipids stabilize the emulsion while the bile salts destabilize the substrate interface, which leads to increased availability of tri-and diacylglycerols for lipolysis by the pancreatic lipase-colipase complex (Borgström *et al.*, 1985). Alone, pancreatic lipase is however strongly inhibited by the bile salt covering the interface (Borgström and Erlanson, 1973), but in presence of colipase this inhibition is overcome. Colipase is secreated as a 10 kDa procolipase from pancreas and processed in duodenum by trypsin through cleavage of an N-terminal pentapeptide, enterostatin, to its mature form colipase (Borgström *et al.*, 1979; Erlanson-Albertsson, 1981). Colipase binds to lipase in a 1:1 molar ratio and also binds to the bile-salt covered triacylglycerol interface, and in this

way anchors the enzyme-complex to the substrate (Erlanson-Albertsson, 1992b). Colipase activates lipase by stabilizing an amphiphilic lid in the open conformation, exposing a hydrophobic surface and the active site to the underlying di-and triglycerides (van Tilbeurgh *et al.*, 1993).
A gastric colipase, identical to pancreatic procolipase, has also been identified in chief cells in rat stomach (Sörhede *et al.*, 1996b; Winzell *et al.*, 1998). Since gastric lipase is independent of colipase, this gastric colipase may act complementary to pancreatic colipase in the activation of pancreatic lipase. Recently, D'Agostino et al described a procolipase deficient (-/-) mouse (D'Agostino *et al.*, 2002). These mice had decreased postnatal survival and weight gain, a steatorrhea when eating high-fat diet, and reduced body weight even when eating standard diet, demonstrating an important role for procolipase in dietary fat digestion.

Fat absorption

The fat digestion is a very efficient process, and more than 95% of the digested fat is absorbed (Miled *et al.*, 2000). The products of pancreatic lipase digestion are 2-monoglycerides and free fatty acids. Only 30% of the triglycerides are completely hydrolyzed to glycerol and free fatty acids. The products are poorly soluble in water and very slowly absorbed from the luminal aqueous environment (Sanford, 1992). A more efficient digestion and complete and rapid absorption is achieved by formation of micelles with capability of incorporating the digestion products in a water-soluble form. The micelles are formed from bile acids, and together with monoglycerides, free fatty acids and fat-soluble vitamins they form mixed micelles. The micelles form complexes with colipase at the oil-water interface, which brings the micelle close to the site of hydrolysis, facilitating the removal of the end products and preventing feedback inhibition. A further important function of the micelles is to carry the fatty acids and monoglycerides to the absorptive surface of the intestine (Sanford, 1992). The fat digestion products are absorbed mainly in the duodenum and proximal jejunum after release from micelles. The low pH in the unstirred water layer at the luminal face of the intestine contributes to fatty acid absorption by reducing the solubility of fatty acids in micelles. The uptake of long-chain fatty acids (LCFA) over the enterocyte membrane is mediated by a fatty acid binding protein (Stremmel, 1988), in combination with passive diffusion, while short- and medium-chain only enter by passive diffusion and pass directly to the portal blood. Fatty-acid chain length and number of double bonds influence fat-

absorption. Medium-chain fatty acids are better absorbed than long-chain fatty acids and unsaturated fatty acids are more efficiently absorbed than saturated fatty acids (Ramirez *et al.*, 2001).
Inside the enterocyte the absorbed LCFAs are re-esterified in the smooth endoplasmatic reticulum. Triglycerides, phospholipides, cholesterol and apoproteins together form chylomicrons, which are secreted to the lymph, which is emptied into the blood stream through the thoracic duct. In peripheral tissues, triacylglyerol is taken up, cleaved by lipoprotein lipase and the chylomicron remnants are gradually reduced in size and eventually taken up by the liver (Ramirez *et al.*, 2001 51).

Thermogenesis

Energy expenditure can be subdivided broadly into two categories of thermogenesis: *obligatory* and *adaptive* (facultative). Obligatory thermogenesis is essential for the survival of all cells of the body and for the maintenance of normal and constant body temperature (endothermy). The largest component of obligatory thermogenesis is provided by the basal metabolic rate, which represents 60-70 % of the total energy expenditure. Basal metabolic rate is measured in the resting and postabsorbtive state in a thermoneutral environment (Argyropoulos and Harper, 2002). One component in the obligatory thermogenesis is the diet-induced heat production that results from the digestion, absorption and metabolism of dietary nutrients. The most important endocrine factors regulating the obligatory thermogenesis are the thyroid hormones.
In contrary to the obligatory thermogenesis that occurs continuously in all organs of the body, the adaptive thermogenesis can be switched on and off and occurs mainly in brown adipose tissue (BAT) and skeletal muscle (Argyropoulos and Harper, 2002). The energy expenditure during exercise occurs mainly in the skeletal muscle. During exposure to cold, heat is produced by shivering thermogenesis in the muscle and non-shivering thermogenesis in BAT. BAT thermogenesis is important in the adaptive thermogenesis in many mammals and its activity is mainly regulated by noradrenalin and the sympathetic nervous system. BAT is a highly sympathetic innervated interscapular tissue, present in several mammals, i.e. rodents and newborn animals and is particularly important for hibernators. Non-shivering thermogenesis could also be induced by high-fat food, called diet-induced thermogenesis.

Uncoupling proteins (UCPs)

ATP has to be synthesized continuously in the mitochondria to be used in ATP-dependent processes like the Na^+/K^+ pump (20% of ATP consumption), protein turnover (12-25%) and the Ca^{2+} pump (4-6%). The substrates for production of ATP are fat, carbohydrates and proteins that are metabolized, resulting in the production of NADH and $FADH_2$. Subsequently NADH and $FADH_2$ are oxidized to NAD^+, FAD and H^+ in the respiratory chain in the inner mitochondrial membrane and the protons are, during the oxidation reaction, transported to the intermembrane space (fig. 4). The generated proton gradient causes protons to flow back to the mitochondrial matrix through F_1F_0-ATP synthase, and the energy thus generated is used to transform ADP to ATP. In this way, substrate oxidation is coupled to the formation of ATP (fig. 4).

The coupling between substrate oxidation and ATP formation is not 100% efficient. The proton gradient can be reduced by proton leakage (fig. 4). Thereby the efficiency of ATP synthesis from substrate oxidation is diminished. In this proton leakage, uncoupling proteins (UCP) are involved. The uncoupling proteins are a family of proteins located in the inner mitochondrial membrane. The name uncoupling protein arises from the ability of the protein to uncouple the proton gradient created over the inner mitochondrial membrane from the synthesis of ATP. Thereby heat is produced instead of ATP. These proteins could either transport protons into the mitochondrial matrix or transport free fatty acid anions out of the matrix, both processes reducing the proton gradient.

UCP1

The uncoupling protein present in brown adipose tissue (BAT) mitochondria (UCP1) was discovered 25 years ago (Nicholls *et al.*, 1978) and was considered as an adaptation of mammalian tissue to non-shivering heat production (adaptive thermogenesis). UCP1 is only expressed in BAT and the expression of UCP1 is upregulated by cold exposure through activation of the sympathetic nerve system and noradrenalin release (Himms-Hagen *et al.*, 1994). The importance of UCP1 is shown in the UCP1 knockout mouse which are unable to keep the body temperature during cold exposure (Enerbäck *et al.*, 1997). It has been proposed that free fatty acids are activators of the translocation of protons over the mitochondrial membrane by UCP1 (Klingenberg, 1999; Rial and Gonzalez-

Barroso, 2001). This explains the upregulation of UCP1 seen during high-fat feeding, the so called diet-induced thermogenesis (Portillo *et al.*, 1998), (Rippe *et al.*, 2000). This upregulation is thought to be a defense mechanism for diet-induced obesity by producing energy as heat instead of ATP, and thus UCP1 is an important regulator of energy balance in animals having BAT (Himms-Hagen, 1990; Rothwell and Stock, 1979). UCP1 is inhibited by the purine nucleotides GDP and ADP. In humans, BAT is present in newborns, while human adults have small deposits of BAT, although brown adipose cells may be present within the white adipose tissue (Lean *et al.*, 1986). Consequently UCP1 is probably not a potential regulator of energy balance in humans (Erlanson-Albertsson, 2002; Lean *et al.*, 1986). However, mitochondrial proton leak has been observed in tissues other than BAT, and was supposed to account for up to 50% of the oxygen consumption of some tissues, and up to 30% of the whole body metabolic rate in rat (Brand *et al.*, 1994; Rolfe and Brand, 1997).

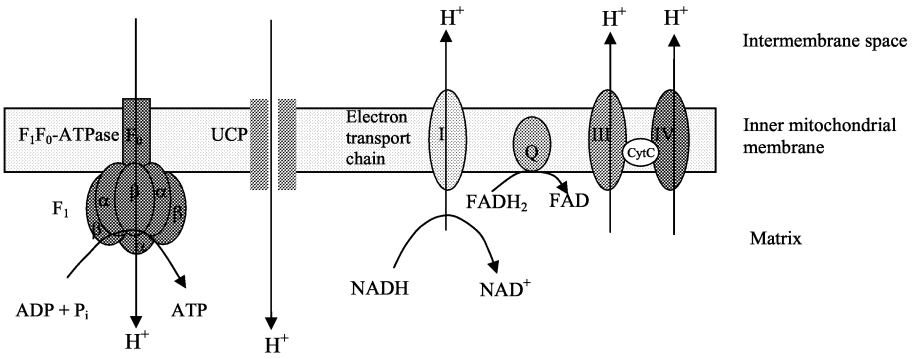

Figure 4. Proton transport across the inner mitochondrial membrane. A proton circuit is created by the coupling of fuel oxidation to proton translocation from the inside to the outside of the inner mitochondrial membrane. Transport of electrons through the respiratory chain complexes I, ubiquinon (Q), III and IV are driven by the oxidation of NADH and $FADH_2$. The electron transport is associated with pumping of protons from the mitochondrial matrix to the intermembrane space, creating en electrochemical gradient. F_1F_0-ATPase utilizes energy from the proton gradient to promote phosphorylation of ADP to ATP. Uncoupling proteins (UCPs) allow leakage of H^+ across the membrane, thus decreasing the electrochemical proton gradient.

UCP2

In 1997, Fleury *et al.* (Fleury *et al.*, 1997) found a mitochondrial protein with 59% identity with UCP1 which was named UCP2 (with the consequence that former UCP in BAT was named UCP1). In contrast to UCP1, the UCP2 mRNA was shown to be widely expressed (Fleury *et al.*, 1997). However, with specific antibodies reliable amounts of UCP2 protein has been detected only in spleen, lung, stomach and WAT (Pecqueur *et al.*, 2001). UCP2 was initially thought to produce heat and to be a thermoregulatory protein like UCP1 based on its ability to dissipate a proton gradient when expressed in yeast (Fleury *et al.*, 1997). However, in contrast to UCP1, the uncoupling activity of UCP2 expressed in yeast was not sensitive to fatty acids (Rial and Gonzalez-Barroso, 2001). Therefore, the role for UCP2 as a thermogenic uncoupler has been questioned and instead other physiological roles of UCP2 have been proposed.
Even though UCP2 is not a thermoregulatory protein it is still controversial if UCP2 is able to affect the energy metabolism and energy balance. Association between the UCP2 gene and energy expenditure is in general not connected to obesity (Elbein *et al.*, 1997), indicating that UCP2 does not have an important role in regulating energy balance (Schrauwen and Hesselink, 2002). The UCP2 involvement in energy balance was first described by the upregulation of UCP2 in white adipose tissue in response to fat feeding in the high-fat resistant A/J mouse strain, whereas no upregulation was seen in obesity prone B6 mouse (Fleury *et al.*, 1997). However, later it has been shown that even if there is a high cellular expression of UCP2 in BAT, as in UCP1-deficient mouse, there is no indication that the isolated brown-fat mitochondria are uncoupled (Nedergaard *et al.*, 2001). Moreover, UCP2-deficient mice showed normal body weight after both cold exposure and HF diet (Arsenijevic *et al.*, 2000). Interestingly, the expression of UCP2 mRNA is stimulated by starvation (Cadenas *et al.*, 1999; Millet *et al.*, 1997). This might be due to an increased level of circulating FFA during starvation.

When UCP2 was found, it was shown to be highly expressed in macrophages and organs of the immune system like spleen and thymus (Fleury *et al.*, 1997). Several reports have also described a relationship between the mitochondrial UCP2 protein and production of reactive oxygen species (ROS) (Negre-Salvayre *et al.*, 1997) (Echtay *et al.*, 2002). UCP2 has been postulated to decrease the production of ROS by disruption of the proton gradient over the inner mitochondrial membrane. Free oxygen radicals are produced in the electron transport chain and production is stimulated in situations with abundance of

oxygen in combination with a limited utilization of the proton gradient (Erlanson-Albertsson, 2002). The free radicals are reactive and may transform the structure of both proteins and nucleic acids within the cell (Papa and Skulachev, 1997), which for example is utilized in the defense of macrophages. For most cells, it is important to limit the formation of ROS for protection of the cells. One postulated mechanism for UCP2 is to protect the organism against free reactive oxygen species by increasing the proton leak and thereby decrease the proton gradient and reduce the oxygen supply. This hypothesis is supported by a study of the UCP2 knockout mouse (Arsenijevic *et al.*, 2000): Mice without UCP2 were infected with *Toxoplasma gondii* (an intracellular protozoan brain parasite that multiply to form cysts), but the UCP2 (-/-) mice were completely resistant to infection in contrast to wild type (WT) mice. The macrophages in the UCP2 (-/-) mice generated 80 % more ROS than WT, supporting a role of UCP2 in the production of ROS. Furthermore, Echtay *et al.* recently showed that superoxide interacts with UCP1, UCP2 and UCP3, which leads to an increase in proton conductance. The superoxid-induced uncoupling requires fatty acids and is inhibited by purine nucleotides (Echtay *et al.*, 2002). This interaction of superoxide with UCP further supports a mechanism for UCP in the regulation of ROS. In another study, insulinoma INS-1 cells transfected with UCP2 cDNA were protected from oxidative stress, whereas exposure to antioxidants reduced UCP2 mRNA expression. Oxidizing agents induced were found to reduce UCP2 and in parallel with increased cell death (Li *et al.*, 2001). In conclusion, UCP2 has in several studies been shown to be upregulated in pancreatic islets by exposure to high concentrations of fatty acids (Lameloise *et al.*, 2001; Li *et al.*, 2002). It is speculated that this upregulation primarily is a protection of the cell to limit ROS production induced by oxidation of the high concentrations of FFA.

One postulated function of UCP2 is in the regulation of ATP-dependent processes, e.g. insulin secretion (Erlanson-Albertsson, 2002). Overexpression of UCP2 in clonal β-cells and rodent islets have been shown to decrease the glucose induced insulin secretion (Chan *et al.*, 2001; Chan *et al.*, 1999; Hong *et al.*, 2001). Increased UCP2 expression reduces the ability of glucose to increase the cellular ATP content in the β-cells, which leads to a reduction in insulin secretion (Chan, 2002; Patane *et al.*, 2002). Joseph *et al.* further demonstrated the importance of UCP2 in the regulation of insulin secretion in the UCP2 knockout (-/-) mice (Joseph *et al.*, 2002). They showed that UCP2 (-/-) mice have enhanced glucose induced insulin secretion after HF feeding compared to WT mice. These mice did not show any of the typical signs of insulin resistance

usually seen after long-term exposure to HF feeding. Instead, they had increased islet sensitivity to glucose, improved glucose tolerance in peripheral tissue, normal plasma glucose and plasma insulin levels and enhanced insulin content in the β-cells (Joseph *et al.*, 2002). It thus seems like than an inhibition of UCP2 function could be an effective way to improve β-cell function in type 2 diabetes. On the other hand, reduced UCP2 expression would probably lead to increased ROS production, which is known to be involved in the β-cell destruction during type 1 diabetes.

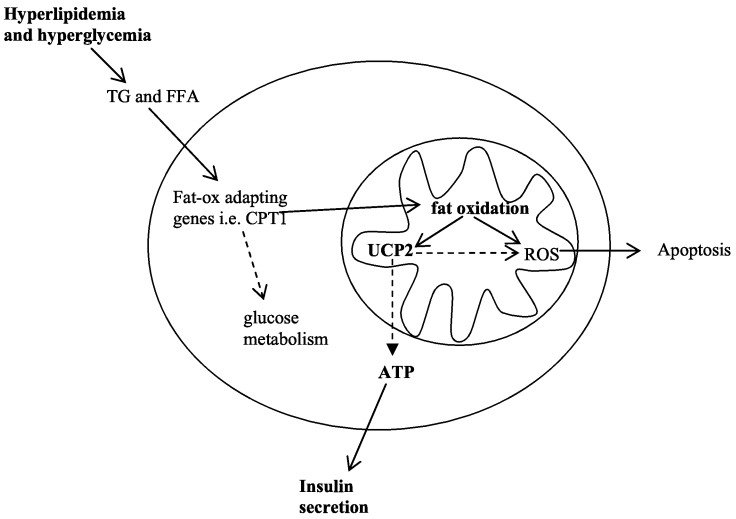

Figure 5. A proposed role for the fatty acid induced expression of UCP2 in the regulation of insulin secretion. Hyperlipidemia in combination with hyperglycemia induces the adaptation of fat oxidation in the β-cell, for example by induction of the fatty acid transport protein carnitine palmitoyl transferase-1 (CPT1), and instead reduces the glucose metabolism. Increased fat oxidation in turn increases the production of reactive oxygen species (ROS), which causes an induction of UCP2 expression to inhibit ROS production to protect the cell from apoptosis. As a consequence the intracellular ATP will decrease, which in turn cases decreased insulin secretion (Chan, 2002). Solid arrows represent an increase; dashed arrows represent a decrease in expression and/or function.

The mechanism by which UCP2 regulates insulin secretion is not completely understood. UCP2 is known to be upregulated during exposure to high concentrations of FFA and glucose. Probably this upregulation is an effect to provide protection from apoptosis due to increased production of ROS during enhanced substrate oxidation (fig. 5). The subsequent effect in the β-cell is an ATP-dependent reduction in insulin secretion. A mechanism in this regulation has been proposed by Chan (Chan, 2002). According to this theory, a high concentration of FFA and glucose result in increased substrate oxidation and subsequent increase of ROS, which in turn induces apoptosis in the cell. Elevated plasma FFA in combination with elevated glucose promote induction of adaptive genes necessary for FFA translocation, like carnitine palmitoyl transferase-1 (CPT-1), which further increases fat oxidation and decreases the glucose oxidation, with subsequent increased plasma glucose level. To protect the cell from ROS, UCP2 is upregulated. The induced UCP2 expression decreases the coupled respiration and thus decreases the intracellular ATP. The low ATP/ADP ratio inhibits the closure of K^+-channels and subsequent Ca^{2+} influx, and insulin release is inhibited (Chan, 2002). Altogether, UCP-2 thus seems to be an important negative regulator of beta-cell insulin secretion (Chan *et al.*, 2001).

UCP3

A third uncoupling protein, UCP3, was discovered soon after UCP2 (Boss *et al.*, 1997; Vidal-Puig *et al.*, 1997). The gene identity of UCP3 was 57% compared to the UCP1 gene and 73% to the UCP2 gene. UCP3 is specifically expressed in skeletal muscle and BAT, and since both these tissues are important in regulated energy expenditure, UCP3 was thought to be important in adaptive thermogenesis. When expressed in yeast, UCP3 was able to dissipate the proton gradient, like UCP1 and UCP2 (Boss *et al.*, 1998). In mice with disrupted skeletal muscle UCP3 gene, the respiratory state 3/state 4 ratio was higher due to decreased state 4 respiration. In UCP3 (-/-) mice, the muscle mitochondria were thus more coupled compared to WT, indicating that UCP3 has uncoupling activity. In spite of the increased coupling shown in the UCP3 (-/-) mice, they were not obese and had normal resting energy expenditure and thermoregulation (Vidal-Puig *et al.*, 2000). Neither were there any differences in body weight, triglyceride content or food intake. UCP3 expression was not upregulated in cold and they did not show cold sensitivity, altogether indicating that UCP3 is not required for normal body weight regulation or thermoregulation.

Mitochondria lacking UCP3 produce more ROS than WT, which suggests that one of the functions of UCP3 could be to prevent the formation of free oxygen radicals within the skeletal muscle (Vidal-Puig *et al.*, 2000).
An upregulation of UCP3 mRNA in skeletal muscle was observed after high-fat feeding (Samec *et al.*, 1998; Schrauwen *et al.*, 2001) and fasting (Millet *et al.*, 1997; Weigle *et al.*, 1998). If the refeeding after starvation is a low-fat (high-carbohydrate) diet there is a downregulation of the skeletal muscle UCP2 and UCP3 mRNA, while refeeding with iso-caloric high-fat diet causes a preserved high expression of both UCP2 and UCP3 mRNA (Samec *et al.*, 1999). UCP3 may hence act as a regulator of lipid as energy substrate which is further evidenced by the upregulation of UCP3 by fatty acids *in vivo* and *in vitro* (Hwang and Lane, 1999; Weigle *et al.*, 1998). A suggested role for UCP3 is a regulator of the fatty acid metabolism (Vidal-Puig *et al.*, 2000).
In addition, mice overexpressing UCP3 have reduced plasma glucose and insulin levels and showed an increased glucose clearance rate (Clapham *et al.*, 2000). Recently overexpression of UCP3 in a muscle cell line showed increased glucose uptake through an induced recruitment of the glucose transporter GLUT4 to the cell surface (Huppertz *et al.*, 2001), and in another study UCP3 and GLUT4 mRNA increased parallel after endurance exercise (Tsuboyama-Kasaoka *et al.*, 1998). These studies provide evidence that skeletal muscle UCP3 has the potential to influence metabolic rate and glucose homeostasis.

UCP4 and UCP5/BMCP1

Both UCP4 and UCP5 (also called brain-specific mitochondrial carrier protein 1, BMCP1) are predominantly expressed in the brain and to a minor degree in other tissues like liver and testis (Mao *et al.*, 1999; Sanchis *et al.*, 1998; Yu *et al.*, 2000). Both genes have most sequence similarity with the UCP3 gene, even though UCP5 cDNA only has a sequence identity of 35% with UCP3, thus being more different from other members of the uncoupling family (Sanchis *et al.*, 1998). UCP2, UCP4 and UCP5 are all expressed in the brain, but UCP5 is the one that is most abundantly expressed in several parts of the brain, i.e. cortex, amygdala and hypothalamus (Erlanson-Albertsson, 2002). Whether UCP4 and UCP5 are involved in energy balance in the brain is not clear. Neither UCP4 nor UCP5 mRNA in the brain are altered by high-fat diet or fasting. In contrast they are both increased during cold exposure (Yu *et al.*, 2000). HF diet was found to cause an increase of liver UCP5 mRNA in the obesity resistant A/J mouse but had no effect in the obesity prone C57Bl/6J mouse. The role of these uncoupling

proteins thus is not clear. Present experiments suggest that they are involved in the tissue-specific thermoregulation or in the regulation of ROS production in the brain and/or metabolic changes associated with nutritional status.

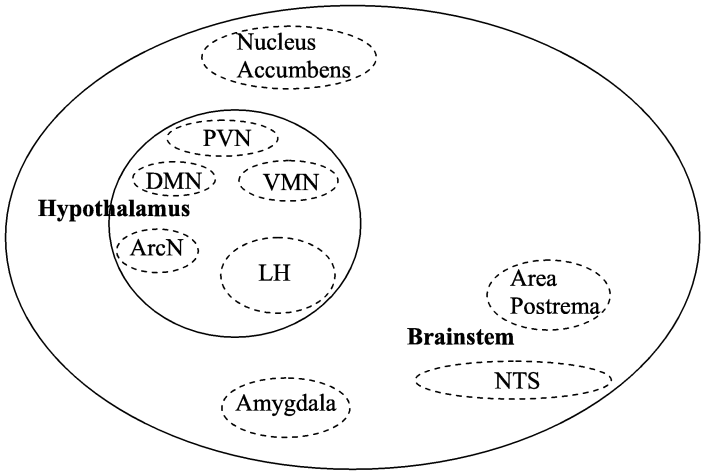

Figure 6. A schematic figure of the brain sites most important in the regulation of food intake. The nuclei in hypothalamus are central in appetite regulation. They express receptors for many different neuropeptides, neurotransmitters and nutrients active in the regulation of food intake. In addition, hypothalamus collects information from other parts of the brain. NTS and area postrema receive information from the gastrointestinal tract via vagus. Nucleus Accumbens express dopamine receptors and opiate receptors, and is important in the reward system. Amygdala is involved in the control of feeding and in the reward system. (PVN=paraventricular nucleus, DMN=dorsomedial nucleus, VMN=ventromedial nucleus, ArcN=arcuate nucleus, LH=lateral hypothalamus, NTS=nucleus tractus solitarius).

Regulation of food intake

The fact that most of us remain in a state of energy balance for most of our lives is quite remarkable, considering the day-to-day variation in energy intake. The body exerts a strong defense against undernutrition and weight loss, but has a much weaker resistance to overconsumption and weight gain. There is a high degree of redundancy in these systems, serving to preserve body mass in the periods of starvation. The genetic inheritance influences how appetite-control

operate, and this partly explains why some are so prone to weight gain. The onset and termination of eating episodes are subject to stimulatory and inhibitory physiological processes, partly regulated by environmental risks and habitual routines (Blundell and King, 1996).

The systems that control feeding behavior and energy balance appear to be comprised of a short-term and a long-term system (fig. 7). The short-term system regulates the meal pattern and feeding throughout the day, while the long-term system balances food intake and energy expenditure and thus plays a role in regulation of the energy stores of the body. Energy balance is mainly regulated by the central nervous system (CNS), which senses the metabolic status from endocrine and neuronal signals, and control energy intake (fig. 7). These systems are also affected by other factors like sight, smell, texture and memory of food as well as the social situation (Wilding, 2002). Much of the integration of all signals occurs in the hypothalamus, which has been shown to be a key brain region for regulation of metabolism and energy expenditure. Some areas of the hypothalamus are very sensitive to specific nutrients that reach these areas; e.g. there are neurons being especially sensitive to glucose and other that are sensitive to amino acids or fatty acids. Some other brain regions also play a role in the energy balance, particularly the NTS and area postrema in the brainstem, parts of the limbic system, the amygdala and cerebral cortex. These brain areas send the information they receive to hypothalamus (Wilding, 2002).

There are numerous substances affecting food intake, both within the nervous system and peripherally (table 3). They all affect appetite and/or food intake in different ways and by using different pathways. Some of them are known to regulate a specific macronutrient, i.e. fat, carbohydrate and protein (Erlanson-Albertsson, 2000). Some of the peptides and neurotransmitters have been shown to affect the energy expenditure by a perturbation of the thermogenesis induced by sympathetic activation. Sympathetic activity and food intake are often reciprocally related, also seen in the effect of several peptides regulating food intake including enterostatin (Bray, 2000).

In the following, I will briefly describe the food regulating effects of some of the peptides and neurotransmitters involved in the appetite and food-intake regulation with effects similar to, or effects interacting with, those of enterostatin.

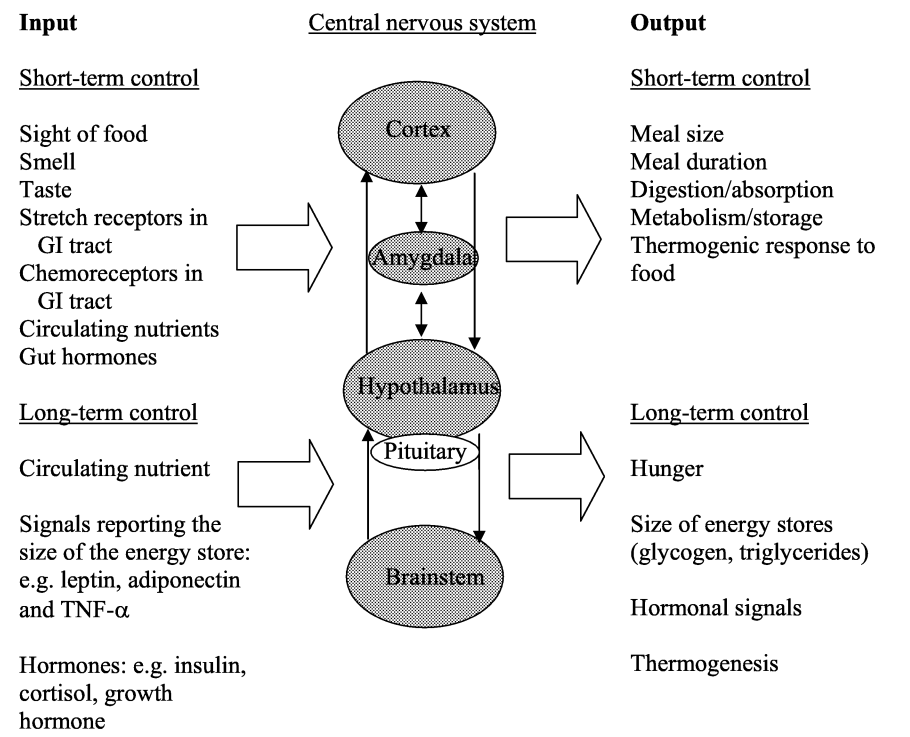

Figure 7. Short and long-term control of the energy balance is mainly regulated by the CNS which senses signals and controls energy intake and thermogenesis. Figure modified from Wilding (Wilding, 2002).

Neurotransmitters

Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter, synthesized from tryptophan. Several receptor subtypes have been identified, but only some of them seem to be involved in appetite regulation: the presynaptic 5-HT_{1A}, the postsynaptic 5-HT_{1B} (5-$HT_{1D\beta}$ in humans) and the 5-HT_{2C} receptor

(Halford, 2001). Serotonin has an anorectic effect, and at least the 5-$HT_{1B/1D\beta}$-receptor in hypothalamic PVN selectively suppresses fat-intake. Blundell *et al.* have suggested that serotonin activation may cause a selective suppression of fat intake, probably through pre-absorptive mechanisms (Blundell *et al.*, 1995). Serotonin acts by influencing the pattern of eating behavior and appetite motivation, resulting in fewer and shorter meals, thus promoting satiety as well as satiation (Hoebel *et al.*, 1989; Meguid *et al.*, 2000). Dexfenfluramine is a selective serotonin agonist that inhibits serotonin re-uptake in presynaptic terminals and stimulates serotonin release from nerve endings. Chronic treatment of dexfenfluramine leads to a decreased caloric intake and induces weight loss by a mechanism assumed to be hypophagia caused by an increase of serotonin in the hypothalamic synapses (Smith *et al.*, 1998). It has been shown that dexfenfluramine can readily reduce the intake of high fat foods in a three-choice macronutrient paradigm, supporting that serotonin activation can lead to a selective avoidance of fat in the diet (Smith *et al.*, 1998).

Table 3. Endogenous substances involved in the complex regulation of food intake.

Endogenous substances that increase food intake	**Endogenous substances that decrease food intake**
Agouti-related peptide (AGRP)	α-melanocyte-stimulating hormone (αMSH)
Dopamine	Apolipoprotein A-IV
Galanin	Bombesin/gastrin-releasing peptide (GRLP)
Ghrelin	Cocaine and amphetamine-regulated transcript (CART)
Insulin*	Cholecystokinin (CCK)
Melanin concentrating hormone (MCH)	Corticotrophin releasing hormone (GRH)
Nitric oxide (NO)	Dopamine
Noradrenalin (NA)	Enterostatin
Neuropeptide Y (NPY)	Glucagon
Orexins	Glucagon-like peptide-1 (GLP1)
Opioids (μ and κ-agonists)	Leptin
	Neurotensin
	Noradrenalin (NA)
	Serotonin
	Tumour necrosing factor α (TNF-α)
	Interleukin 1 (IL-1)

*When given peripherally

Dopamine

Dopamine is a neurotransmitter synthesized from phenylalanine or tyrosine in the central and peripheral nervous systems and involved in the regulation of food intake by modulating the food reward system. Dopamine is normally increased in hypothalamic ventromedial nuclei (VMN) during spontaneous eating and this increase is more pronounced in obese rats (Meguid *et al.*, 2000). The release has particularly been demonstrated after exposure to palatable stimuli like high-fat food and sugar (Baker *et al.*, 2001; Colantuoni *et al.*, 2002; Colantuoni *et al.*, 2001). The dopamine level is associated with the number of meals and meal duration and is thought to be required to initiate a meal.
The dopamine receptors are predominantly postsynaptic, of D_1 and D_2 subtypes. D_1 receptors stimulate while D_2 inhibits adenylyl cyclase, and since hypothalamic neurons express both receptor subtypes, dopamine can either activate or inhibit intracellular activity, which in turn results in either stimulation or inhibition of food-intake (Meguid *et al.*, 2000) (fig. 8). D_1 receptors are more extensively expressed in VMN of obese rats, while D_2 are more abundant in lean rats, and this divergent expression of the receptor subtypes seem to be directly involved in the regulation of meal size and number (Meguid *et al.*, 2000).

Noradrenalin (NA)

Noradrenalin is a neurotransmitter produced from dopamine in the adrenal medulla and stored in the noradrenergic neurons. Alterations of brain NA can either increase or decrease eating, depending on the site of secretion and other variables. NA is a ligand for a variety of adrenergic receptors (all G-protein coupled) in the central and peripheral nervous system, including α_1, α_2, β_1 and β_2 subtypes. Among these, α_2–adrenergic receptors in PVN have been linked to the NA-stimulatory effect on eating, while the α_1–adrenergic receptors decrease eating (Wellman, 2000). The activation of α_2–adrenergic receptors inhibits satiety, resulting in enhanced eating and increased meal size. NA is thought to stimulate eating by inactivation of PVN cells that are activated to suppress eating by activation of α_1–adrenergic receptors (fig. 8). Thus it is proposed that adrenergic receptors localized on NA neurons in PVN, are organized in an antagonistic fashion such that activation of α_1–adrenergic receptors activates feeding inhibitory fibers, resulting in suppression of food intake, whereas activation of α_2–adrenergic receptors increases feeding by inhibition of these fibers (Wellman *et al.*, 1993). In rats, secretion of NA peaks just before the onset

of the dark period and thus the onset of eating, and this is correlated with an increase in the number of α_2–adrenoceptors within PVN (Jhanwar-Uniyal *et al.*, 1986). It is also proposed that the action of NA on eating may be due to an indirect interaction with leptin and NPY (Wellman, 2000) (fig. 8).

Neuropeptides

Opiates

The most well-known property of opiates is the analgetic effect, which has been known since prehistoric times in human culture, and the dual action in mediating pain suppression and euphoria have been very useful. Opiates are a family of peptides divided into the three classes, μ, κ and δ, depending on receptor type affinity. The receptors differ in pharmacologic properties and tissue distribution and belong all to the G_i-coupled family of receptors, with subsequent inhibition of adenylyl cyclase or ion-channels. Opioids are produced in the central and peripheral nervous system as well as in the gastrointestinal tract and pancreatic islets. They are produced as larger precursor molecules: pro-opiomelanocortin (POMC) for β-endorphin and other endorphins, proenkephalin for met-enkephalins and leu-enkephalin, and prodynorphin for dynorphins. The natural ligand for μ-receptors is β-endorphin, for δ-receptors the enkephalins and for κ-receptors the dynorphins.
In general, opioid-agonists stimulate feeding, while antagonists suppress it (Glass *et al.*, 1999) (fig. 8). Specifically, μ- and κ-opiate agonists seem to stimulate fat and sugar consumption (Colantuoni *et al.*, 2002; Romsos *et al.*, 1987; Zhang *et al.*, 1998). Several of the brain sites known to affect food intake are activated by opiates, indicating the opioid-system to be involved in food intake through multiple pathways (Glass *et al.*, 1999; Zhang *et al.*, 1998). Typically, opiates seem to influence the food reward system, probably by the state of pleasure derived from food consumption, and especially from fat- and sugar-rich food (Colantuoni *et al.*, 2002; Zhang *et al.*, 1998). Hence, there seems to be a correlation between taste-palatability and activation of endogenous opioid-system, and indeed, a link between endogenous opioids, like β-endorphin, and obesity has been observed in humans and in animal models (Genazzani *et al.*, 1986; Recant *et al.*, 1980). Nucleus accumbens appears to be the part of the brain involved in the reward effect induced by opioids. When the μ-receptors in the nucleus accumbens are stimulated, other parts of the brain are activated, shown by induction of enhanced Fos immunoreactivity in several

parts of hypothalamus and activation of the ingestion (Zhang and Kelley, 2000). In addition to stimulation of food intake, opioids have been reported to mediate other metabolic effects like decreased insulin secretion (Green *et al.*, 1983; Vettor *et al.*, 1994), affected insulin mechanism of action (Kim *et al.*, 2000; Vettor *et al.*, 1994) and influence on energy expenditure (Mandenoff *et al.*, 1982; Mandenoff *et al.*, 1991; Vettor *et al.*, 1994). However, these metabolic effects are controversial.

Galanin

Galanin is a neuropeptide distributed in the central and peripheral nervous system, as well as in the gastrointestinal tract (Bishop *et al.*, 1986; Merchenthaler *et al.*, 1993). Overexpression or injection of galanin to hypothalamic PVN or to NTS stimulates the intake of dietary fat (Leibowitz, 1995; Odorizzi *et al.*, 1999). Another study proposes that galanin not specifically increases the fat intake but instead increase the intake of the already preferred macronutrient (Smith *et al.*, 1996). Galanin increases in correlation with fat intake and has thus been identified as a neurochemical marker for fat ingestion and body weight gain (Akabayashi *et al.*, 1994). Currently, three G-protein coupled receptor subtypes have been identified in central and peripheral tissues (Waters and Krause, 2000). The stimulation of food-intake by galanin has been proposed to be a result of increased release of β-endorphin and/or dopamine (fig. 8) in specific brain sites and thus causing an increased reward (Dube *et al.*, 1994) (Leibowitz, 1994). In addition, it has been shown that the food intake stimulating effect of galanin could be inhibited by leptin (Sahu, 1998) and by the opiate antagonist naloxone (Barton *et al.*, 1995). Besides stimulating fat intake, galanin has been shown to decrease energy expenditure through a reduced sympathetic activity, together provoking a positive energy balance (Bray, 2000).

Neuropeptide Y (NPY)

Neuropeptide Y (NPY) is found in high concentrations in the hypothalamus where it is synthesised in cell bodies of the arcuate nucleus and released from PVN (Sawchenko and Pfeiffer, 1988). NPY is a very potent stimulator of food intake, specifically carbohydrate intake (Stanley *et al.*, 1986), and chronic i.c.v. infusion results in the development of obesity with typical endocrine and

metabolic abnormalities (Stanley *et al.*, 1986; Zarjevski *et al.*, 1993). The stimulation of NPY receptors induces opioid release and opioid-antagonists inhibit the NPY-induced feeding, suggesting an opioidergic pathway involved in the NPY response (Kotz *et al.*, 1993). The NPY action has also been shown to be mediated by dopamine and noradrenalin release (Myers *et al.*, 1996) (fig. 8). In addition NPY decreases the sympathetic activity to BAT in rodents, and decreases the metabolic rate (Egawa *et al.*, 1991). Altogether, the effects of NPY results in a positive energy balance.
NPY have at least five receptors but it is not completely clear if it is the Y1 or the Y5 receptor that is responsible for mediating the effect on food intake (Hofbauer, 2002; Wilding, 2002). Surprisingly, NPY knockout of normal mouse had no effect on body weight (Palmiter *et al.*, 1998), and neither knockout of the Y1 and Y5 receptors resulted in hypophagia (Marsh *et al.*, 1998; Pedrazzini *et al.*, 1998). However, NPY knockout in ob/ob mice resulted in a reduction in body weight (Erickson *et al.*, 1996). NPY neurons are a major target for leptin action, and part of the effect of NPY is probably of leptin origin (Schwartz *et al.*, 1996) (fig. 8). Physiologically the role of NPY can be considered as helping co-ordinate the metabolic and behavioural responses to starvation.

Gut- and intestinal peptides

Cholecystokinine (CCK)

CCK is a so called brain-gut peptide, since it is found both in the brain and in the gastrointestinal tract. In the gut, it is mainly present in the proximal small intestinal endocrine cells and enteric nerves (Moran, 2000). CCK is present in several forms; CCK-33, CCK-8 and CCK-4, produced from preprocholecystokinine, of which CCK-8 seem to be the most potent form (Crawley and Corwin, 1994). CCK has many different roles as signal molecule, both centrally and peripherally. Peripherally administered CCK is known to inhibit feeding in most mammals due to decreased time spent eating and reduced meal size (Strohmayer and Greenberg, 1994). In humans CCK-8 infusion has been shown to induce a reduction in calorie intake with a decrease in hunger feelings (Gutzwiller *et al.*, 2000). The stimuli for endogenous CCK release are long-chain fatty acids and amino acids (Sanford, 1992). It has been claimed that a part of the satiety induced by fat and protein probably is related to the CCK release (Degen *et al.*, 2001). In addition, CCK is believed to contribute to the

long-term control of food intake through an interaction with leptin (Matson and Ritter, 1999) (fig. 8).
Two CCK receptor subtypes have been identified, both of the G-protein coupled type. CCK_A is most abundant in GI-tract; in the pancreas, on afferent vagal and enteric neurons, but also in some brain sites. The CCK_A receptor has high affinity for CCK-8 and CCK-33. CCK_B receptors are widely distributed in the brain, but are also found within the stomach, and have affinity for CCK-fragments and gastrin. Deletion of the rat CCK_A receptor gene results in obesity and type 2 diabetes, suggesting the CCK satiety action to be of importance for metabolic regulation (Moran, 2000). Central CCK is released in response to a meal. The central effect is at least partly mediated by the serotonergic system (Langhans, 2001) (fig. 8). A major effect of CCK is to stimulate pancreatic enzyme secretion and gallbladder contraction as well as to inhibit gastric emptying (Langhans, 2001). Regarding appetite regulation it is suggested that low doses of CCK has a direct effect on food intake through the activation of CCK_A receptors present on vagal afferent nerves, while higher doses of CCK act indirect by inhibition of gastric emptying (Moran, 2000).

Apolipoprotein A-IV (Apo A-IV)

Apolipoprotein A-IV is secreted by the small intestine (in rodents also by the liver), and the production is stimulated by lipid absorption and specifically by the formation of chylomicrons (Tso *et al.*, 2001). ApoA-IV is associated to chylomicrons and high density lipoprotein (HDL) together with apoA-I, apoB-48 and apo-C. Until recently, the physiological effect of apoA-IV was largely unknown, but is now thought to contribute to appetite control after fat intake and digestion (Liu *et al.*, 1999). The inhibition of food intake by apoA-IV is observed both after intravenous and intracerebroventricular infusion. Administration of apoA-IV i.c.v. is more potent than i.v. infusion and in addition, the appetite suppressing effect can be abolished by i.c.v. administration of apo A-IV antibodies, indicating that the inhibition of food-intake is centrally mediated. ApoA-IV has actually been identified in cerebrospinal fluid, and since de novo synthesis in the brain is unlikely, apoA-IV is thought to pass through the blood-brain barrier. By immunohistochemistry, apoA-IV has been identified in astrocytes and tanycytes in the form of granulas and perinuclear distribution, suggesting that apoA-IV may be contained in perinuclear organelles or vesicles (Tso *et al.*, 2001) (Langhans, 2001).

The synthesis of apoA-IV is stimulated by insulin and inhibited by leptin, since these two hormones are involved in the regulation of long-term energy homeostasis, apoA-IV may be a part of the long term regulation of food-intake (Liu *et al.*, 1999).
ApoA-IV may also inhibit food intake, through the effect on gastric motility and gastric secretion. Additional information is necessary to understand the mechanism of action for apoA-IV. The identification and location of a receptor for apoA-IV would for instance be helpful.

Adipose tissue derived hormone

Leptin

Leptin is a 16 kDa adipocyte-derived hormone which acts as an afferent signal in the negative feedback regulation of body weight. Leptin is present in plasma at a concentration highly correlated with adipose tissue mass, and acts by reporting the nutritional state of the body, i.e. the size of the energy stores (Friedman, 2002). Leptin does not itself affect the meal pattern and is not increased during a meal. Instead leptin seems to act in the long-term regulation of feeding behaviour, influencing the amount of food consumed relative to the amount of energy that is expended. Leptin is thus not classified as a satiety factor but interacts extensively with other components known to affect the amount of ingested food (Friedman, 2002) (fig. 8). The plasma leptin concentration is a potent signal for the energy state of the body. Low leptin is a response to starvation, while high leptin demonstrates a state of obesity. Mice lacking circulating leptin due to a mutation in the leptin gene, C57BL/6J *ob/ob* mice (Halaas *et al.*, 1995), are genetically obese since they never receive the signal that there are adequate fat stores, and thus become hyperphagic. Five to ten percent of obese humans have low levels of leptin, but generally they have high levels, suggesting association with insensitivity to leptin (Maffei *et al.*, 1995).
The leptin receptor is a member of the cytokine receptor family and consists of a single transmembrane domain on the cell surface. Five splice forms of the receptor have been identified but only one, Ob-Rb, seems to mediate the weight reducing effect. Ob-Rb is abundantly expressed in hypothalamus (Tartaglia *et al.*, 1995), and the brain seems to be the most important target site for leptin that is known to cross the blood-brain barrier (Banks *et al.*, 1996). It is suggested that leptin is sensed by various groups of neurons in the hypothalamus. This in

turn activates behavioural, hormonal and metabolic responses like neuropeptides and neurotransmitters that regulate food intake and body weight. Many different neuropeptides and neurotransmitters are known to respond to the action of leptin, either by increasing or decreasing food intake, i.e. NPY, galanin, αMSH, MCH, CCK and CART (table 3) (Spiegelman and Flier, 1996). Mice with disruption in the leptin receptor, *db/db* mice, are obese and show identical phenotype as *ob/ob*.

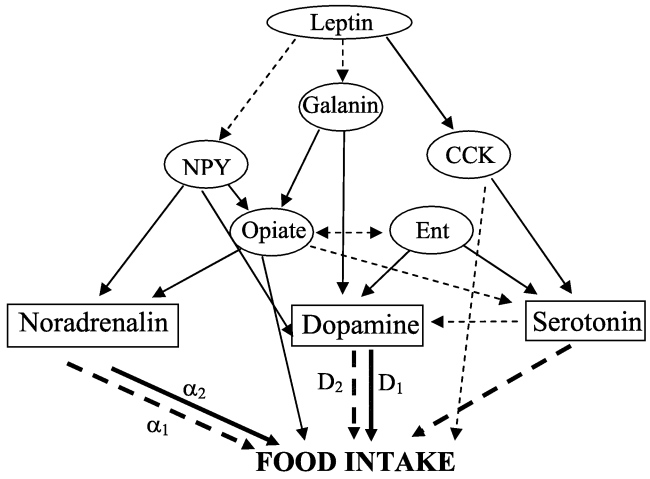

Figure 8. Integration of some of the signals that regulate food intake. For details, see in the text of each substance. Dotted line indicates negative regulation and solid line positive regulation

Enterostatin

Background

Enterostatin is produced in the duodenum after tryptic cleavage of pancreatic procolipase (Borgström *et al.*, 1979) (fig. 9), but also in minor amount after cleavage by pepsin and acid of gastric procolipase (Sörhede *et al.*, 1996b). Enterostatin was thought to be an activation peptide, cleaved from pancreatic procolipase to activate the enzyme. However, the enzyme activity did not increase although it bound better to the phospholipid-covered triglyceride interface after cleavage (Borgström *et al.*, 1979). The amino acid sequence of the N-terminal pentapeptide of porcine colipase was determined to be Val-Pro-

Asp-Pro-Arg (VPDPR) (Borgström *et al.*, 1979). The N-terminal sequence seem to be well conserved between species; VPDPR in pig (Borgström *et al.*, 1979), ox (Rathelot *et al.*, 1975), horse (Julien *et al.*, 1978) and cat (Rippe and Erlanson-Albertsson, 1998), while the sequence is Ala-Pro-Gly-Pro-Arg (APGPR) in chicken (Bosc-Bierne *et al.*, 1984) and man (Sternby and Borgstrom, 1984). In rat, the sequence was first determined to be VPDPR (Erlanson-Albertsson, 1981) but has recently been revised to be APGPR in both rat and mouse (Rippe and Erlanson-Albertsson, 1998; Wu *et al.*, 2002). Therefore, most studies on rats have been done with VPDPR.

In order to produce antibodies against the cleaved pentapeptide, Erlanson-Albertsson surprisingly noticed that the peptide caused weight loss when injected to rabbits (Erlanson-Albersson, 1994; Erlanson-Albertsson, 1992a). The effect of intraperitoneal injection of VPDPR was further tested on food intake in rats, and the anorectic effect of the peptide was verified when VPDPR decreased food intake in a dose-dependent way (Erlanson-Albertsson and Larsson, 1988a, 1988b). The peptide got the name enterostatin (Erlanson-Albertsson *et al.*, 1991b) and its effects and mechanisms have been scrutinized accordingly to understand the physiological role of enterostatin. In addition to the duodenum, enterostatin has been identified in the endocrine cells of the gastric and intestinal mucosa (Sörhede *et al.*, 1996a), and as its precursor form procolipase in the chief cells of the gastric mucosa (Sörhede *et al.*, 1996b). Enterostatin has also been identified in the cerebrospinal fluid in humans (Zhao *et al.*, 2001).

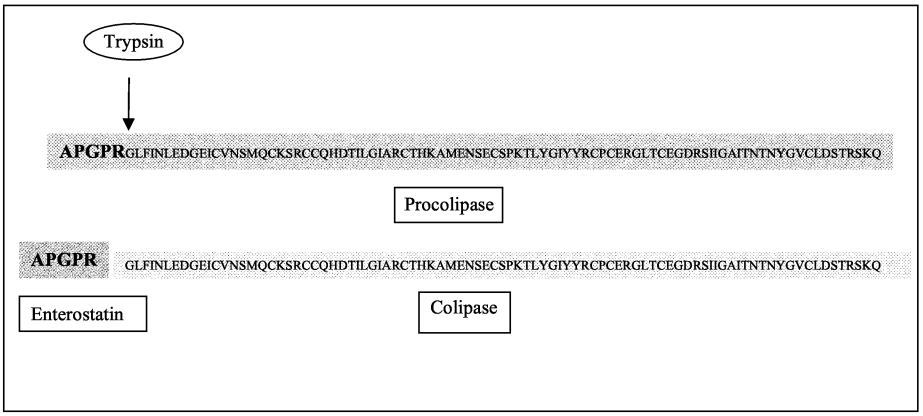

Figure 9. Cleavage of procolipase to enterostatin and colipase. Procolipase (here the rat sequence) is cleaved in the N-terminal by trypsin, forming colipase and enterostatin. Colipase acts as an obligatory cofactor for pancreatic lipase during triglyceride digestion and enterostatin is an endogenous satiety factor with specificity for fat.

Feeding-suppressing effect of enterostatin

Injection of enterostatin, both centrally and peripherally, has been shown to decrease food intake, and specifically fat intake, with a dose-dependent and U-shaped response, inhibiting food intake at low doses and stimulating food intake at high doses. All administration routes of enterostatin, with the exception of intravenous injection, have a rapid effect within 30 minutes (table 4). When rats were offered a three-choice macronutrient diet of protein, carbohydrate and fat, enterostatin specifically inhibited the fat intake (Okada *et al.*, 1991; Okada *et al.*, 1992). In a choice between low-fat (LF) and high-fat (HF) diet, enterostatin only reduced the intake of the HF diet (Lin *et al.*, 1997; Mei and Erlanson-Albertsson, 1992). Rats given a single diet, either LF or HF, only decreased the intake of HF diet as a response of enterostatin (Lin *et al.*, 1993a; Rippe *et al.*, 2000). For efficient inhibition of HF diet by enterostatin an adaptation to HF food is required. It is suggested that a signal related to the chronic ingestion of dietary fat is a requirement for enterostatin action (Lin and York, 1998b). The behaviour of rats after injection of enterostatin has been shown to be the same as after natural satiety, which is reduced time spent on eating, grooming and physiological activity, while the time spent sleeping and resting increased (Lin *et al.*, 1993b). This suggests that enterostatin may mediate its effect by giving an early satiety. In Osborne-Mendel (OM) rats, having a high voluntary intake of fat, enterostatin efficiently decreased the fat intake, while in the dietary fat-resistant S5B/Pl rat, enterostatin had no effect on food intake (Okada *et al.*, 1992). It was also found that OM rats had lower levels of colipase than S5B/P1 rats, indicating that endogenous enterostatin levels are lower in OM rats. The inverted relationship between obesity and procolipase levels is further supported by the genetically obese Zucker fa/fa rat having low levels of colipase mRNA (Okada *et al.*, 1993a).

Effects of peripheral administration of enterostatin

Dietary fat intake is reduced by intragastric (i.g.) (White *et al.*, 2000), oral (Rippe *et al.*, 2000), intraduodenal (i.d.) (Mei and Erlanson-Albertsson, 1996b) and intraperitoneal (i.p.) (Erlanson-Albertsson *et al.*, 1991a; Tian *et al.*, 1994) administration of enterostatin (table 4). All these administration routes are close to the production sites of enterostatin. The response to gastrointestinally administered enterostatin is dependent on an afferent vagal signalling pathway indicated by several studies (fig. 10). Thus transsection of the hepatic vagus

completely blocked the inhibitory response of i.p. injection of enterostatin on HF diet consumption in rats (Tian *et al.*, 1994). In addition, pretreatment with capsaicin, which causes degeneration of vagal neurons, abolished the inhibitory response of enterostatin after near-celiac arterial injection (Lin *et al.*, 2000). The importance of neuronal transmission was further supported by the finding that intraduodenal enterostatin had no effect on food intake after simultaneous infusion of the local anesthetic tetracain (Mei and Erlanson-Albertsson, 1996b). In addition, neuronal transmission of enterostatin response from the intestine to the brain is demonstrated by peripheral enterostatin inducing c-Fos protein immunoreactivity in specific brain sites (nucleus tractus solitarius (NTS), nucleus ambiguus, supraoptic nucleus (SON), suprachismatic nucleus, paraventricular nucleus (PVN), and pontine nucleus), which was abolished after selective hepatic vagotomy (Tian *et al.*, 1994).

Enterostatin also reduces HF diet after intravenous (i.v.) injection (Lin *et al.*, 2000; Mei and Erlanson-Albertsson, 1992) and after intra-arterial injection (Lin *et al.*, 2000). Enterostatin given intravenously has a delayed effect (> 1 h) compared to other administration routes (table 4). The reason for the late response is not known at present time, but enterostatin has been shown to bind to plasma proteins, i.e. albumin, and this might limit the uptake to the brain (Wu *et al.*, 2002). The intra-arterial injections done by Lin *et al.* (Lin *et al.*, 2000) showed that a near celiac arterial infusion and intracarotid enterostatin both gave an immediate response at low doses (2 and 0.5 nmol respectively). After near-celiac infusion the enterostatin effect was inhibited by vagal afferent inhibition demonstrating a local gastrointestinal response dependent on vagus transmission, while intracariotic injection was consistent with a central site of action.

Effects of central administration of enterostatin

Reduction of fat intake after intracerebroventricular injection of enterostatin has been shown in rat, sheep and baboons (Lin *et al.*, 1997; Mei and Erlanson-Albertsson, 1992; Miner *et al.*, 1994; Weatherford *et al.*, 1992) (fig. 10). Most studies have been performed by injection to the right lateral ventricle in the brain (Lin *et al.*, 1997; Lin and York, 1998b; Mei and Erlanson-Albertsson, 1992) (table 4). Hypothalamus is the major center for regulating feeding behaviour and metabolic responses. Specifically, enterostatin has been shown to reduce HF intake after microinjection locally to hypothalamic PVN and to the

extrahypothalamic site amygdala (fig. 6). There is no effect after injection to the VMN of hypothalamus or NTS (Lin and York, 1997b). Lin *et al.* has also shown that the feeding suppression after injection of enterostatin to PVN and amygdala resulted from decreased meal size and reduced meal duration, confirming an induced satiation and early satiety by enterostatin (Lin and York, 1998a).

Table 4. Threshold concentrations and response times for enterostatin to decrease high-fat food intake in rats.

	Threshold (nmol)	Response time (min)	Reference
Intragastric	100	<30	(White *et al.*, 2000)
Intraduodenal	11*	<30	(Mei and Erlanson-Albertsson, 1996b)
Intraperitoneal	40	15	(Lin *et al.*, 1993b; Okada *et al.*, 1992)
Intravenous	13	60-120	(Lin *et al.*, 2000; Mei and Erlanson-Albertsson, 1992)
Intracarotid arterial	2	<5	(Lin *et al.*, 2000)
Near celiac arterial	2	<5	(Lin *et al.*, 2000)
Intracerebroventricular	0.3	<30	(Lin *et al.*, 1994)
Paraventricular nucleus	0.1	<10	(Lin and York, 1997b)
Amygdala	0.01	<5	(Lin and York, 1997b)

* $kg^{-1}min^{-1}$

It has been speculated how enterostatin reaches the site of action in the brain. Local production of procolipase in the brain has been proposed but never confirmed (Okada *et al.*, 1993c). Rippe *et al.* have recently showed that procolipase is actively taken up across the blood-brain barrier (BBB) (Rippe *et al.*, 1998). A specific mechanism is suggested to mediate the uptake of procolipase across the BBB, and this might be an alternative way for enterostatin to reach the targets in brain since enterostatin is easily degraded in intestine and serum by peptidases (Bouras *et al.*, 1995). Recently, Koizumi *et al.*

for the first time showed that enterostatin itself can pass from the circulation into the brain (Koizumi *et al.*, 2002). Labelled enterostatin was given intravenously in physiologic concentration (106 nmol) and was detected (1.6-6.6 pmol) in several parts of the brain, including hypothalamus, indicating an uptake of enterostatin across the BBB. The radioactivity in the brain regions continued to increase until the endpoint of the experiment (120 min). This pattern of uptake correlates with the delay in response after intravenous injection of enterostatin. In addition to brain, enterostatin has recently been detected in human cerebrospinal fluid (Zhao *et al.*, 2001).
Central action of enterostatin requires low concentrations (<1 nmol) compared to peripheral administration, and if the peptide is injected directly to the brain the response is rapid (table 4).

Regulation of the production of enterostatin

Enterostatin production is primarily regulated through production of the parent molecule procolipase. Enterostatin is thought to be at least partly regulated by a feedback mechanism seen by an increased production of procolipase/enterostatin in pancreas and stomach after HF feeding (Mei *et al.*, 1993a). The increased procolipase synthesis occurs in proportion to the amount fat ingested within 24 hours after the presentation of a HF diet (Wicker and Puigserver, 1987). Conversely, fasting initially (within 48h) reduced pancreatic procolipase activity levels in rats and did thereafter increase (Erlanson-Albertsson and York, 1997). Rippe *et al* (Rippe *et al.*, 1998) have shown an active uptake of procolipase from the blood stream to the pancreas and gastrointestinal tract and Koizumi *et al* (Koizumi *et al.*, 2002) have demonstrated an uptake of enterostatin from the blood to the pancreas. Both might be feedback mechanisms in the regulation of procolipase/enterostatin.
Some different substances active in energy metabolism have been shown to regulate the procolipase synthesis. Insulin regulates procolipase production negatively by inhibition of procolipase mRNA expression (Duan *et al.*, 1991), while cAMP stimulates the mRNA expression of procolipase (Duan and Erlanson-Albertsson, 1992). Also the gastric inhibitory peptide (GIP), released during fat ingestion, has been shown to increase procolipase mRNA. Adrenalectomy (no endogenous production of corticosterone) in obese Zucker (fa/fa) rats with previously low production of procolipase, resulted in increased procolipase production while no effect was seen in the lean (fa/-) rats (Okada *et al.*, 1993b).

Metabolic effects of enterostatin

Enterostatin has several metabolic effects related to appetite regulation and energy metabolism of which the most studied is the decreased insulin secretion (fig. 10). This effect has been observed both *in vivo* and *in vitro*. *In vivo*, chronic infusion of enterostatin both i.p. (Mei and Erlanson-Albertsson, 1996a) and i.c.v. (Okada *et al.*, 1993a) decreased plasma insulin levels. The inhibition of glucose-induced insulin secretion has also been demonstrated in isolated rat islets (Mei *et al.*, 1993b; Ookuma and York, 1998), in perfused islets (Erlanson-Albertsson *et al.*, 1994), and in perfused pancreas (Silvestre *et al.*, 1996). Ookuma *et al.* suggested that one possible mechanism for enterostatin reducing insulin secretion is through reduction of cAMP, since enterostatin reduces the increase in cAMP induced by the κ-opiate agonist U50, 488 in parallel with decreased insulin secretion (Ookuma and York, 1998). The decreased insulin secretion seen after central infusion is probably due to the autonomic nerve system affecting the pancreatic β-cell secretion, while the peripheral effect probably is a direct effect on the β-cell (Erlanson-Albertsson and York, 1997).

Another hormone affected by enterostatin is corticosterone, produced in the adrenal cortex (fig. 10). Enterostatin has been shown to increase the levels of serum corticosterone after both central and peripheral chronic injection (Mei and Erlanson-Albertsson, 1996a; Okada *et al.*, 1993a). Adrenalectomy abolishes the endogenous production of glucocorticoids. After adrenalectomy of rats the feeding response of exogenous enterostatin to HF feeding is eliminated (Okada *et al.*, 1993b). Another effect of adrenalectomy is reduced plasma insulin levels and blockage of the response of plasma insulin to enterostatin treatment (Mei and Erlanson-Albertsson, 1996a). The production of procolipase mRNA and thereby enterostatin is stimulated by adrenalectomy, indicating that corticosterone is able to modify the expression of procolipase. The importance of corticosterone in facilitating the action of enterostatin on HF feeding has also been demonstrated by Mizuma *et al.* (Mizuma *et al.*, 1994) showing an increase in the inhibition of total caloric intake by enterostatin following corticosterone treatment.

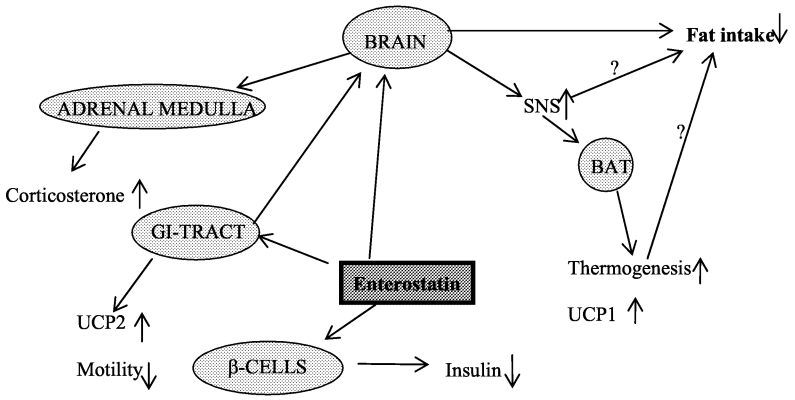

Figure 10. Schematic presentation of sites of action and effects of enterostatin. The reduction of fat-intake by enterostatin is caused either by targets in the brain or through afferent vagal signals from the gastrointestinal (GI) tract to the brain. According to the thermostatic theory, food intake could also be reduced by increased thermogenesis. Enterostatin increases thermogenesis and UCP1 in brown adipose tissue (BAT) after high fat feeding, probably by activation of the sympathetic nervous system. Enterostatin also induces UCP2 expression in the gastrointestinal tract after HF feeding. Peripherally, enterostatin decreases insulin secretion from pancreatic β-cells and increases corticosterone plasma levels.

In addition to its endocrine effects, enterostatin influences energy metabolism through its activation of the sympathetic drive to interscapular brown adipose tissue (BAT), which might increase thermogenesis (Nagase *et al.*, 1996) (fig.10). Interestingly, the increase of sympathetic firing of enterostatin was only seen in rats fed with HF food, while in chow (LF) fed rats, which are generally unresponsive to the enterostatin inhibition of food intake, enterostatin gave no sympathetic response. The stimulation of the nerves had the same effect pattern of enterostatin as in the suppression of food intake, i.e. a dose-response curve with a peak effect at 1 nmol enterostatin and smaller effects with lower or higher concentrations (Nagase *et al.*, 1996). Rippe *et al.* recently reported enterostatin to increase the mRNA expression of UCP1 in BAT and UCP2 in gut, in rats fed HF in a thermoneutral (29°C) environment (fig. 10). At least the increase of UCP1 expression is postulated to be an effect of stimulation of sympathetic activity to BAT (Rippe *et al.*, 2000).

Gastric emptying and intestinal motility are important parameters in satiety regulation. The reduction of fat intake by enterostatin does not occur in parallel with slow gastric emptying (Lin and York, 1997a). Fat is known to increase the intestinal transit time, but after intraintestinal infusion of enterostatin in pig, the intestinal transit time was shown to be increased due to a prolonged inactive phase between the contractions (Pierzynowski *et al.*, 1994) (fig.10).

Site and mechanism of action

Enterostatin is assumed to have both central and peripheral sites of action, based on the response times, threshold doses and other parameters observed after central and peripheral administration routes. However, the cellular targets and mechanisms of enterostatin are not yet elucidated and are under investigation.

Central administration of enterostatin is most potent when given in the amygdala and in the PVN, both sites known to be active in the control of feeding (fig. 6). Intraperitoneal or gastrointestinal infusion of enterostatin is dependent on intact afferent vagal transmission for effect and for inducing c-Fos immunoreactivity in specific brain sites known to be activated in feeding. Thus, neuronal transmission of the enterostatin response from the intestine to the brain is a prerequisite if enterostatin is not entered into the brain by direct injection or transport from the blood across the blood-brain barrier. Physiologically, enterostatin is produced mainly in the duodenum and can thus exert its effects locally or by transport to the surrounding capillaries. The local effect in the intestine might be directly on nerve endings in the muscle layer of the intestine, or by stimulation of a paracrine system and thus stimulation of the afferent nerves indirectly (Langhans, 2001). The identities of the target molecules or paracrine mechanisms are not known. Enterostatin also exerts a direct effect on the pancreatic β-cell with a subsequent decrease in insulin secretion. The target and intracellular effects are being investigated.

Enterostatin has been shown to bind specifically to crude rat brain membranes (Lin *et al.*, 1998; Sörhede *et al.*, 1993). The study by Sörhede et al proposed a two-site model with one high (30≈nM) and one low (0.5≈nM) K_d-value, while Lin *et al.* in their binding study identified a low affinity binding site (K_d≈0.1 μM). These authors also postulated that another high-affinity binding is probable for the action of enterostatin. The two-site affinity model may explain the biphasic (U-shaped) response curve of enterostatin. The identity of the target

protein(s) in the brain membranes was not elucidated in either of the mentioned studies.

Several reports have suggested enterostatin to interact with μ- and κ-opiate receptor pathways, and indeed, enterostatin in many ways acts as an opiate antagonist (fig. 8). The κ-opiate agonist U50,488 blocks the inhibitory effect of i.c.v. enterostatin on food intake (Ookuma *et al.*, 1997). However, U50,488 was unable to displace the binding of enterostatin to crude rat brain membranes and thus did not directly interfere with the same receptor (Lin *et al.*, 1998). The binding of enterostatin to brain membranes was however possible to displace by the, β-casomorphins $_{1\text{-}7,\ 1\text{-}5,\ 1\text{-}4}$, peptides with μ-opiate-like activity, suggesting a μ-opioid or μ-opioid-like receptor to be of importance (Lin *et al.*, 1998). In the same report enterostatin was shown to inhibit the β-casomorphin$_{1\text{-}7}$–induced stimulation of HF intake in a dose-dependent way.

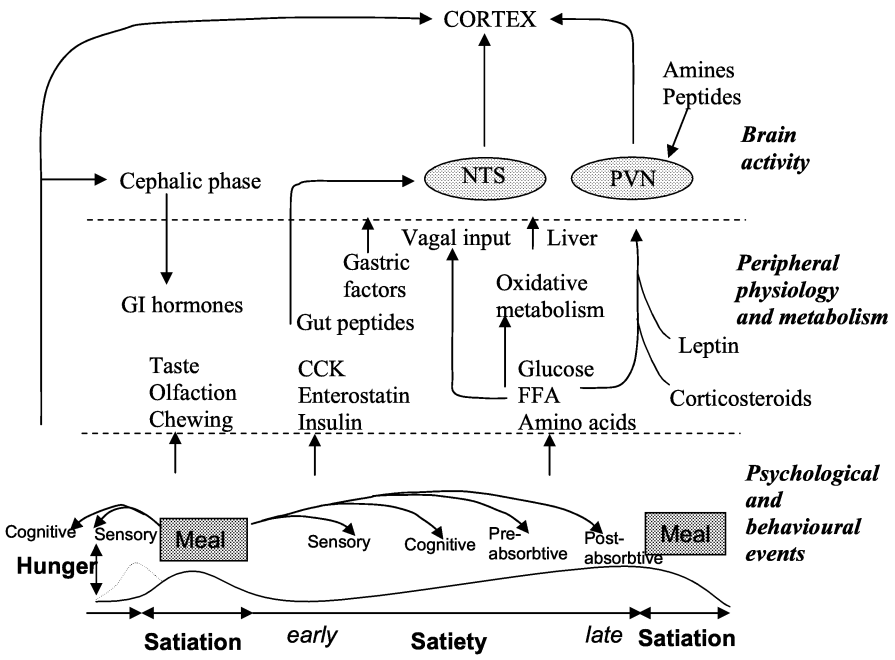

Figure 11. The satiety cascade in relation to the three levels of the psychobiological system of appetite: the behavioural pattern, peripheral physiology and metabolism, and brain activity. Appetite is a synchronous operation on all three levels. Figure modified from Blundell (Blundell, 1991) and Kovacs (Kovacs, 2002).

The antagonistic effect of enterostatin and μ-opiates was further supported in a study by Takenaka *et al.* who showed that i.c.v. injection of enterostatin could inhibit the analgetic effect induced by the μ-opiate agonist morphine. The anti-analgetic effect of enterostatin was, however, not seen after analgesia induced by a κ-opiate agonist or a δ-opiate agonist (Takenaka *et al.*, 2001). In addition, enterostatin was shown to improve amnesia induced by scopolamine in mice, which is in agreement with some other anti-opiates that has been shown to improve memory (Takenaka *et al.*, 2001).

The stimulation of food-intake by galanin and NPY is actually inhibited by enterostatin, but no direct interactions with their receptors have been found (Lin *et al.*, 1993a). The fat intake reducing effect of enterostatin also appears to be mediated through the serotoninergic and dopaminergic systems, both known to regulate food intake (fig. 8). Enterostatin has been shown to increase the turnover of serotonin in hypothalamic sites important for the regulation of feeding behaviour (Erlanson-Albertsson and York, 1997). In addition, the concentration of both serotonin and dopamine in the lateral hypothalamic area (LHA) in rats were shown to be increased after injection of enterostatin to LHA (Koizumi and Kimura, 2002). The authors therefore propose the central feeding effect of enterostatin to be due to a serotonergic and dopaminergic response. Further evidence for a serotonin pathway mediating enterostatin response is indicated by abolishment of enterostatin response by the serotonin antagonist metergoline (Erlanson-Albertsson and York, 1997). One proposal is that enterostatin may modulate the opioidergic activity through a serotonin pathway (Erlanson-Albertsson and York, 1997).
Adaptation to HF food seem to be important for enterostatin to act as an inhibitor of fat feeding, indicating there must be a secondary signal related to dietary fat for the response of enterostatin. Ingestion of high levels of dietary fat induces a range of endocrine, metabolic and neurochemical changes, but the specific parameter important for the enterostatin action is still unknown.

How to treat obesity

The escalating obesity problem and the increasing understanding of the complex systems regulating body weight, have led to efforts to develop new drugs to reduce body weight. Actually, this is a more difficult task than immediately apparent since a change in one regulation system can be compensated by

adaptations and regulations of other systems. Biology also has a much weaker resistance to overconsumption and weight gain. Another problem in development of obesity drugs is that many of the peptides involved in food intake also affects other systems besides appetite, e.g.. reproduction and sleep-wake cycle (Wilding, 2002).

However, the only way to treat obesity is to obtain a negative energy balance, i.e. energy intake should be less than energy expenditure. It is easy to understand that healthy eating and increased physical activity would solve the problem, but unfortunately that does not always work in practice since there is an additional biological component. The genes and the environment are not compatible in this case. To decrease the energy intake, it is possible to either reduce appetite, especially appetite for energy dense food, or inhibit the absorption of energy. To increase energy expenditure it is possible to either increase physical activity or increase thermogenesis.
There are four general classes of anti-obesity drugs: 1) Inhibitors of food intake (appetite suppressants) that reduce hunger perception and increase the feeling of fullness. 2) Inhibitors of fat absorption. 3) Enhancers of energy expenditure through increase of thermogenesis without increase in physical activity. 4) Stimulators of fat mobilisation (Campfield *et al.*, 1998).
Only a couple of drugs have been developed for the treatment of obesity. One of them, Sibutramine (Reductil®) reduces appetite (class 1), while one, Orlistat (Xenical®), inhibits the digestion of fat (class 2). So far, there are no drugs in class 3 and 4, however leptin, categorised in class 4, are under development as a drug (Campfield *et al.*, 1998; Dhillon *et al.*, 2001; Heymsfield *et al.*, 1999). In class 3, a β_3-adrenoceptor agonist would be a possible candidate drug (Hauner, 2001; Scheen and Lefebvre, 2000).

Sibutramine (Reductil®)

Sibutramine is a re-uptake inhibitor of serotonin and noradrenalin, but does not stimulate the release of serotonin from nerve endings. Sibutramine has been shown to cause weight loss in humans and rats, by enhancing satiety (Connoley *et al.*, 1999). The suppressant effect of sibutramine on food intake has been shown to be due to activation of β_1–adrenoceptors, 5-$HT_{2A/2C}$ receptors and particularly α_1–adrenoceptors (Jackson *et al.*, 1997). The ability of sibutramine to cause weight loss has been shown not only to be an effect of reduced food intake, but also as a consequence of increased metabolic rate, i.e. sibutramine

has additional thermogenic properties (Connoley *et al.*, 1999). The thermogenic effect was demonstrated by increased oxygen consumption, increased body temperature and an eighteen fold increase in brown adipose tissue in rats treated with 10 mg kg^{-1} sibutramine, resulting in a 30% increase in thermogenesis. These increases are due to central stimulation of efferent sympathetic nerves that activate thermogenesis via β_3–adrenoceptors (Connoley *et al.*, 1999). In addition, sibutramine has also been shown to improve the glycemic control in patients with type 2 diabetes (Fujioka *et al.*, 2000).

Orlistat (Xenical®)

Orlistat (tetrahydrolipstatin) is a chemically synthesised hydrogenated derivate of lipstatin, a naturally occurring lipase inhibitor produced by *Streptomyces toxytricini* (Weibel *et al.*, 1987). Orlistat covalently blocks lipases by reacting with the serine residue in the catalytic triad in the active site (Hadvary *et al.*, 1991), thereby inhibiting the hydrolysis of dietary triglycerides to absorbable free fatty acids and monoglycerides. Orlistat acts only locally in the gastrointestinal tract to inhibit gastric and pancreatic lipases, cholesterol esterase and other gastrointestinal lipases that are serine hydrolases (Borgström, 1988). The systemic absorption of orlistat is negligible (Zhi *et al.*, 1995), and therefore does not inhibit lipases like lipoprotein lipase and hormone-sensitive lipase *in vivo*. The recommended dose of 120 mg three times a day with the main meals reduces fat-absorption to about 30% (Zhi *et al.*, 1994). Treatment with orlistat together with a mildly hypocaloric diet has been shown to promote a weight loss of about 10% over a one year period (Sjöström *et al.*, 1998). The weight reduction by orlistat is associated with positive changes in several risk factors like dyslipidemi, hyperinsulinemi, glucose intolerance, type 2 diabetes and blood pressure (Ballinger and Peikin, 2002). Reduced postprandial plasma CCK levels have been reported after orlistat treatment, and this has been associated with an increase in hunger and reduced fullness by the patients (Feinle *et al.*, 2001). Few side-effects have been observed by orlistat. The most common is gastrointestinal side effects like steatorrhoea, seen after a diet high in fat. There might be a risk of decreased uptake of fat soluble vitamins, but generally these remain within the clinical reference range (Sjöström *et al.*, 1998). However, the safety of orlistat has not yet been established beyond two years that is the maximum time for treatment with orlistat (Ballinger and Peikin, 2002).

PRESENT INVESTIGATIONS

Aim

The aim of the present study was primarily to find, identify and characterise the receptor or target molecule(s) for the endogenous peptide enterostatin, and to further evaluate the subsequent intracellular mechanisms after targeting.

Studies were performed to:

- Find a possible receptor protein in a neuronal cell line, and evaluate the possible antagonistic action of opiates according to this protein.

- Purify enough amounts of the target protein from rat brain membranes to be able to identify the protein, and to further evaluate the effects of opiates on the enterostatin binding to the target protein. Examine if the same protein could be identified in an insulin-producing cell line, and study the intracellular and physiological consequences of the targeting.

- Study the effects after intravenous infusion of enterostatin and the opiate β-casomorphin on the high-fat food intake in rats and to study the binding properties of enterostatin to rat brain membranes and to the target protein F_1F_0-ATP synthase in absence or presence of β-casomorphin.

- Study the relationship between the fatty acid induced UCP2 mRNA expression and insulin secretion after long time exposure of substances known to influence the insulin secretion in the insulin producing cell line INS-1.

Results and Discussion

The target molecule for enterostatin (paper I, II and III)

Background

The search for a receptor or cellular target for enterostatin has been in focus since enterostatin was found to be a peptide active in appetite regulation. Since enterostatin is most potent when injected into the brain, and gastrointestinal injection is dependent on afferent vagal transmission for effect, a first postulated receptor for enterostatin is supposed to be of neuronal origin. Several experiments have been done to elucidate the target molecule for enterostatin and some proposed interactions, like with galanin- and NPY-receptors, have been excluded as candidate receptors for enterostatin (Blundell *et al.*, 1993 72). Two independent experiments showed a binding of enterostatin to crude rat brain membranes, however, the size or identity of the target molecule was not investigated (Lin *et al.*, 1998; Sörhede *et al.*, 1993). Both studies proposed dissociation constants for the binding. In the study by Sörhede *et al.*, a two-site affinity binding model was postulated, one high affinity and one low affinity binding, with K_d of 0.5 nM and 30 nM respectively (Sörhede *et al.*, 1993). Lin *et al.* also suggested enterostatin to have one high and one low affinity site, and proposed a K_d of around 1 nM for the high affinity binding and about 100 nM for the low affinity (Lin *et al.*, 1998). Since the response curve of enterostatin is U-shaped, it has been proposed that the high affinity receptor inhibits and the low affinity receptor stimulates fat intake (Lin *et al.*, 1998).

Paper I

Since previous studies had shown a binding of enterostatin to brain membranes we screened neuronal cell lines for *in vitro* studies. The human neuroepithelioma cell-line SK-N-MC was the only out of five cell lines tested of neuronal origin, that showed affinity for enterostatin. What property that differs SK-N-MC cells from other neuronal cell-lines that make them special for enterostatin is not known.

In the SK-N-MC cells, the specific binding of ^{3}H-enterostatin was found to be saturated at 50 nM (fig. 12A). A Scatchard plot of the binding indicated a two affinity binding model, in agreement with previous findings in rat brain membranes (Sörhede *et al.*, 1993). K_d were estimated to 0.5-1.5 for the high affinity and 15-30 nM for the low affinity, also in agreement with previous results (Sörhede *et al.*, 1993). It was possible to displace bound ^{3}H-enterostatin

with unlabelled enterostatin and IC_{50} was estimated to 0.3 µM (fig. 12B). To be able to radiolabel enterostatin with iodine for autoradiography, a prolonged peptide with an N-terminal tyrosine was constructed (YGGAPGPR). This peptide (called enterostatin analogue) was shown to bind to SK-N-MC cells, but with a single-site binding pattern with a K_d of about 40 nM, hence close to the Kd of the low affinity binding site. A protein with affinity for enterostatin was revealed after affinity chromatography of solubilized SK-N-MC cells and subsequent SDS-PAGE. The enterostatin-binding protein had an estimated size of 53 kDa. A protein with the same size was found by affinity cross-linking of ^{125}I-labelled enterostatin analogue to SK-N-MC cells. A protein with an estimated size of about 53 kDa was visualised after SDS-PAGE and autoradiography. The binding of enterostatin was abolished with addition of an excess of unlabelled enterostatin during the incubation.

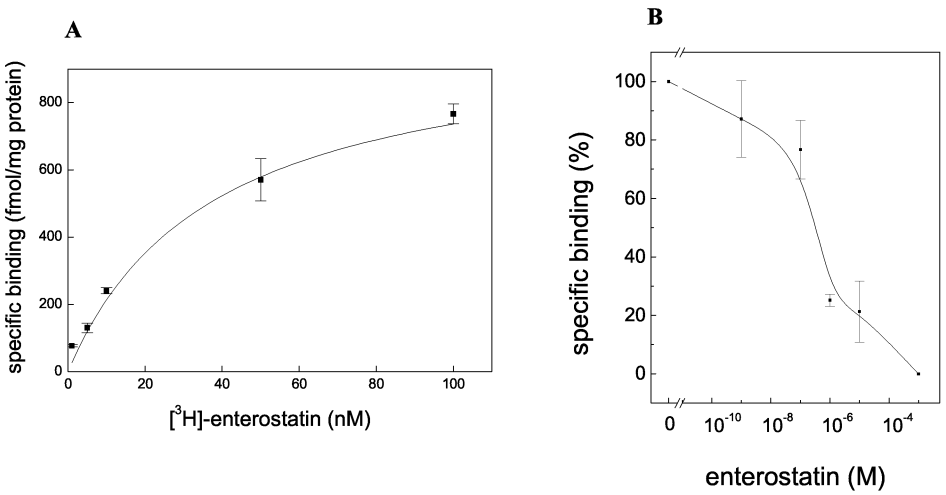

Figure 12. The specific binding of ^{3}H-enterostatin to SK-N-MC cells was saturated at about 50 nM **(A)** and displaced with unlabeled enterostatin **(B)**.

Paper II

Rat brain membranes were used to purify enough amounts for identification of the enterostatin binding protein. Intracellular proteins disturbed the interaction between enterostatin and the membrane proteins, hence a further purification step was necessary. Membrane proteins were purified using a detergent/polymer aqueous two-phase system (Sivars and Tjerneld, 2000). The membrane protein fraction was subjected to enterostatin affinity chromatography and specifically eluted with an excess of enterostatin. The eluate was separated on a SDS-PAGE, and the main 52 kDa band (fig. 13A) was excised and analysed with MALDI-TOF-MS. Unexpectedly, the protein was identified as the β-chain of the F_1F_0-ATP synthase. The interaction was confirmed using purified bovine F_1-ATP synthase and the binding of labelled enterostatin was displaced in a dose-dependent way by unlabelled enterostatin, supporting a specific binding (fig. 13B). An interaction of enterostatin to a protein with the same size as the β-chain of the F_1F_0-ATP synthase was confirmed in the insulinoma cell line INS-1 (fig. 13C).

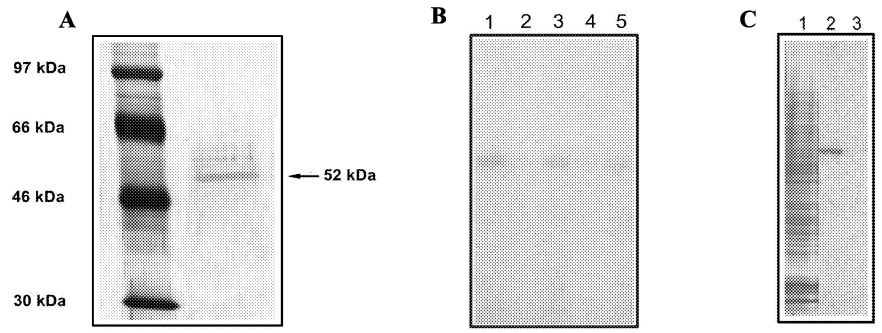

Figure 13. A. Proteins from rat brain membranes were separated on SDS-PAGE after purification and enterostatin affinity chromatography. The 52 kDa protein revealed was identified as the β-subunit of the F_1F_0-ATP synthase. **B.** The binding of enterostatin to β-subunit of F_1F_0-ATP synthase was verified in pure F_1-ATPase separated on SDS-PAGE (lane 1). The binding was abolished in a concentration dependent way after addition of two different concentrations of enterostatin analogue (lane 2 and 3) and two different concentrations of enterostatin (lane 4 and 5). **C.** Proteins from INS-1 cells were separated on SDS-PAGE (lane 1). Enterostatin showed affinity for a protein with the same size (52 kDa) as the β-subunit of F_1F_0-ATP synthase (lane 2), and the binding was completely displaced by an excess of unlabelled enterostatin (lane 3).

F_1F_0-ATP synthase is an enzyme that until recently was thought to be exclusively expressed in the inner mitochondrial membrane. The total molecular size is ≈530 kDa. The protein consisting of one membrane-bound F_0 portion, which is involved in the proton translocation, and one F_1 portion on the matrix side of the inner membrane, that catalyses the ATP hydrolysis. F_1-ATPase has nine subunits; 3α, 3β, γ, δ and ε, with masses (in *E. coli*) of about 55, 50, 31, 19 and 14 kDa, respectively. A stalk composed of subunits from both F_1 and F_0 connect the two portions. An ATP synthase of similar structure is found in all organisms that form or cleave ATP coupled to proton translocation. The β-subunit from different species show exceptionally strong sequence homology, whereas the other subunits show more sequence and size variation between species (Boyer, 1997). The enzyme has a rotation mechanism in which the proton gradient drives the proton translocation through the F_0 portion, which causes a rotation of the F_1 γ-subunit. The rotation leads to sequential conformational changes in the β-subunits with subsequent release of the bound ATP (Boyer, 1997) (fig. 4). For production of ATP, the complete F_1F_0-ATP synthase within the membrane and a proton gradient is required, but the F_1-ATPase itself can exhibit activity in the reverse reaction, i.e. the hydrolysis of ATP to form ADP.

As mentioned above, the identification of F_1F_0-ATP synthase as the target molecule was surprising. Since G-protein coupled receptors located in the plasma membrane are most common for peptides regulating food intake, a target molecule in the inner mitochondrial membrane was unexpected. The major question was how enterostatin could pass through three membranes to target a protein inside the mitochondrial matrix. However, studies have reported the presence of F_1-ATPase at the cell surface on lymphocytes and human endothelial cells (Chang *et al.*, 2002; Das *et al.*, 1994; Moser *et al.*, 2001; Moser *et al.*, 1999). Also recently, Martinez *et al.* reported that the β-chain of F_1F_0-ATP synthase, expressed on the surface of hepatocytes, was a receptor for apolipoprotein A-I (apoA-I) (Martinez *et al.*, 2003). ApoA-I is a lipoprotein present on the surface of high density lipoprotein (HDL), acting as a ligand for the HDL receptor. HDL particles mediate the transport of cholesterol from peripheral tissues to the liver for further metabolism. The receptors for HDL on hepatocytes are thus important in the cholesterol homeostasis.
Another apolipoprotein, apoA-IV, is also present in HDL particles. ApoA-IV has been shown to suppress appetite specifically after digestion of fat. Enterostatin and apoA-IV share a number of features and have recently been

compared in a review article (Liu *et al.*, 1999). The receptor for apoA-IV is still unknown.

In addition, the α/β-subunits of F_1F_0-ATP synthase, expressed on the surface of endothelial cells, have been shown to act as a binding site for angiostatin, a proteolytic product of plasminogen (Moser *et al.*, 2001; Moser *et al.*, 1999). Angiostatin is a potent angiogenetic substance and has been suggested to act by steric hindrance inhibition of ATP synthesis by binding to the α/β-subunits and inhibiting the required conformational changes (Moser *et al.*, 2001). Another peptide, the C-terminal domain of p43, endothelial monocyte-activating peptide II (EMAP II), is known to bind to the α-subunit of F_1F_0-ATP synthase on the surface of endothelial cells (Chang *et al.*, 2002).

Other substances, for example the antibiotic peptide efrapeptin (Abrahams *et al.*, 1996; Cross and Kohlbrenner, 1978) and the endogenous inhibition protein IF_1 (Cabezon *et al.*, 2000), are also known to bind to and thus inhibit, the catalytic F_1-part of F_1F_0-ATP synthase of the mitochondrial located enzyme.

The ectopic expression of F_1F_0-ATP synthase on the cell surface is not a general feature of all cells (Martinez *et al.*, 2003). It may be that the K-N-MC cells have

er I).

this enzyme on the surface (pap

ostatin to F_1-ATPase was further investigated in
n enterostatin and F_1-ATPase was studied in an
tem (Albertsson *et al.*, 1990; Patton *et al.*, 1978).
n of enterostatin between the top-phase and the
in the presence of F_1-ATPase, it was possible to
ng. The stronger binding between ligand and
hange in the partition coefficient of the ligand in
rtition coefficient for enterostatin in the absence
e 1.44, while F_1-ATPase alone was to 100%
e. In the presence of F_1-ATPase, the partition
eased from 1.44 to 0.61, hence demonstrating a
g was estimated to 170 nM based on a single
lt is approximately in agreement with Lin *et al.*
nM (Lin *et al.*, 1998).

Paper III

The binding properties of enter
paper III. The targeting betwee
aqueous two-phase partition sys
By measurement of the partitio
bottom-phase in the absence or
calculate the K_d of the bindi
protein, the more pronounced c
presence of F_1-ATPase. The pa
of F_1-ATPase was found to b
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coefficient for enterostatin decr
binding. The K_d of the bindin
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who proposed a K_d of about 100

Enterostatin as an opiate antagonist (paper I, II and III)

Background

Several studies have shown interaction of enterostatin with the μ- and κ-opioid pathways regarding high-fat intake and binding to brain membranes (Barton *et al.*, 1995; Lin *et al.*, 1998; Ookuma *et al.*, 1997; White *et al.*, 2000). The κ-opioid agonist U50, 488 is known to stimulate high-fat feeding. U50, 488 injected i.c.v. together with enterostatin has been shown to reverse the selective inhibitory effect of enterostatin on HF food intake in Sprague-Dawley rats (Barton *et al.*, 1995), and enterostatin has been shown to inhibit the stimulation of HF intake by U50, 488 (Ookuma *et al.*, 1997). In addition, the stimulation of insulin secretion in isolated islets induced by U50, 488 was completely abolished by enterostatin (Ookuma and York, 1998). These studies support that enterostatin attenuates fat intake through inhibition of a κ-opioid pathway although no interaction directly with κ-opioid receptors has been found (Ookuma *et al.*, 1997). U50, 488 was neither able to displace the binding of enterostatin to crude rat brain membranes (Lin *et al.*, 1998).

β-casomorphins are small peptides with μ-opioid agonist activity, produced during digestion of the milk-protein β-casein (Teschemacher *et al.*, 1997). It should be noted that β-casomorphin$_{1\text{-}5}$ (YPFPG) and enterostatin (APGPR) is similar in structure and size with proline residues in position two and four. Centrally and intragastrically administered β-casomorphin$_{1\text{-}5}$ and β-casomorphin$_{1\text{-}7}$ have been shown to specifically stimulate the intake of HF food, but not the intake of LF food. The peptides hence act contradictory to enterostatin regarding HF intake (Lin *et al.*, 1998; White *et al.*, 2000). β-casomorphins (β-CM$_{1\text{-}4}$, β-CM$_{1\text{-}5}$ and β-CM$_{1\text{-}7}$) were all able to displace the binding of enterostatin to crude rat brain membranes with an IC_{50} estimated to 7μM (Lin *et al.*, 1998). It has been postulated that β-casomorphin has affinity for the low-affinity binding site for enterostatin. The low affinity receptor is proposed to stimulate the HF food intake seen by high concentrations of enterostatin (White *et al.*, 2000).

Paper I

The binding of enterostatin to SK-N-MC cells was possible to displace by addition of two different opiates. The binding of 20 nM ^{3}H-enterostatin was displaced by 1 μM and 100 μM of met-enkephalin (YGGFM) and β-casomorphin (YPFPG) in a concentration dependent manner (fig. 14A). Met-enkephalin is known to bind primarily to δ-opioid receptors, but also to μ-receptors, while β-casomorphin mainly binds to μ-receptors. This result

indicates that μ-opioids might act on the same target molecule as enterostatin or vice versa. The results are in agreement with Lin *et al.* who demonstrated that β-casomorphin could displace the binding of enterostatin to brain membranes (Lin *et al.*, 1998).

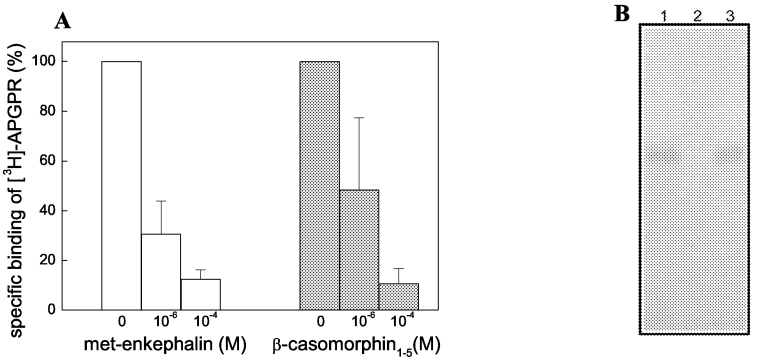

Figure 14. A. The binding of ^{3}H-enterostatin to SK-N-MC cells was displaced in a concentration dependent way by the opiates met-enkephalin and β-casomorphin. **B.** The binding of enterostatin to pure F_1-ATPase was displaced by the μ-opiate β-casomorphin (lane 2) but not by the κ-opiate U50, 488 (lane 3).

Paper II

The apparent targeting of enterostatin to F_1F_0-ATP synthase was further verified by binding to purified bovine F_1-ATPase. The iodinated enterostatin analogue was found to bind to a protein with the size of the β-subunit in the F_1-ATPase. The binding was possible to abolish with addition of an excess of unlabelled enterostatin or enterostatin analogue as well as with addition of an excess of the μ-opioid β-casomorphin (fig. 14B). However, addition of the κ-opioid agonist U50, 488 did not inhibit the binding of enterostatin to F_1-ATPase (fig. 14B), indicating that μ-opioids, but not κ-opioids interfere with the targeting of enterostatin.

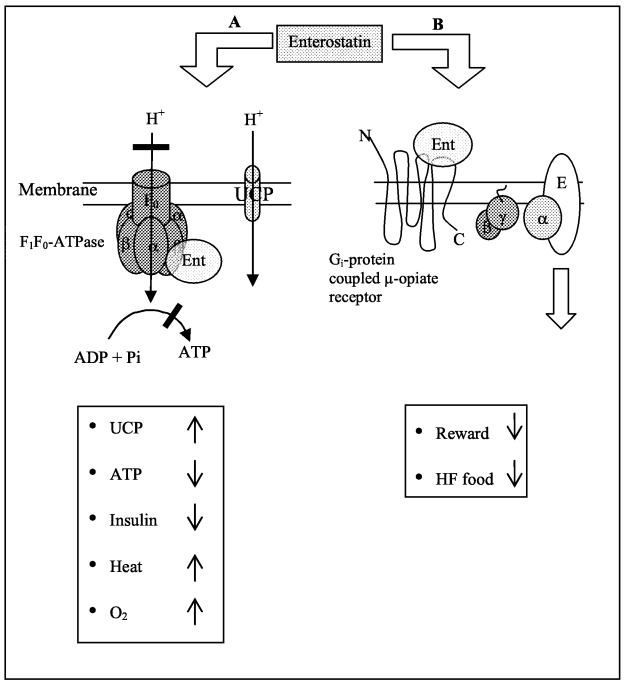

Figure 15. Two postulated cellular target proteins for enterostatin. In pathway **A**, enterostatin targets the β-subunit of F_1F_0-ATP synthase which probably perturbs the ATP synthesis and subsequently decreases the transport of protons through the ATPase. The protons will instead pass through the uncoupling protein (UCP) in the membrane causing an increase of in UCP expression with consequent increased heat production and oxygen consumption. The decreased ATP production will affect ATP-dependent processes such as decreased insulin secretion in pancreatic β-cells. In **B**, a complementary pathway for enterostatin is proposed to target a μ-opiate receptor or a μ-opiate like receptor, which is of G_i-protein coupled type, inhibiting the effector protein (E) that might be either adenylyl cyclase or K^+-channel. The targeting will result in decreased intake of high-fat food, probably through a decreased reward mediated by inhibition of μ-opiates.

Paper III

The effect of intravenous injection of enterostatin and β-casomorphin, alone or together, on high-fat feeding was studied in Sprague-Dawley rats. A low dose (9 nmol) of enterostatin did not affect the HF food (table 5) intake while 38 nmol decreased, and the higher dose 76 nmol instead increased the HF intake. β-

casomorphin increased the HF food intake after intravenous injection of all tested concentrations. The combination of equimolar 38 nmol injection of enterostatin and β-casomorphin did not show any effect on HF food intake compared to control. When given together in the higher dose, 76 nmol of each peptide, an increase in HF food intake was observed. These results verify the U-shaped response of enterostatin and confirm the antagonistic effect of enterostatin and β-casomorphin, at a limited concentration, also after intravenous administration. At higher doses, there was instead a synergistic effect with stimulation of HF food intake.

Table 5. Composition and energy content of the diets used in the feeding experiments

Nutritional content		**Low-fat (LF, chow) diet** weight (g/100g)	**High-fat (HF) diet** weight (g/100g)
Protein		21	26
Carbohydrate		47	41
Fiber		10,5	6
Fat		5	18
of which	Corn oil	-	18
	Lard	3.5	-
	Linoleic acid	1.5	-
Vitamins		1	1
Minerals		3.6	4
Percent of energy as:			
Protein		28	24
Carbohydrate		57	38
Fat		15	38
Energy	kJ/100g	1260	1800
	kcal/100g	300	430

The competition between enterostatin and β-casomorphin was further verified in crude rat brain membranes. β-casomorphin partially displaced the binding of enterostatin (IC_{50}=10 μM). However the displacement with β-casomorphin did not occur to the same extent as with enterostatin itself.

The properties of the binding of enterostatin to F_1-ATPase were studied in a two-phase partition system as described above. The estimated K_d between iodinated enterostatin and F_1-ATPase was 170 nM in absence of β-casomorphin, but in addition of β-casomorphin the apparent K_d for enterostatin was increased to 500 nM. This result suggests that the binding of enterostatin to F_1-ATPase could be partly blocked by an excess of the μ-opioid β-casomorphin, thus supporting previous results.

Altogether, this study further supports interference of the opioid pathways in the action of enterostatin both *in vivo* and *in vitro* (fig.15). Additionally, we show in this study that the dissociation constant of enterostatin to F_1-ATPase is disturbed in presence of β-casomorphin, indicating that μ-opiates might target F_1F_0-ATP as well.

Enterostatin in the regulation of energy metabolism (paper II)

Background

Enterostatin has in previous studies been shown to affect the regulation of energy metabolism in different ways: Activation of the sympathetic drive to interscapular BAT was shown after i.c.v. injection of enterostatin to rats fed with HF diet (Nagase *et al.*, 1996). However, no increased sympathetic firing after enterostatin injection was seen in rats fed with low fat diet (Nagase *et al.*, 1996). This result might indicate an increased energy expenditure by increased thermogenesis in BAT after enterostatin treatment during HF feeding. Stimulation of UCP1 in BAT after enterostatin treatment and HF food intake is further supported in a study in mice housed in thermoneutral environment, by Rippe *et al.* (Rippe *et al.*, 2000). In the same study, an increase of UCP2 mRNA expression in the gastrointestinal tract was induced by enterostatin, while UCP2 mRNA expression in BAT was decreased.

Enterostatin has previously been shown to decrease glucose- or lipid-induced insulin secretion in isolated islets, perfused pancreas and *in vivo* in rats (Erlanson-Albertsson *et al.*, 1994; Mei *et al.*, 1997; Ookuma and York, 1998; Rodriguez-Gallardo *et al.*, 1999).

Paper II

The surprising observation that enterostatin specifically binds to the β-chain of the F_1F_0-ATP synthase was thought to influence intracellular ATP production and intracellular events dependent on ATP production. Intracellular effects after targeting of enterostatin were studied in the insulin-producing cell-line INS-1, since previous observations have shown that enterostatin can regulate insulin secretion. In these cells enterostatin was shown to target a protein in the same size as the β-chain of the F_1F_0-ATP synthase. ATP was measured with a luminometric assay after 2 or 15 minutes incubation with enterostatin in INS-1 cells. Enterostatin was initially shown to decrease the ATP production, but after 15 minutes the ATP concentration was returned to the same level as in the control. Our hypothesis is that the cell rapidly compensates for the decreased ATP production with increased substrate oxidation and subsequent increased proton gradient and finally restored ATP production. The increased substrate oxidation and the accompanying increased proton gradient may induce the augmented thermogenesis and oxygen consumption observed in INS-1 cells after enterostatin incubation (fig. 15). The increased thermogenesis in cells in the presence of enterostatin was measured with a microcalorimeter and estimated to a 4.6 % increase lasting for two hours. This increased thermogenesis may be due to an increased proton leak, perhaps through UCP2, to protect the cells from increased production of free oxygen species as a consequence of the increased proton gradient.

The effect of enterostatin to reduce insulin secretion was here confirmed in the rat insulinoma cell-line INS-1. The glucose-induced insulin secretion is an ATP-dependent process. It is hypothesised that the metabolism of glucose increases intracellular ATP which in turn closes K^+-channels leading to increased influx of Ca^{2+}, which is a stimulus for exocytosis of insulin-containing vesicles. The consequence of a reduced ATP-production by binding of enterostatin to the F_1F_0-ATP is hence a decreased glucose induced insulin secretion in insulin producing cells (fig. 15). The decrease in insulin secretion induced by enterostatin might also be a consequence of induced thermogenesis and decreased ATP production following an increased uncoupling, for example by induced UCP2 expression (Chan, 2002). Another proposed mechanism for enterostatin to reduce insulin secretion is through a reduction in the intracellular cAMP levels. The cellular target for this mechanism is however not elucidated (Ookuma and York, 1998).

The effect of enterostatin regarding the regulation of energy metabolism was further visualized in an animal experiment. Body weight gain was compared to

energy intake (i.e. feed efficiency = g body-weight/kcal) in rats during intraperitoneal administration of enterostatin during HF (38 % of calories as fat) and LF (15 % of calories as fat) food intake. HF diet caused a hyperphagia and increased body weight gain compared to LF diet. Enterostatin abolished the body weight gain seen in high-fat fed animals, and also significantly decreased the feed efficiency. The previously observed effect of enterostatin in reducing the intake of HF food and reducing body weight gain after HF feeding might, at least hypothetically, be due to an increased energy expenditure through targeting of enterostatin to F_1F_0-ATP synthase.

Uncoupling protein 2 and insulin secretion (paper IV)

Background

Type 2 diabetes is associated with obesity and increased levels of circulating free fatty acids and high plasma glucose. Several studies have proposed that long-term exposure of β-cells to FFAs decreases insulin secretion in response to glucose, and hence alters the energy metabolism of the β-cell (Haber *et al.*, 2003; Randle *et al.*, 1994; Zhou and Grill, 1994) (fig. 5). Long-term exposure to fatty acids or dietary fats is also known to increase the expression of UCP2 in several tissues, including the β-cell (Lameloise *et al.*, 2001; Zhang *et al.*, 2001). UCP2 is supposed to decrease the glucose-induced ATP production by uncoupling of the proton gradient over the inner mitochondrial membrane and thus decreasing the oxidative phosphorylation. Since insulin is an ATP dependent process, insulin secretion is decreased in β-cells with increased expression of UCP2 (fig. 5). The increased insulin secretion in the UCP2 null mice (Zhang *et al.*, 2001) and the decreased insulin secretion in rat islets overexpressing UCP2 (Chan *et al.*, 2001; Chan *et al.*, 1999) further support this. Long-time exposure of high-fat diet in animals lacking the gene for UCP2 show lower blood-glucose and higher plasma-insulin levels compared to wild-type mice, indicating that UCP2 might be a linkage between fatty acids and impaired insulin secretion (Joseph *et al.*, 2002). It is, however, not known if the inhibited insulin secretion caused by fatty acids is automatically associated with changes in UCP2 expression.

Paper IV

The insulin secretion and UCP2 mRNA expression was compared in INS-1 cells exposed to oleic acid for 72h, and as expected, oleic acid caused an increased UCP2 expression and a decreased insulin secretion (fig. 16A and B). The same parameters were measured after incubation with forskolin or TNFα with and without oleic acid. Oleic acid induced UCP2 mRNA expression also in the presence of forskolin (fig. 16A), but the forskolin-induced insulin secretion was not affected by oleic acid (fig 16B), in spite of the increased UCP2 expression. This result indicates that cAMP bypasses the UCP2-mediated decreased insulin secretion, maybe by a direct stimulation of exocytosis of insulin. TNFα, on the other hand, was shown to decrease UCP2 expression (fig.16A), whereas in presence of oleic acid there was no change in the UCP2 expression compared to control in presence of oleic acid. In spite of the induced UCP2 expression by oleic acid in the presence of TNFα, insulin secretion was not reduced (fig.16B), indicating that TNFα counteracts the reduction of insulin secretion associated with increased UCP2 expression. It thus seems that increased UCP2 expression is not always associated with decreased insulin secretion.

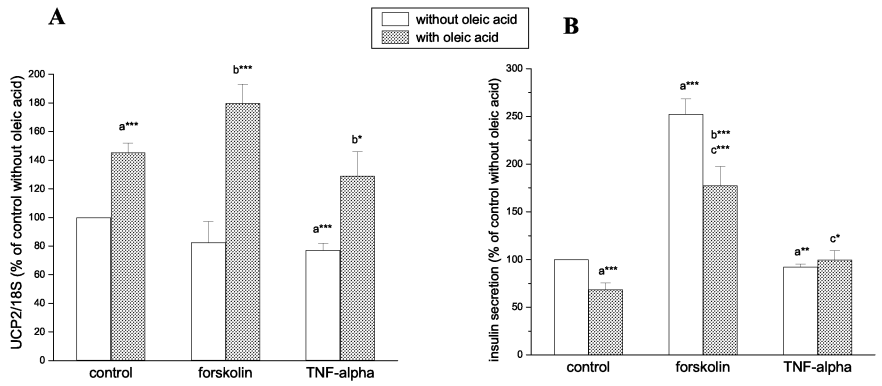

Figure 16. UCP2 expression **(A)** and insulin secretion **(B)** in INS-1 cells after long-term (72h) exposure to oleic acid, in combination with forskolin and TNFα.
Data are presented as mean of percent of control in the absence of oleic acid ± SE, from three independent experiments analyzed in triplicates. *≤0.05, **≤0.01 and ***≤0.005. a=compared to control without oleic acid, b=compared to the same substance without oleic acid, c=compared to control with oleic acid.

Major conclusions

- A target protein for enterostatin has been identified as the β-chain of the F_1F_0-ATP synthase in neuronal cells, and an equally sized protein with affinity for enterostatin has also been identified in the insulinoma cell-line INS-1.

- In INS-1 cells, the targeting of enterostatin was shown to affect ATP-production, thermogenesis, oxygen consumption and insulin secretion. The effect of enterostatin in the regulation of energy metabolism was further confirmed in an animal experiment when enterostatin was shown to decrease the energy efficiency (weight gain/ ingested kcal) in high fat fed rats.

- The antagonistic properties of enterostatin and β-casomorphin were demonstrated in several ways: Simultaneous intravenous injection of enterostatin and β-casomorphin counteracted the inhibitory effect on high-fat food intake by enterostatin. The specific and reversible binding of enterostatin to crude rat brain membranes was partly inhibited by addition of β-casomorphin, and in addition, the binding of enterostatin to F_1-ATPase was inhibited by different μ-opiate agonists but not by a κ-opiate agonist.

- Fatty acid-induced UCP2 overexpression in INS-1 cells impaired insulin secretion independently of increased cAMP, and cAMP thus exerting its effect on insulin secretion separately from UCP2. On the other hand, TNFα seems to counteract the inhibitory influence of UCP2 expression on insulin secretion, indicating that TNFα perturbs the effect of UCP2 expression on insulin secretion.

Concluding remarks and Future perspectives

We have for the first time identified a target protein for enterostatin. The targeting of enterostatin to the β-chain of the F_1F_0-ATP synthase was initially surprising, but turned out to fit very well with many of the observed effects of enterostatin, such as inhibited insulin secretion and increased UCP expression. However, the complete mechanism of action and especially the food regulating mechanism, for enterostatin is still not clear. The antagonistic or competitive effects of opiates, and particularly μ-opiates, are not explained, although μ-opiates were shown to abolish the binding of enterostatin to F_1-ATPase.

Several reports have postulated a two-affinity binding site for enterostatin, one high-affinity and one low-affinity. Whether this should be due to two different target proteins or the same protein with diverging responses to different concentrations of the peptide is still not known. It has previously been proposed that a μ-opioid receptor is involved in the low-affinity targeting and in that case maybe F_1F_0-ATP synthase could act as a high-affinity target for enterostatin.

There are however some objections to F_1F_0-ATP synthase as a receptor for enterostatin: Firstly, the mitochondrial enzyme is expressed in every cell of the body while enterostatin only exerts its effect on a few cell types, i.e. some types of neuronal cells and pancreatic β-cells. Secondly, it seems difficult, although maybe possible, for enterostatin to reach the mitochondrial matrix for targeting. Actually both these problems might be solved by the very recent report from Martinez *et al.* (Martinez *et al.*, 2003), which demonstrates an ectopic expression of β-chain of ATP synthase on the cell surface, acting as a receptor for apolipoprotein A-I. Expression of subunits or holo-enzyme of F_1F_0-ATP synthase, acting as target for peptides has actually also been shown previously. The expression of F_1F_0-ATP synthase on the cell surface is however only seen in some kinds of cells, which could be an explanation for the selective targeting for enterostatin. In addition, it has been shown that a μ-opioid could bind to the brain mitochondrial fraction, and that morphine, another μ-opioid, can decrease the oxidative phosphorylation and oxygen consumption, both linked to ATP synthesis. The importance of these facts according to enterostatin is, however, not known.

Yet, the appetite and HF food regulating effects of enterostatin might at least partly be explained by the targeting to F_1F_0-ATP synthase. Increased thermogenesis following enterostatin targeting could act as a satiety signal

according to the thermostatic theory. An enhanced thermogenesis has been suggested to act as a satiety signal since animals terminate the meal in order to avoid hyperthermia (Brobeck, 1985). This has been further confirmed in several studies where heat, produced by uncoupling or increased metabolism has been shown to decrease food intake (Friedman, 1998; Himms-Hagen, 1995). This thermogenic effect of enterostatin might be especially important during fat-intake, since fat has a weaker effect on satiety and lower diet-induced thermogenesis compared to protein and carbohydrates (Blundell *et al.*, 1993; Westerterp-Plantenga *et al.*, 1999).

Even though enterostatin has a thermoregulatory effect on HF food intake there is clearly a sensory component related to the reward system of feeding. The sensory component is believed to involve the μ-opioid pathway. Several experiments, including some presented in this thesis, have suggested a competition between enterostatin and μ-opiate peptides. With an excess of opiates, a stimulation of fat intake will occur and with an excess of enterostatin an inhibition of fat intake will occur. Enterostatin is thus thought to serve as an "anti-opiate"-factor, probably by inhibition of the reward effect caused by opiates induced by palatable food. Both galanin and NPY stimulates feeding and induce opioid release and thus a reward effect (Dube *et al.*, 1994; Kotz *et al.*, 1993). Enterostatin has been shown to inhibit both galanin- and NPY-induced feeding, although not by interaction with their receptors (Lin *et al.*, 1993a). This further supports that enterostatin modulates the feeding by interaction with the opioidergic system (fig. 8).

The mechanism of action for enterostatin is thus thought to occur both through interaction with F_1F_0-ATP synthase and μ-opiate pathways (fig. 15). Further studies are, however, necessary to elucidate the exact mechanisms regarding both the appetite effect and the metabolic effects of enterostatin. The interaction properties of enterostatin with F_1-ATPase are under investigation and hopefully the interaction will be analysed by crystallography. In addition, the effects of enterostatin on ATP-production and oxygen consumption as well as UCP and ROS production will be further evaluated *in vitro* as well as *in vivo*.

POPULÄRVETENSKAPLIG SAMMANFATTNING
(SUMMARY IN SWEDISH)

De flesta människor bibehåller en relativt konstant kroppsvikt genom livet trots att mängden mat och motion varierar från dag till dag. En konstant kroppsvikt betyder att energiintag (mat och dryck) och energiutgifter (värme och rörelse) i genomsnitt är lika stora, d.v.s. kroppens energibalans är i jämvikt. Övervikt är ett resultat av positiv energibalans, vilket innebär att energiintaget varit större än energiåtgången under en längre tid och att energiöverskottet har lagrats som fett (triglycerider) i kroppens fettväv. Förmågan att lagra överskottsenergi i form av fett har varit genetiskt fördelaktigt när tider av överflöd varvats med svältperioder. Det har medfört att det finns starka regleringssystem och försvars-mekanismer mot undernäring och viktnedgång, medan försvarsmekanismerna mot övervikt är mycket svagare.

De senaste decennierna har övervikt och fetma utvecklats till en global epidemi. Detta dilemma är inte längre begränsat till västvärlden utan också i u-länderna är fetma ett växande problem. Orsaken till denna snabbt ökande fetma är att våra gener inte är anpassade för vår livsstil med allt mer stillasittande i kombination med lättillgänglig och kaloririk mat. Fetma är till ca 40 % ärftligt. Det finns ingen specifik fetma-gen utan ett flertal gener verkar kunna spela roll vid utvecklingen av fetma. Aktuella gener reglerar så väl lagring av fett, reglering av energiåtgång och reglering av aptit och mättnad. Övervikt och fetma är starkt relaterat till många olika sjukdomstillstånd såsom typ 2 diabetes och hjärt- och kärl- sjukdomar.

Aptiten är ett komplicerat samspel mellan många olika faktorer. Signaler i nervsystemet, t.ex. efter syn, lukt eller tanke på mat, stimulerar till måltid. Under måltiden uppstår mättnad när näringsämnena når magsäcken och tunntarmen. Detta signaleras via nervsignaler och hormoner som utsöndrats som svar på måltiden. Flera psykologiska faktorer är också inblandade vid mättnad. Synen och doften av mat är inte längre lockande i slutet av en måltid. När maten så småningom är nedbruten och näringsämnena kommer ut i blodbanan inträffar den andra fasen i mättnaden. Denna fas pågår fram till dessa näringsämnen är slut och man får förnyad aptit och inleder en ny måltid.

Näringsämnena tas upp från blodet till kroppens celler där de bryts ner ytterligare till den slutgiltiga energirika molekylen ATP, som är nödvändig för alla energikrävande processer i cellerna. Den energimängd som bildas i form av ATP överensstämmer inte helt med den mängd energi vi fått i oss via födan

eftersom en del energi ”läcker” ut i form av värme. Det finns specifika proteiner som kallas urkopplande proteiner (UCP) som utför detta ”energi-läckage” och på så sätt ökar energiomsättningen, och det bildas då värme istället för ATP. Dessa värmeproducerande proteiner finns framför allt i brun fettväv och kallas UCP1. Man har nyligen funnit liknande proteiner i kroppens övriga vävnader, dessa benämns UCP2 t.o.m. UCP5. Funktionen av dessa proteiner är emellertid ännu inte fastställd, men de verkar inte ha samma värmeproducerande förmåga som UCP1, även om de på olika sätt verkar vara inblandade i energiomsättningen.

Reglering av födointaget och energibalansen består dels av en kortsiktig reglering som påverkar t.ex. antalet måltider per dag och måltidens storlek, medan den långsiktiga regleringen balanserar födointaget och energiomsättningen för att upprätthålla kroppens energireservoarer. Det finns ett stort antal kroppsegna substanser som alla påverkar olika delar av dessa regleringssystem. Vissa påverkar intaget av ett specifikt födoämne, t.ex. fett, och ett exempel på en sådan är den kroppsegna peptiden enterostatin. Enterostatin bildas i tunntarmen genom klyvning av procolipas till enterostatin och colipas, som är ett protein som är nödvändigt vid nedbrytning av fett i tarmen. Enterostatin har visat sig minska födointaget, och specifikt fettintaget, hos råttor både efter injektion i hjärnan och perifert i kroppen t.ex. i buken. I hjärnan har enterostatin visat sig ha effekt framför allt i hypotalamus som är hjärnans aptitregleringscentrum. Enterostatin har också andra effekter på energiomsättningen, t.ex. genom att hämma insulinsekretionen från beta-cellerna i bukspottskörteln. Dessutom har enterostatin visat sig kunna öka energiomsättningen hos råttor som äter mat med högt fettinnehåll genom att öka mängden UCP1 och UCP2.

Trots att vi vet mycket om effekterna av enterostatin så vet vi fortfarande inte mekanismen bakom eller hur mottagarmolekylen (receptorn) för enterostatin ser ut eller hur den verkar. Tidigare försök har visat att enterostatin binder till hjärnmembraner och att bindningen går att hämma med opiater. Opiater är morfinliknande ämnen som stimulerar vårt belöningssystem. Denna motverkande effekt mellan enterostatin och opiater har också bekräftats i djurförsök där opiater hindrar enterostatinets aptithämmande effekter och istället stimulerar fettintaget.

I denna avhandling har jag identifierat en mottagarmolekyl (receptor) för enterostatin, och jag har också studerat vad som händer inne i cellen efter att enterostatin har fäst till denna molekyl. Jag har också undersökt de motverkande effekterna mellan enterostatin och olika opiater såväl i celler som i djurförsök. Dessutom har jag studerat sambandet mellan fettsyror, UCP2 och

insulinsekretionen för att försöka förstå funktionen av UCP2 vid utvecklingen av den fetma-relaterade sjukdomen typ 2 diabetes.

I **delarbete I** fann jag att enterostatin band specifikt till en klonad nervcell. Bindningen av enterostatin var möjlig att hämma genom att tillsätta två olika opiater som båda binder till s.k. my-opiat receptorer. Ett protein som binder specifikt till enterostatin "fiskades" fram genom att hälla en lösning bestående av proteiner från nervcellerna över en gel på vilken jag fäst enterostatin, och jag kunde på så sätt fiska upp de proteiner i lösningen som bara binder till enterostatin (en metod som kallas affinitets-kromatografi). Efter storleksseparation av dessa proteiner fann jag ett sådant protein. Ett protein med samma storlek kunde också synliggöras genom att blanda radioaktivt märkt enterostatin med cellproteinerna och separera proteinerna med avseende på storlek.

I **delarbete II** ville jag identifiera det enterostatin-bindande protein jag tidigare funnit. För att få tillräckligt med material använde jag mig av hjärnor från råtta. Membraner från hjärncellerna separerades fram och därifrån renade jag fram de proteiner som sitter bundna i membranen. För att finna det protein som enterostatin binder till använde jag mig återigen av affinitetskromatografi och storleksseparation (se ovan), och fann även nu ett protein i samma storlek. När jag fått fram tillräckligt stora mängder (c:a 100 ng) kunde det överraskande identifieras som en del av ATP-syntas. ATP-syntas är det enzym som bildar ATP i mitokondrien (cellens energikraftverk) och som bär energi med hjälp av energirika bindningar. Identifieringen till ATP-syntas kunde också verifieras genom bindning till ett renat enzym. Bindningen kunde dessutom bekräftas i en insulinproducerande cell-linje, INS-1. Även denna bindning visade sig kunna hämmas med en my-opiat. Enterostatinets bindning till ATP-syntas gav även effekter i INS-1 celler. Vi kunde observera en påverkan på ATP produktionen, en ökad värmeproduktion och en ökad syrekonsumtion, vilket tillsammans tyder på en ökad energiomsättning i cellen. Vi visade också att enterostatin, även i INS-1 celler, kunde hämma insulinsekretionen. Enterostatinets förmåga att öka energiomsättningen visades också i försök på råttor som fick mat med högt fettinnehåll i kombination med enterostatin, och de visade sig då få en ökad energiomsättning.

Bindningsegenskaperna mellan enterostatin och ATP-syntas studerades fortsättningsvis i **delarbete III**. Bindningen studerades i ett s.k. två-fas system för att kunna bestämma en dissociationskonstant, som är ett mått på

bindningsstyrkan. Dissociationskonstanten bestämdes till 170 nM, men i närvaro av my-opiaten β-casomorfin ökade den till 500 nM, vilket innebär att my-opiaten stör inbindningen av enterostatin till ATP-syntaset. Detta bekräftades dessutom i hjärnmembraner från råtta, där inbindningen av enterostatin delvis kunde hämmas av β-casomorfin. Den motverkande effekten mellan enterostatin och β-casomorfin studerades även i en djurstudie. β-casomorfin som injicerades i blodet visade sig stimulera fettintaget hos råttor medan enterostatin, inom ett begränsat koncentrationsintervall, hämmade fettintaget. Vid samtidig injektion av både enterostatin och β-casomorfin eliminerades dock effekten. Dessa studier tyder på att enterostatin och my-opiater, åtminstone delvis, verkar via samma regleringsmekanism.

I **delarbete IV** undersökte vi om den ökning av UCP2 som man ser vid långvarig exponering av fettsyror, som t.ex. vid övervikt, alltid följs av en parallell hämning av insulinsekretionen. INS-1 celler odlades i närvaro av oljesyra i tre dygn, vilket ledde till en ökning av UCP2 mRNA (messenger(budbärar)-RNA, som är en förlaga till proteinet). Denna ökning följdes också som väntat av en hämmad insulinutsöndring. Genom att tillsätta två substanser, forskolin och TNFα, som båda påverkar insulinutsöndringen genom olika mekanismer, ville vi se om vi kunde förändra denna relation mellan UCP2 och insulin. Vi fann att insulinsekretionen som stimulerades av forskolin inte påverkades av det ökade UCP2 uttrycket. Däremot tycktes TNFα kunna motverka den hämningen av insulin som beror på ökat UCP2. Slutsatsen var alltså att ökat UCP2 mRNA inte alltid behöver innebära hämmad insulinutsöndring.

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