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*Published in:*  
Physiology & Behavior

*DOI:*  
[10.1016/j.physbeh.2004.08.040](https://doi.org/10.1016/j.physbeh.2004.08.040)

2004

[Link to publication](#)

*Citation for published version (APA):*

Berger, K., Sörhede Winzell, M., Mei, J., & Erlanson-Albertsson, C. (2004). Enterostatin and its target mechanisms during regulation of fat intake. *Physiology & Behavior*, 83(4), 623-630.  
<https://doi.org/10.1016/j.physbeh.2004.08.040>

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# Enterostatin and its target mechanisms during regulation of fat intake

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## **Abstract**

A high-fat diet easily promotes hyperphagia giving an impression of an uncontrolled process. Fat digestion itself however provides control of fat intake through the digestion itself, carried out by pancreatic lipase and its protein cofactor colipase, and through enterostatin, a peptide released from procolipase during fat digestion. Procolipase (-/-) knockout mice have a severely reduced fat digestion and fat uptake, pointing to a major role of the digestive process itself. With a normal fat digestion enterostatin basically restricts fat intake by preventing the overconsumption of fat. The mechanism for enterostatin is an inhibition of a mu - opioid-mediated pathway, demonstrated through binding studies on SK-N-MC-cells and crude brain membranes. Another target protein of enterostatin is the beta-subunit of F<sub>1</sub>F<sub>0</sub>-ATPase, displaying a distinct binding of enterostatin, established through an aqueous two-phase partition system. The binding of enterostatin to F<sub>1</sub>-ATPase was partially displaced by  $\beta$ -casomorphin, a peptide stimulating fat intake and acting competitively to enterostatin. We hypothesize that regulation of fat intake contains a reward component, which is an opioidergic pathway.

*Keywords:* F<sub>1</sub>F<sub>0</sub>-ATP synthase, beta-casomorphin, two-phase partition, brain membranes, food intake

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## 1. Introduction

When fat is administered intraduodenally it reduces food intake, suggesting a regulated process [1]. Yet, dietary fat is often associated with overconsumption and obesity. The term “passive overconsumption of fat” instead suggests an unregulated process and that dietary fat has a weak effect on satiation [2]. Under certain conditions fat intake is however highly regulated, whereas this regulation could easily be distorted and turned into an unregulated process. In this article we want to discuss the conditions and mechanisms for a regulated fat intake, viewed through the mechanism of action for enterostatin, a peptide that in experimental animal studies has been found to regulate fat intake.

## 2. Enterostatin – a peptide released from procolipase during fat digestion

Dietary triacylglycerol is hydrolyzed by pancreatic lipase and its protein cofactor pancreatic colipase. The importance of colipase for dietary fat digestion has been illustrated by the procolipase *-/-* mice, who displayed a severely reduced fat digestion and fat absorption when fed a high-fat diet [3]. What was a surprise to us was the secretion of colipase as a precursor form, named procolipase [4]. During characterization of procolipase we observed that procolipase was easily activated by trypsin, to yield colipase on one hand and a pentapeptide, enterostatin, on the other hand [5]. Enterostatin was not important for the digestion of fat, but instead turned out to display an appetite regulating effect with a specificity against high-fat diet or fat as opposed to carbohydrate or protein [6]. The structure of enterostatin is a bridged proline structure, P-X-P, well preserved across species [7, 8]. Enterostatin was found to act as a satiety signal with specificity for fat in rat and mouse. Since enterostatin as well as its precursor molecule procolipase increased with high-fat diet it was suggested to act as a negative feedback regulator during fat intake [4]. The

anorectic effect, originally observed after central and peripheral injection, was also observed after intra-intestinal administration, hence at a site where enterostatin is also produced [9, 10]. For the transmission of the satiety effect of enterostatin intact vagus afferent innervation was important [11].

In identifying the mechanism of action for enterostatin various binding studies were performed. Such studies demonstrated that <sup>3</sup>H-enterostatin bound to brain membranes with one high-affinity binding site ( $K_d=0.5$  nM) and one low affinity binding site ( $K_d=30$  nM), both in crude brain membranes [12, 13] and in the human neuroepithelioma cell line SK-N-MC [14]. Such a two-affinity model could explain the U-shaped dose-response effect of enterostatin on high-fat food intake [12, 15, 16]. While low doses suppressed high-fat intake, higher doses either had no effect or even stimulated high-fat intake.

The specific binding of enterostatin to SK-N-MC cells was displaced by  $\beta$ -casomorphin<sub>1-5</sub> and Met-enkephalin, two opioid peptides having affinity to  $\mu$ -opioid receptors [14]. Which then is the receptor or target protein for enterostatin involved in the regulation of fat intake?

## 4. Identification of target pathways for enterostatin

In the identification of the target pathways for enterostatin both central and peripheral sites of action have been suggested. Centrally enterostatin is most efficient when injected into the amygdala and the paraventricular nucleus in decreasing food intake [17]. For the peripheral effect of inhibiting food intake either intraduodenal, intragastric or intraperitoneal administration of peptide is efficient, provided vagal signaling pathways are intact [10, 18, 19]. Based on animal studies with the OLEFA rat, lacking the CCK-A receptor, it was concluded that the enterostatin response is mediated through or dependent on peripheral as well as on

central CCK-A receptors [20]. A requirement for enterostatin to be effective in animals, no matter of administration route, is that the animals are adapted to high-fat diet [21]. A behavioral or metabolic feature thus seems to be of great importance for the enterostatin effect. Enterostatin has a rapid effect (<30 min) after administration by all routes, central as well as peripheral, with the exception of i.v. injection, where there is a delay in the response of 60-120 min. This implicates that there are local targets both in the gastrointestinal region and in the brain. The slow effect seen after i.v. injection indicates a slow uptake of enterostatin to the brain from the circulating blood, may be because enterostatin binds to plasma proteins [22]. Recently Koizumi et al [23] reported that enterostatin was taken up across the blood brain barrier and the highest concentration in the brain was seen after a delay of 120 minutes [23].

#### 4. The opioid pathway for regulation of fat intake

In targeting the pathways for regulation of fat intake and the mechanism of action for enterostatin, two different domains were of interest - reward and thermogenesis. In understanding the reward pathway several studies have proposed enterostatin to interact with opioid receptors or opioid pathways [13, 14, 16, 24]. The opioid receptors involved in feeding are of the  $\mu$ - and  $\kappa$ -subtypes, the  $\mu$ - and  $\kappa$ -agonists in general stimulating feeding, while the antagonists suppress it. The  $\kappa$ -opioid agonist U50, 488 was specifically found to stimulate high-fat feeding and block the inhibitory effect of enterostatin on fat intake when given intracerebroventricularly [24]. In spite of the antagonistic effect on feeding, U50, 488 was however not able to displace the binding of enterostatin to crude rat brain membranes, indicating that the receptor identified in brain membranes was not a  $\kappa$ -opioid receptor [13]. The binding of enterostatin was however displaced by  $\beta$ -

casomorphin [13], suggesting the  $\mu$ -opioid receptor to be of importance. We have however not been able to show that enterostatin binds to any form an opioid receptor.  $\beta$ -casomorphin has in several studies been shown to specifically stimulate the intake of high-fat food and act contradictory to enterostatin [13, 16].  $\beta$ -casomorphins are a group of digestion fragments from the milk protein  $\beta$ -casein found to act as  $\mu$ -opioid receptor ligand with opioid activity. These opioidergic peptides have been found in fragments from human, bovine, ovine and water buffalo  $\beta$ -casein [25].  $\beta$ -casomorphin<sub>1-5</sub> with the sequence YPFPG is released from bovine  $\beta$ -casein and there is an obvious sequence similarity between enterostatin and  $\beta$ -casomorphin<sub>1-5</sub> containing five amino acids with proline residues in position two and four (P-X-P).

To further clarify the relationship between enterostatin and  $\beta$ -casomorphin we investigated the effect of intravenous administration of enterostatin, in the presence and absence of  $\beta$ -casomorphin, on high-fat intake in rat. Intravenous administration was chosen based on the finding that enterostatin is found in the circulation during a meal [26]. As demonstrated in figure 1a enterostatin significantly decreased high-fat food intake in female Sprague-Dawley rats after intravenous injection of enterostatin at a dose of 38 nmol. At a higher dose, 76 nmol, enterostatin instead stimulated food intake during the first hour after injection compared to control rats. The lower dose of enterostatin, 9 nmol, had no effect on food intake compared to control. In a similar set-up intravenous injection of  $\beta$ -casomorphin increased high-fat food intake at all concentrations tested, 9.5, 38 and 76 nmol (fig 1b). When injecting the two peptides together at 38 nmol there was no effect on food intake compared to control (fig 2a). In a further experiment, enterostatin and  $\beta$ -casomorphin were both given at a higher dose (76 nmol) resulting

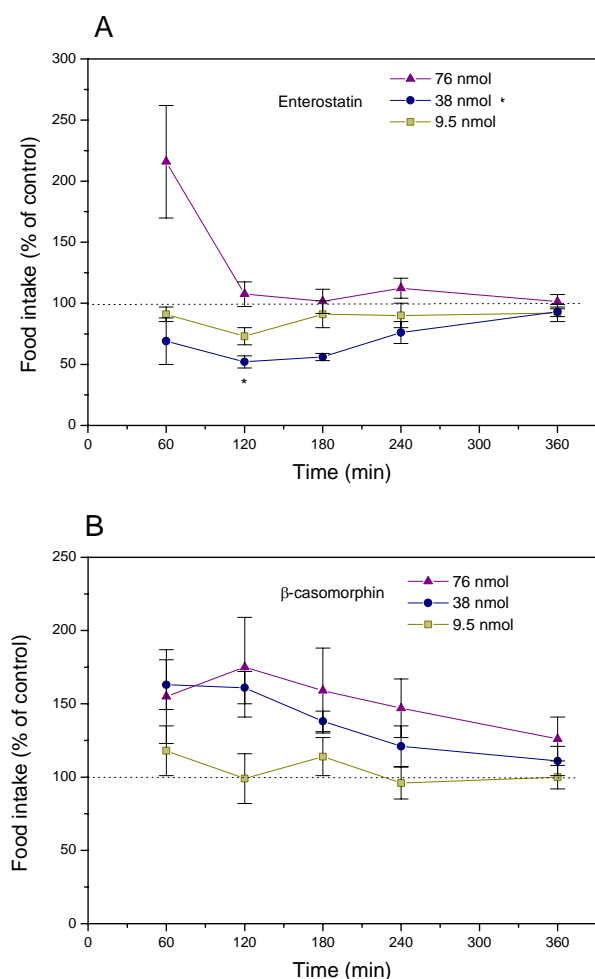


Fig. 1. Dose-response curves on high-fat food intake for intravenously injected enterostatin (A) and  $\beta$ -casomorphin (B). The food intake in control rats was set to 100% (dotted line). Data represent mean  $\pm$ SEM. A. Enterostatin at a low dose, 9.5 nmol, did not affect high-fat food intake compared to control. 38 nmol enterostatin decreased the high-fat food intake significantly compared to control ( $p=0.037$ , repeated measure ANOVA). At 38 nmol, there was a significant decrease at time point 120 min ( $p=0.02$ , Bonferroni test). At a higher dose, 76 nmol, there was no difference in food intake compared to control, even though there was an initial, but not significant, increase (\*  $p<0.05$ ). (B).  $\beta$ -casomorphin at 9.5 nmol did not change the intake of high-fat intake compared to control, while the higher doses, 38 and 76 nmol increased high-fat food intake with increasing dose but without significance.

in an increased food intake compared to control (fig 2b). Taken together these experiments demonstrate that enterostatin and  $\beta$ -casomorphin act in an antagonistic way at certain doses, the effect of

enterostatin to reduce food intake being abolished by  $\beta$ -casomorphin.

We also investigated whether enterostatin binding to crude brain membranes could be displaced by  $\beta$ -casomorphin. Competition studies indicated that  $\beta$ -casomorphin competitively inhibited the binding of  $^3\text{H}$ -enterostatin to brain membranes (Fig. 3). The  $\text{IC}_{50}$  value for  $\beta$ -casomorphin was calculated to 10  $\mu\text{M}$ . These studies hence confirmed a competition between the peptides enterostatin and  $\beta$ -casomorphin for binding to brain membranes. The experiments hence support a two-site affinity model for enterostatin binding, with the binding of high-affinity receptors, leading to a decreased food intake and with the binding to a low-affinity receptor, leading to an increased food intake. Whether this receptor is indeed a u-receptor could not be concluded at present time.

### 5. $\beta$ -subunit of $\text{F}_1\text{F}_0$ -ATP synthase as a target protein for enterostatin

In a search for the target protein of enterostatin we found that SK-N-MC cells specifically bound enterostatin as opposed to a couple of other neuroblastoma cell lines [14]. Due to scarcity of material from SK-N-MC cells and since crude brain membranes showed a two-site affinity binding for enterostatin, competing with  $\beta$ -casomorphin we performed a large scale purification of receptor protein, starting with rat brain membranes. These were extracted and purified in an aqueous two-phase partition system followed by affinity chromatography specifically eluted with enterostatin as described[27]. One major band was visualized on SDS-PAGE and identified by MALDI-TOF mass spectrometry as the  $\beta$ -subunit of  $\text{F}_1\text{F}_0$ -ATP synthase [27]. We were surprised by the identification of this protein, but repeated the purification several times and arrived at the same result.  $\text{F}_1\text{F}_0$ -ATP synthase is a mitochondrial enzyme that produces ATP in the inner mitochondrial membrane,

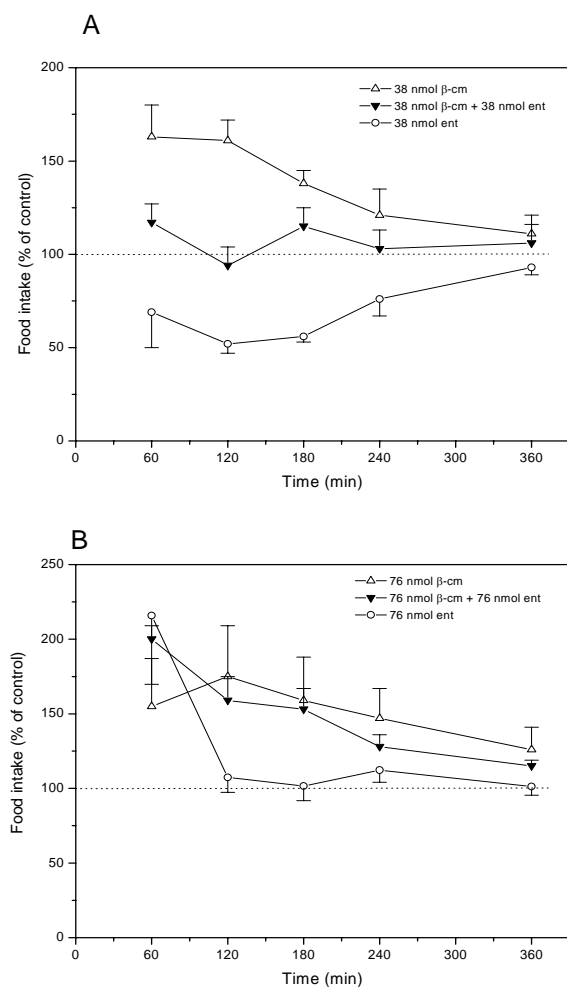


Fig. 2. Cumulative food intake after intravenous administration of enterostatin and  $\beta$ -casomorphin, alone or together. Food intake in control group was set to 100% (dotted line). Data represent mean  $\pm$ SEM. (A) At a dose of 38 nmol, enterostatin significantly reduced high-fat food intake, while  $\beta$ -casomorphin stimulated high-fat food intake. When given together there was no change in food intake. (B) At a higher dose, 76 nmol, enterostatin did not suppress high-fat food intake, instead it slightly stimulated food intake one hour after injection.  $\beta$ -casomorphin at this dose stimulated high-fat food intake. When the peptides were given together (76 nmol of each), an increase in food intake was observed.

using the energy from the proton gradient created during the passage of the electrons from NADH and FADH<sub>2</sub> to oxygen.

Our first experiment was to verify the binding between F<sub>1</sub>-ATPase and enterostatin using pure enzyme. We got a purified heart bovine preparation of F<sub>1</sub>-ATPase[28] and could by cross-linking demonstrate a binding between enterostatin

and the enzyme, specifically the  $\beta$ -subunit of F<sub>1</sub>F<sub>0</sub>-ATP synthase[27]. We also wanted to investigate the cellular effects of such a binding. Using a pancreatic  $\beta$ -cell line (INS-1) we demonstrated a targeting of the beta subunit of F<sub>1</sub>-ATPase and a decreased ATP production after enterostatin treatment, increased thermogenesis, increased oxygen consumption and a decreased insulin secretion[27]. We concluded that enterostatin “disturbed” the ATP-production, hence could be important for an ATP-dependent pathway or in raising thermogenesis. We also found to our surprise that the binding of enterostatin to purified F<sub>1</sub>-ATP synthase was inhibited by the presence of  $\beta$ -casomorphin, while the  $\kappa$ -opioid agonist U50, 488 failed to affect the binding [27]. Just after the publication of the beta subunit of F<sub>1</sub>-ATPase as a target protein for enterostatin[27] the same protein was identified as a target protein for apolipoprotein A-I (apoA-I) [29] on the surface of hepatocytes. This ectopic localization of components of the ATP synthase complex and the presence of ATP hydrolase activity at the hepatocyte cell surface [29] was an important discovery in view of the fact that any ligand needs to be transported through two membranes to reach the mitochondrial ATP synthase. We then imagined that the F<sub>1</sub>-ATPase targeted by enterostatin rather was localized to the plasma membrane than to the mitochondria. Thus, it appears that membrane-bound ATP synthase has a previously unsuspected role in modulating not only lipoprotein uptake, but also regulation of fat intake.

To substantiate the binding between enterostatin (APGPR) and F<sub>1</sub>-ATPase a two-phase partition method was used as has been described for protein-protein interaction or protein-ligand interaction[30, 31]. In a two-phase system the partition coefficient ( $K_c$ ) of a chemical substance is defined as the ratio of its concentration in the top phase (t) to its

concentration in the bottom phase (b). If enterostatin and  $F_1$ -ATPase interact to form a complex, then a change in the partition will occur and a  $K_d$  estimated. Measurement of the partition of enterostatin showed a partition coefficient of 1.44, when partitioned alone in the absence of  $F_1$ -ATPase. The partition of  $F_1$ -ATPase was found to be 100% located to the bottom phase. It could hence be inferred that any binding of enterostatin to the ATPase would decrease the partition coefficient of enterostatin. We found that the partition coefficient of enterostatin decreased to 0.61 in the presence of  $F_1$ -ATPase. Assuming a molar binding between enterostatin and  $F_1$ -ATPase, a dissociation constant,  $K_d$ , was estimated to  $1.7 \times 10^{-7}$  M. In the presence of  $\beta$ -casomorphin at three different concentrations,  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  M, the apparent  $K_d$  for iodinated enterostatin was increased to  $5.0 \times 10^{-7}$  M. The binding experiments hence substantiate a binding between pure enterostatin and purified  $F_1$ -ATPase when mixed together in buffer and that this binding was disturbed by the presence of  $\beta$ -casomorphin.

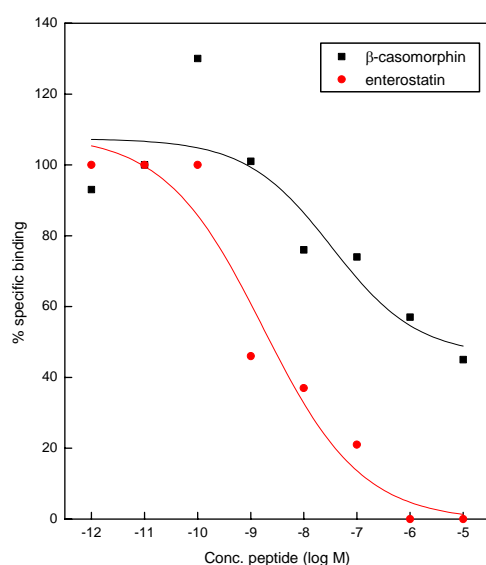


Fig. 3. Binding competition studies with unlabeled enterostatin and  $\beta$ -casomorphin to crude brain membranes incubated with  $^3H$ -enterostatin.  $\beta$ -casomorphin was able to inhibit the binding of  $^3H$ -enterostatin to rat brain membranes ( $IC_{50}=10\mu M$ ).

## 6. Uncoupling proteins, high-fat diets and enterostatin

In understanding the over consumption of high-fat diet and the reduced thermogenesis during high-fat diet the discovery of the uncoupling protein family and the induced expression of these proteins following high-fat diet was a turbulent point in the history of diet-induced thermogenesis [32, 33]. There are to date at least five different uncoupling proteins, being expressed in various tissues[34]. The role of these proteins in regulating energy expenditure during high-fat diets is not clear. The knockout mice lacking UCP1 [35] and UCP2[36] did not show any obesity following high-fat diet, which may be interpreted as a consequence of other thermogenic pathways substituting the role of these proteins in raising thermogenesis during high-fat diet. We were interested in the role of the UCP1, present only in brown adipose tissue and the UCP2, present in several tissues including the gastrointestinal tract. We fed mice with high-fat diet at two different surrounding temperature and found that mice defended themselves during high-fat diet by raising UCP1 expression provided the surrounding temperature was around  $23^{\circ}C$ [37]. With a temperature of around  $27^{\circ}C$ , the animals became obese and the expression of UCP1 was not increased[37]. It was hence clear that the animals defended themselves against high-fat diet induced obesity by raising thermogenesis. Enterostatin added to the diet further increased the expression of brown adipose tissue UCP1 during high-fat diet. This effect may be an indirect effect of enterostatin on UCP1 expression, based on the finding that enterostatin activates the sympathetic drive to brown adipose tissue[38].

We also investigated the expression of UCP2 in the gastrointestinal tract[39]. It was found that high fat diet actually decreased the expression of UCP2 in the stomach and in the duodenum, while the expression of UCP2 was increased in

adipose tissue, in agreement with previous findings[32]. Enterostatin added to the diet increased the UCP2 expression in the gastro-intestinal tract, but had no effect on the adipose tissue expression[37]. While UCP1 has a distinct role in raising thermogenesis, the role of UCP2 is less clear. It seems that UCP2 rather serves as a protection against free radical oxygen species during high-fat diet. The exact role of enterostatin, if any, in relation to the function of UCP1 and UCP2 expression during high-fat feeding is not known.

## 7. Discussion

In this work we have found that enterostatin when given i.v. inhibited high-fat food intake in a narrow concentration range (fig. 1a), confirming the U-shaped dose-response curve for enterostatin previously seen after intravenous administration [40]. This bimodal response of enterostatin in regulating fat intake confirms earlier observations on enterostatin, when given intraintraintestinally [16] and centrally [13, 41]. We also found that  $\beta$ -casomorphin<sub>1-5</sub> stimulated the intake of high-fat diet in a dose dependent way (fig.1b). A stimulation of high-fat food intake by  $\beta$ -casomorphin has been described to occur following central [13] as well as intragastric [16] administration. We show here that this occurs also after intravenous administration of  $\beta$ -casomorphin. Furthermore we observed that an equimolar (38 nmol) dose of  $\beta$ -casomorphin blocked the inhibiting effect of enterostatin on the intake of high-fat food (fig. 2a), while at higher dose the effect was abolished (fig. 2b). The mutual interaction of enterostatin and  $\beta$ -casomorphin, two structurally related peptides, suggests similar pathways during regulation of fat intake.

In this work we also demonstrated a binding of enterostatin to crude rat brain membranes and that this binding was partially displaced by  $\beta$ -casomorphin<sub>1-5</sub> (fig. 3). Since the receptor of  $\beta$ -

casomorphin is a  $\mu$ -opioid receptor we believe that enterostatin might well interact with a  $\mu$ -opioid pathway. This is in agreement with earlier studies where the binding of enterostatin to neuronal SK-N-MC cells was displaced by  $\beta$ -casomorphin and Met-enkephalin, suggesting a  $\mu$ -opioid pathway for enterostatin [14].

In the search for a target protein for enterostatin, we identified the  $\beta$ -subunit of mitochondrial F<sub>1</sub>F<sub>0</sub>-ATP synthase as a candidate molecule [27]. In this work we performed a binding study using purified F<sub>1</sub>-ATP synthase and enterostatin in an aqueous two-phase partition study. We hence demonstrated a distinct binding of enterostatin to F<sub>1</sub>-ATP synthase judged by a changed partition of enterostatin in the presence of F<sub>1</sub>-ATP synthase compared to the partition of enterostatin when alone. A K<sub>d</sub> was calculated based on a single molecular interaction, and found to be  $1.7 \times 10^{-7}$  M. In our previous binding studies we have found that enterostatin binds with a two affinity-binding mode to brain membranes and to SK-N-MC cells, the K<sub>d</sub> being  $0.5 \times 10^{-9}$  M and  $3.0 \times 10^{-8}$  M, respectively. Lin et al have proposed a two-site affinity binding of enterostatin and based on binding studies identified a low affinity binding site of around  $1 \times 10^{-7}$  M [13]. It can thus be argued that the high-affinity binding receptor for enterostatin is a  $\mu$ -opiate receptor while the low affinity receptor might be the F<sub>1</sub>F<sub>0</sub>-ATP synthase. The binding of enterostatin to F<sub>1</sub>F<sub>0</sub>-ATP synthase could be partially blocked by an access of  $\beta$ -casomorphin, as demonstrated here and previously [27].

A hypothetical scheme for enterostatin mediation of regulation of fat intake would be as follows: After release of enterostatin into the intestinal lumen during high-fat feeding, enterostatin activates a  $\mu$ -opioid pathway or a mitochondrial F<sub>1</sub>F<sub>0</sub>-ATP synthase in the intestine (Fig. 4). The  $\mu$ -opioid pathway most probably influences the reward system, while the interaction



with  $F_1F_0$ -ATP may influence ATP-production. In addition to the local effect in the intestine enterostatin may be absorbed into the blood and transported to target cells in the brain and other peripheral tissues [42]. In<sup>H</sup> the brain enterostatin increases the release of serotonin as well as dopamine, may be through opiate-receptor signaling [43]. The importance of the interaction with  $F_1F_0$ -ATP synthase is not known [27].

$\beta$ -casomorphin<sub>1-5</sub> is released in the intestine following digestion of bovine  $\beta$ -casein. It seems as  $\beta$ -casomorphin and enterostatin work through similar pathways in an opposite way, as many of their effects are contradictory. For example  $\beta$ -casomorphin stimulates, while enterostatin suppresses fat intake [16],  $\beta$ -casomorphin stimulates insulin secretion [44], while enterostatin decreases insulin secretion [45], furthermore enterostatin increases thermogenesis while opioids decreases

thermogenesis [39, 46]. We believe that  $\beta$ -casomorphin may also be absorbed and interact with enterostatin target molecules i.e. the  $\mu$ -opiate receptor and  $F_1F_0$ -ATP synthase. The concerted action of enterostatin and  $\beta$ -casomorphin may explain an increased insulin secretion following carbohydrate meal complemented with milk compared to water [47].

In conclusion enterostatin influences fat intake by restricting an over consumption of fat intake. This occurs through interaction with a postulated u-opioid pathway affecting reward and through interaction with the beta subunit of  $F_1F_0$ -ATP synthase affecting ATP-production (fig. 4). Further studies are needed to understand the role of  $F_1F_0$ -ATP synthase in appetite regulation, since this protein is targeted not only by enterostatin, but also by  $\beta$ -casomorphin, another protein affecting fat intake.

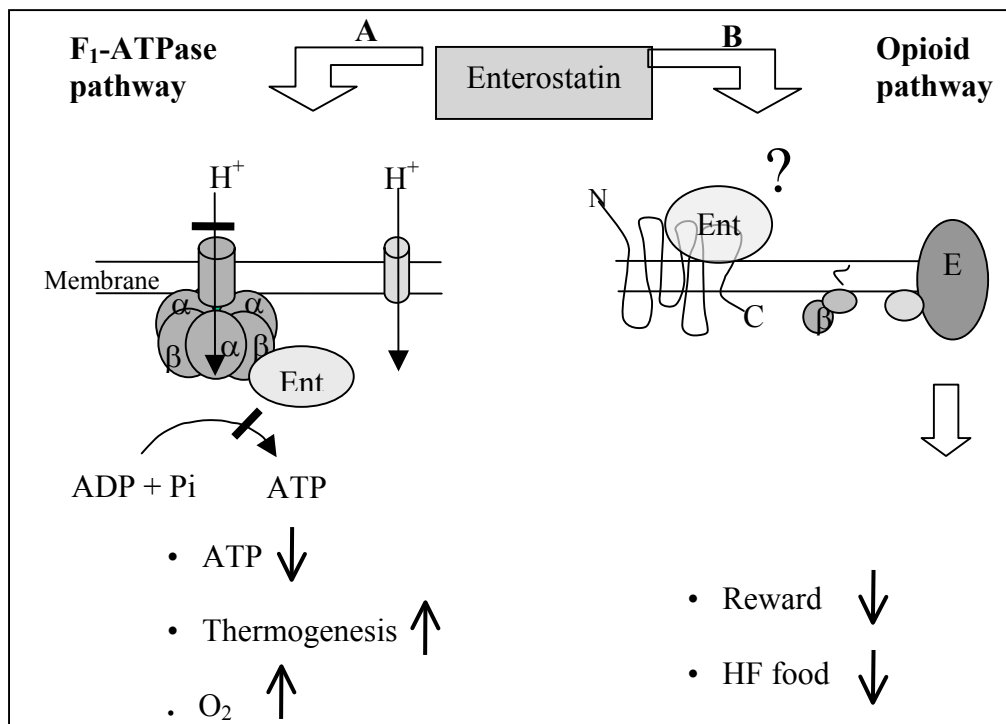


Fig. 4. Two postulated target proteins for enterostatin. In pathway A, enterostatin targets the  $\beta$ -subunit of  $F_1F_0$ -ATP synthase, perturbing ATP synthesis. When in mitochondria the protons may instead pass through the uncoupling protein (UCP) in the membrane causing increased thermogenesis and oxygen consumption. In pathway B enterostatin is proposed to target a U-opiate receptor or a u-opiate like receptor, of G-protein coupled nature, affecting the effector protein (E), which might be adenylate cyclase or a potassium channel. The targeting results in a decreased reward.

## Acknowledgements

We thank Professor Per-Åke Albertsson, Dept of Biochemistry, University of Lund for assistance with two-phase partition experiments and Ulla Gülich for excellent technical assistance. This research was

supported by the Swedish Medical Research Council (K2003-03X-007904-16C), Dr A. Pålssons Foundation, Swedish Nutrition Foundation, Royal Physiographic Society, Johanna Andersson Foundation and Dr Carl Trygger Foundation

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