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Regulation of leptin distribution between plasma and cerebrospinal fluid by cholecystokinin receptors

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1 Cholecystokinin (CCK) is a postprandial hormone that elicits a satiating effect and regulates feeding behaviour. CCK has been shown to enhance the effect of leptin in several experimental paradigms.

2 The goal of this work was to characterize the effect of endogenous CCK on plasma leptin content by using CCK receptor antagonists. Therefore, we administered SR-27,897, a selective CCK_1 receptor antagonist, and L-365,260, a selective CCK_2 receptor antagonist, to fed and food-deprived rats and determined plasma leptin concentration by enzyme immunoassay. Plasma insulin and glucose concentration as well as food intake were also determined.

3 Under our conditions, SR-27,897 increased plasma concentration of leptin both in fed and food-deprived rats. It also increased food intake as well as plasma concentration of insulin in fed animals. L-365,260 increased plasma leptin concentration only in fed rats.

4 In animals receiving exogenous leptin, CCK-8 increased the ratio between the concentration of leptin in cerebrospinal fluid and plasma.

5 These results show that CCK receptor antagonists increases plasma concentration of leptin and suggest that endogenous CCK may facilitate the uptake of plasma leptin to the cerebrospinal fluid.
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Abbreviations: BBB, blood – brain barrier; CCK, cholecystokinin; CCKR, cholecystokinin receptor; CSF, cerebrospinal fluid

Introduction

Cholecystokinin (CCK) is a gastrointestinal hormone that plays a key role in the regulation of both endo- and exocrine pancreas, and also a neurotransmitter in mammalian central nervous system (CNS). CCK-related peptides bind to two different receptors: the CCK_1 receptor (CCK_1R), which mediates most peripheral and hormonal actions of CCK; and the CCK_2 receptor (CCK_2R), which is the most abundant form in the brain and identical to the receptor of gastrin in peripheral organs (Crawley & Corwin, 1994; Noble *et al*., 1999). CCK is a postprandial hormone that elicits a satiating effect involving both CCK_1Rs (Moran & McHugh, 1992) and CCK_2Rs (Dourish *et al*., 1989). This effect seems to be linked to the activation of vagal afferents that synapse in brain stem areas with neurons projecting to the hypothalamus (Corp *et al*., 1993; Rinaman *et al*., 1995). Although the effect of CCK in reducing meal size is well documented (Smith *et al*., 1981), the importance of this hormone on both body weight and metabolism regulation remains to be characterized. Interestingly, Otsuka Long – Evans Tokushima Fatty (OLETF) rats, which are spontaneous mutants having a disrupted CCK_1R gene, are obese (Funakoshi *et al*., 1995) and exhibit an altered feeding behaviour (Moran *et al*., 1998; Moran, 2000). These antecedents suggest that CCK could play a role in energy balance (Matson & Ritter, 1999) and be integrated in a larger regulatory system. In this context, the interaction between CCK and other hormones also involved in regulating satiety and energy expenditure, such as insulin or leptin, has been the goal of several number of studies (Verspohl *et al*., 1986; Barrachina *et al*., 1997).

CCK has been shown to stimulate *in vitro* insulin release from rat pancreatic islets through CCK_1Rs (Verspohl *et al*., 1986; Karlsson *et al*., 1998). In accordance, CCK_1R antagonists decrease insulin immunoreactivity in rats treated with exogenous CCK-8 that were challenged with oral glucose (Verspohl *et al*., 1988) or with an intraduodenal meal (Rossetti *et al*., 1987). More recently, a large body of literature focused on OLETF rats show that these animals develop a noninsulin-dependent diabetes-like syndrome associated with hyperinsulinaemia and hyperleptinaemia (Funakoshi *et al*., 1995; 1996; Takiguchi *et al*., 1998).

Concerning the interaction between leptin and CCK, pharmacological studies show a synergic interaction between these hormones on the control of food intake (Barrachina *et al*., 1997) and metabolic activity (Matson *et al*., 2002). The effect of CCK on circulating leptin has been extensively studied by Bado's group. These authors have reported that exogenous CCK-8, administered to fasted rats, increases plasma leptin in a dose-dependent manner and enhances leptin release from isolated perfused rat stomach (Bado *et al*., 1998).

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The goal of this work was therefore to clarify the effect of endogenous CCK on circulating concentrations of both insulin and leptin. Since CCK is a postprandial hormone, our study has been performed by administering CCK_1R (SR-27,897)- and CCK_2R (L-365,260)-selective antagonists to rats just before the food-intake period occurring during the early dark phase of the circadian cycle (Akana *et al*., 1994). SR-27,897 and L-365,260 were used at a single dose assessed as selective in previous works (Ruiz-Gayo *et al*., 2000 and references cited therein). We have compared the effect of this treatment with a group of animals that were food deprived during this period.

Methods

Animals

Male Wistar rats (CRIFA, Spain), weighing 180 – 230 g at the time of the experiment, were housed under a light/dark cycle (12/12 h) in a temperature-controlled room ($22\pm1°C$), with standard rat chow (Panlab, Spain) and water available *ad libitum*. Lights were switched off at 1700. Animals were handled daily, for at least 1 week, to avoid stress by manipulation on the day of the experiment. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) for the care and use of laboratory animals.

Chemicals

The CCK_1R antagonist, SR-27,897 (1-[[2-4-(2-chlorophenyl)thiazol-2-yl-aminocarbony]-indolyl]-acetic acid), was kindly provided by Sanofi-Synthelabo, France (Poncelet *et al*., 1993). The CCK_2R antagonist, L-365,260 ((3*R*)-(+)-*N* -(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-3-methylphenylurea)), was a gift of Merck Sharp and Dohme Research Laboratories, U.S.A. (Chang *et al*., 1989). CCK-8, leptin and other chemicals and solvents were from Sigma, U.S.A.

Food-deprivation conditions

Animals were food deprived during 210 min. Briefly, food was withdrawn 120 min before the lights were switched off and rats were maintained food deprived until the end of the experiment, 90 min after the lights were switched off. Water was available *ad libitum*. Food-deprived rats were maintained in the same room than *ad libitum* fed rats.

Drug treatment and procedure for experiments with CCKR antagonists

Experiments were carried out simultaneously in fed and food-deprived rats. SR-27,897 and L-365,260 were administered subcutaneously at 0.3 and 1 mg kg^{-1}, respectively, 120 min before the lights were switched off. Vehicle was 4% carboxymethylcellulose. Animals were killed by decapitation 90 min after the lights were switched off and trunk blood was collected in chilled tubes containing heparin, then centrifuged at 4°C for 20 min at 3000 r.p.m. and plasma stored at −20°C until assay.

Drug treatment and procedure for experiments with exogenous leptin and CCK-8

These experiments were carried out only in fed rats. Leptin (0.1 mg kg^{-1}) was administered in saline buffer to all animals 120 min before the lights were switched off. CCK-8 (10 $\mu g\,kg^{-1}$) was administered in saline 30 min after leptin. A sample of blood was obtained from the caudal artery 90 min after the lights were switched off and handled as described below. Rats were then immediately anaesthetized and cerebrospinal fluid (CSF) was obtained by puncturing the cisterna magna.

Plasma samples analysis

Plasma leptin concentration was analysed by using a specific enzyme immunoassay (EIA) kit for rat leptin (Assay Designs Inc., U.S.A). Intra- and interassay variations were 11.6 and 11%, respectively. For leptin assay, plasma was diluted 1/5 in buffer assay. Leptin concentration was within the detection range of the kit, that is, 0.06 – 3.6 ng ml^{-1}. Insulin was determined by using a specific EIA kit for rat insulin (Mercodia, Denmark), based on the direct sandwich technique in which two monoclonal antibodies are directed against separated antigenic determinants on the insulin molecule. Plasma samples were within the detection range of the assay 0.7 – 5.5 $\mu g\,ml^{-1}$ (1.8% intraassay variation, 3.8% interassay variation). Glucose was measured by a spectrophotometric method (Glucose Trinder Method, Roche, Spain) (Barham & Trinder, 1972).

Feeding experiments

Food was weighed 120 min before the lights were turned off. Drugs were administered at the same time. Food intake was determined during a period of 210 min. Food intake was expressed as % of the control group.

Statistical analysis

Individual group comparisons were made using a two-way ANOVA. The factors of variation were treatment and food availability. The effect of drugs within a given group (fed or food deprived) were analysed by using a one-way ANOVA, followed by a Newman – Keuls' test.

Results

Effect of the CCKR antagonists on plasma concentration of insulin

In the case of SR-27,897, two-way ANOVA indicated a significant effect of fasting ($F_{(1,31)}=32.503$; $P<0.01$). The effect of treatment as well as the interaction between treatment and food deprivation were not significant. As illustrated in Figure 1a, treatment of fed rats with SR-27,897 (0.3 mg kg^{-1}) raised plasma insulin concentration from 1.9 ± 0.2 to $3.2\pm0.8\ \mu g\,dl^{-1}$. In the case of L-365,260 (1 mg kg^{-1}), two-way ANOVA also detected an effect of fasting ($F_{(1,28)}=27.469$; $P<0.01$) but not of treatment. The interaction between them was not significant (Figure 1b).

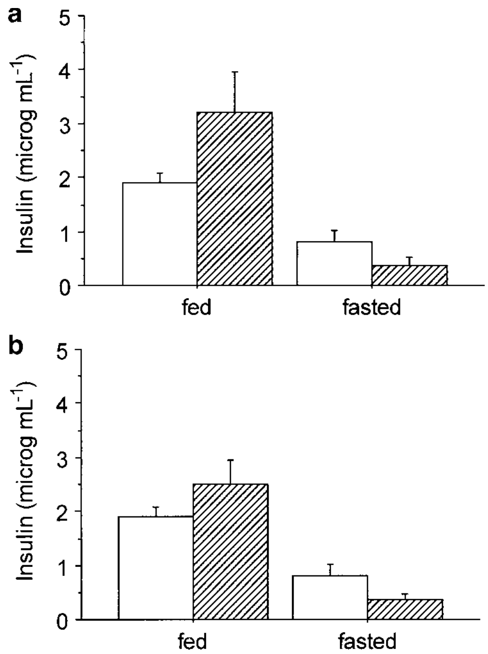

Figure 1 (a) Effect of SR-27,897 (0.3 mg kg^{-1}) on insulin plasmatic concentration. Vehicle (open columns) or SR-27,897 (*hatched columns*) were administered 120 min before the lights were off. Values are means ± s.e.m. of 8 – 10 animals. (b) Effect of *L-365,260* (1 mg kg^{-1}) on insulin plasma concentration. Values are means ± s.e.m. of 8 – 10 animals.

Table 1 Effect of SR-27,897 (0.3 mg kg^{-1}) and L-365,260 (1 mg kg^{-1}) on glucose (mg dl^{-1}) plasma concentration determined 210 min after food deprivation

Group	*Glucose* (mg dl^{-1})
Fed + vehicle	149 ± 3.2
Fed + SR-27,897	131.5 ± 4.3**
Fed + L-365,260	148.0 ± 7.0
Fasted + vehicle	132.4 ± 3.7#
Fasted + SR-27,897	128.6 ± 4.6
Fasted + L-365,260	130.0 ± 3.5

Vehicle, SR-27,897 or L-365,260 were given 120 min before the lights were off. **$P<0.01$, compared to the fed + vehicle group. #$P<0.05$, compared to the fed + vehicle group (Newman – Keuls' test).

Effect of CCKR antagonists on plasma glucose concentration

Table 1 resumes the effect of both SR-27,897 (0.3 mg kg^{-1}) and L-365,260 (1 mg kg^{-1}) on glucose plasma concentration. In the case of SR-27,897, two-way ANOVA revealed a significant effect of both treatment ($F_{(1,35)}=6.826$; $P<0.05$) and food deprivation ($F_{(1,35)}=5.665$; $P<0.05$) without significant interaction between them. In fed rats, SR-27,897 decreased plasma glucose concentration (one-way ANOVA, $F_{(1,21)}=9.536$; $P>0.01$). In the case of L-365,260, two-way ANOVA only indicated a significant effect of food deprivation ($F_{(1,35)}=17.105$; $P>0.01$).

Effect of the CCKR antagonists on plasma concentration of leptin

Figure 2a illustrates the effect of SR-27,897 (0.3 mg kg^{-1}) on plasma leptin concentration, both in fed and fasted rats. Two-way ANOVA revealed significant effect of treatment ($F_{(1,34)}=19.797$; $P<0.01$) and food deprivation ($F_{(1,34)}=13.869$; $P<0.01$) without significant interaction between treatment and fasting. SR-27,897 significantly increased plasma leptin concentration both in fed and fasted rats from 11.2 ± 2.2 to 25.0 ± 1.3 ng ml^{-1} (one-way ANOVA, $F_{(1,18)}=11.193$; $P<0.01$) and from 5.1 ± 1.4 to 12.9 ± 2.3 ng ml^{-1} ($F_{(1,16)}=9.539$; $P<0.01$), respectively.

In the case of the CCK_2R antagonist, L-365,260 (1 mg kg^{-1}), two-way ANOVA also indicated a significant effect of treatment ($F_{(1,35)}=8.407$; $P<0.01$) and fasting ($F_{(1,35)}=14.652$; $P<0.01$) without significant interaction between them. The increase of plasma leptin elicited by L-365,260 was from 11.2 to 20.5 ± 1.1 ng ml^{-1} in fed rats (one-way ANOVA, $F_{(1,19)}=6.104$; $P<0.05$) and from 5.0 ± 1.4 to 9.1 ± 1.9 ng ml^{-1} in fasted rats ($F_{(1,16)}=12.756$; $P<0.01$) (Figure 2b).

Food intake measurement

Figure 3 illustrates the effect of SR-27,897 (0.3 mg kg^{-1}) and L-365,260 (1 mg kg^{-1}) on food intake in a set of animals that were not fasted before the experiment. Under these conditions, one-way ANOVA revealed a significant effect of treatment ($F_{(2,21)}=6.263$; $P<0.01$) in the case of animals treated with

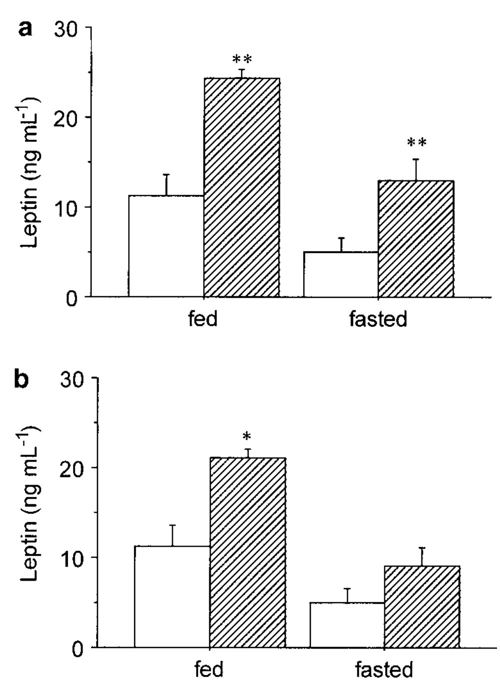

Figure 2 (a) Effect of SR-27,897 (0.3 mg kg^{-1}) on leptin plasmatic concentration. Vehicle (open columns) or SR-27,897 (hatched columns) were administered 120 min before the lights were off. Values are means ± s.e.m. of 8 – 10 animals. **$P<0.01$, compared to the control group (Newman – Keuls' test). (b) Effect of L-365,260 (1 mg kg^{-1}) on leptin plasma concentration. Values are means ± s.e.m. of 8 – 10 animals. *$P<0.05$, compared to the control group (Newman – Keuls' test).

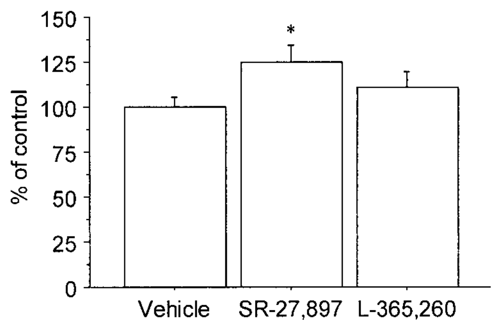

Figure 3 Effect of SR-27,897 ($0.3\,mg\,kg^{-1}$) and L-365,260 ($1\,mg\,kg^{-1}$) on food intake. Values are means±s.e.m. of 8 – 10 animals. *$P<0.05$, compared to the control group (Newman – Keuls' test).

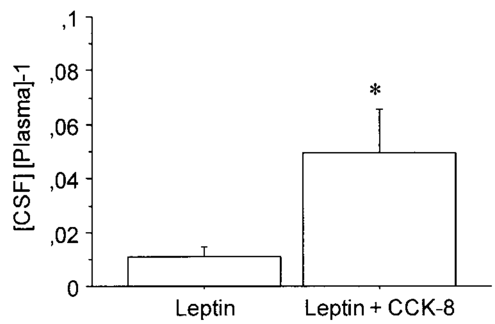

Figure 4 Effect of CCK-8 on the ratio between CSF and plasma leptin concentration. Values are means±s.e.m. of five to six animals. *$P<0.05$ (Newman – Keuls' test).

SR-27,897. In this experimental group, the increase of food intake was 47.3% over the control group.

Effect of CCK-8 on exogenous leptin distribution

CCK-8 decreased the concentration of leptin in plasma (33.6 ± 7.1 vs $22.1\pm0.5\,ng\,ml^{-1}$; $P=0.1$) and increased leptin concentration in CSF (0.4 ± 0.1 vs $1.1\pm0.3\,ng\,ml^{-1}$; $P=0.8$). Figure 4 illustrates the effect of CCK-8 ($10\,\mu g\,kg^{-1}$) on the ratio between the concentration of leptin in CSF and plasma. One-way ANOVA revealed that the ratio was about four to five times higher in the group treated with CCK-8 ($F_{(1,10)}=5.513$; $P<0.05$). Data are expressed as a relative value to minimize variability between different animals.

Discussion

In this work, we describe the effect of CCKRs blockade on plasma concentrations of both insulin and leptin. We used selective CCK_1R (SR-27,897) and CCK_2R (L-365,260) antagonists, administered just before the onset of the dark phase of the circadian cycle, that is, a physiological period of food intake in rodents. These conditions were used to model the typical hormonal/metabolic situation of an early postprandial or feeding period in rats. The variables of our study were pharmacological treatment and food availability. This work was carried out simultaneously in (i) *ad libitum* fed rats and in (ii) rats that were food deprived from 2 h before the dark period. Under these conditions, SR-27,897 and L-365,260 significantly increased leptin plasma concentration both in fed and food-deprived animals, whereas insulin was only slightly increased in fed animals treated with SR-27,897. Such an increase was accompanied by a decrease of plasma concentration of glucose, which could be the consequence of the slight increase of plasma insulin, as food intake was higher in these animals.

A direct effect of CCK on insulin release would be rather unexpected as CCK, by acting on CCK_1Rs, seems to stimulate, rather than inhibit, insulin release (Verspohl *et al*., 1986; 1988; Karlsson *et al*., 1998). Furthermore, the increase in insulin concentration was not observed in food-deprived animals treated with the CCK_1R antagonist. Thus, in the present study, the observed effect on insulin (Figure 1) might be related to the increase of food intake elicited by SR-27,897. In contrast to that, the effect detected on leptin concentration (Figure 2) seems to reflect a direct action of endogenous CCK as leptin increased both in fed and food-deprived rats. These data are in apparent contradiction with previous results in the literature, suggesting a synergistic potentiation between both CCK and leptin (Barrachina *et al*., 1997; Matson & Ritter, 1999; Matson *et al*., 2002). CCK has been shown to increase plasma leptin concentration in fasting/refeeding paradigms with a concomitant decrease of leptin content in the stomach fundus epithelium (Bado *et al*., 1998), indicating a secretagogue effect of CCK on gastric stores of leptin. Under similar conditions, CCK_2R blockade decreases the level of circulating leptin (Attoub *et al*., 1999). Nevertheless, to our knowledge, the effects of CCKRs antagonists under more physiological conditions, such as those used in this study, have not been investigated. As an alternative mechanism CCKRs antagonists might facilitate the release of leptin from fat stores. However, such a possibility seems to be also unlikely as CCK_2R antagonists inhibit rather than stimulate leptin release from rat adipocytes (Attoub *et al*., 1999). In consequence, the effect reported in this study would not be linked to an inhibitory effect of endogenous CCK on the release of leptin from fat or from stomach. Interestingly, SR-27,897 increased plasma leptin levels both in fed and food-deprived rats, suggesting that the effect of this drug would be mediated by a mechanism independent of food intake. Thus, the effect of SR-27,897 in increasing food intake (Ruiz-Gayo *et al*., 2000; this study) is insufficient to explain its effect on plasma leptin concentration.

The effect of CCKR antagonists may therefore be due to the blockade of an eventual regulatory role of CCK on leptin kinetics, that is, metabolism and/or distribution. To test this hypothesis, we administered exogenous leptin and determined the effect of CCK-8 on the concentration of leptin both in plasma and in CSF. The dose of CCK-8 used in this work ($10\,\mu g\,kg^{-1}$) might increase CCK immunoreactivity in plasma until approximately 40 pM (Linden, 1989), which is below the K_D value of CCK-8 for CCK receptors (Durieux *et al*., 1992; Attoub *et al*., 1999), but probably enough to occupy a significant number of CCKRs. Under these conditions, we observed a decrease of plasma leptin concentration together with an increase of leptin concentration in the CSF ($P\approx0.1$). These results taken together with the significant increase of the ratio $(leptin)_{CSF}/(leptin)_{plasma}$ in CCK-8-treated rats, strongly suggest that CCK-8 enhances the permeability of CNS barriers to leptin. Alternatively, the increase of leptin immunoreactivity in CSF might also be due to an eventual effect of CCK-8 on neuronal stores of leptin. This possibility has to be considered as leptin has been recently identified in circumventricular areas of the hypothalamus lacking blood – brain barrier (BBB) (Ur *et al*., 2002).

Leptin enters into the CNS by means of a specific transport system located both in the BBB and in the choroid plexus (Devos *et al*., 1996; Kastin *et al*., 1999; Zlokovic *et al*., 2000). If CCK facilitates the entry of leptin into the CNS, blockade of CCK receptors might increase plasma leptin content by hindering leptin distribution. Such an effect of CCK-8 would be compatible with the secretagogue effect of CCK observed by Bado's group. In this context one could also interpret other studies, which suggest that the hypothalamus is the target for a functional synergy between CCK and leptin (Barrachina *et al*., 1997; Wang *et al*., 1998; Matson *et al*., 2002). Interestingly, experiments carried out in OLETF rats, which lack CCK_1Rs, demonstrate that these animals are resistant to the effect of peripheral leptin, but respond to i.c.v. administered leptin, suggesting a role for CCK in the regulation of the accessibility of leptin to the CNS (Niimi *et al*., 1999).

In summary, our results suggest that CCK may be a mediator for regulating leptin distribution and allow to hypothesize that the synergy between CCK and leptin could be, in part, due to a facilitatory role of CCK on leptin entry into the CNS. In fact, we have detected a robust effect of CCKR antagonist, which points to a physiological role of CCK in regulating plasma leptin concentration. In addition, as intraperitoneal CCK-8 slightly increases plasma concentration of CCK (Linden, 1989; Linden & Södersten, 1990), it can be argued that CCKRs occupancy would not be dramatically higher in animals treated with exogenous CCK than in controls. In consequence, although further studies are necessary, it can be proposed that the interaction between CCK and leptin described in this study can be endowed with physiological sense and may have clinical implications for obese patients.

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