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Short communication

Potential modulation of plasma ghrelin and glucagon-like peptide-1 by anorexigenic cannabinoid compounds, SR141716A (rimonabant) and oleoylethanolamide

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The CB_1 cannabinoid receptor antagonist, *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide (rimonabant; SR141716A), and oleoylethanolamide (OEA) are known to reduce food consumption, by, at least partially, a peripheral regulation of feeding. The effects of systemic SR141716A or OEA (5 mg/kg) administrations on food consumption in 24 h food-deprived and fed rats were investigated. In fasted rats, SR141716A and OEA produced an inhibition in food intake measurable the first 20 min following injection. The increase in ghrelin levels observed in the vehicle-injected rats was abolished in animals receiving OEA and significantly reduced with SR141716A. Neither OEA nor SR141716A modified glucagon-like peptide-1 (7-36) amide portal levels 20 min after the administration. In fed rats, plasma ghrelin levels of SR141716A- and OEA-treated rats were 35 % lower as compared with those of the vehicle-injected rats. These results show an influence of cannabinoid agents on circulating ghrelin levels and suggest that their short-term action on appetite seems to be in accordance with the control of secretion of gastrointestinal orexigenic peptides, mainly expressed in the upper part of the gastrointestinal tract.

Cannabinoids: Gastrointestinal peptides: Appetite: Rimonabant: Oleoylethanolamide

Diseases characterised by impaired energy balance, such as obesity, are among the leading causes of illness and mortality in developed countries (Mokdad *et al.* 2000). Understanding the complex network of central and peripheral factors that influence both appetite and energy expenditure is a major public health goal. There is increased evidence suggesting that the cannabinoid system is involved in the regulation of food intake and appetite (Harrold & Williams, 2003).

Most of the pharmacological effects of Δ^9-tetrahydrocannabinol, the major ingredient of hemp (*Cannabis sativa* L.), are mediated through its interaction with G-protein-coupled receptors, namely the CB_1 and CB_2 cannabinoid receptors. Anandamide (AEA) and 2-arachidonoylglycerol are the most important endogenous cannabinoid agonists. The stimulation of appetite by cannabinoid agonists has been widely reported, both in marijuana consumers and in cachectic patients (Kirkham & Williams, 2001). In contrast, CB_1 cannabinoid receptor antagonists have been shown to reduce appetite and body weight both in animal models and in human subjects (Black, 2004). *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide (rimonabant; SR141716A), a drug developed by Sanofi-Synthelabo, is now in phase III clinical trials in obese patients (Cota *et al.* 2003*a*). First, it was proposed that the CB_1 cannabinoid receptors located in the brain participate in the regulation of appetite (Kirkham & Williams, 2001). However, recent studies support the hypothesis that cannabinoid agents modulate feeding through peripheral CB_1 cannabinoid receptors (Gomez *et al.* 2002): (1) food deprivation produces an important increase in AEA content in the small intestine but not in the brain; (2) CB_1 cannabinoid antagonists such as SR141716A have no effect when directly injected in the brain in food-deprived

Abbreviations: AEA, anandamide; GLP-1 (7-36) amide, glucagon-like peptide-1 (7-36) amide; OEA, oleoylethanolamide; SR141716A, rimonabant or *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide.

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animals. The gastrointestinal localisation of CB_1 cannabinoid receptors detected by Western blotting reveals a significant differential distribution in the mouse gastrointestinal tract, the highest levels of expression being in the stomach and in the colon (Casu *et al.* 2003). In addition, oleoylethanolamide (OEA), an endogenous monounsaturated analogue of AEA, is able to decrease post-deprivation food intake when injected intraperitoneally (Rodriguez de Fonseca *et al.* 2001). Taken together, these results support a role of the peripheral cannabinoid system in appetite control. Cannabinoid receptors are localised in the same parts of the gastrointestinal tract as the peptides that regulate appetite. Among them, ghrelin and glucagon-like peptide-1 are synthesised and secreted by fundus stomach mucosae (Dornonville de la Cour *et al.* 2001) and low intestine endocrine L-cells, respectively (Meier *et al.* 2002). Ghrelin, a circulating twenty-eight-amino-acid peptide, primarily identified as the endogenous ligand of the pituitary growth hormone secretagogue-receptor (Kojima *et al.* 1999), emerges as one of the most powerful physiological orexigenic and adipogenic agents (Tschop *et al.* 2000) able to 'orchestrate' hunger and food-seeking through its fluctuating concentrations in plasma (Cowley *et al.* 2003). Glucagon-like peptide-1 (7-36) amide (GLP-1 (7-36) amide) is a thirty-amino-acid peptide secreted from the L-cells mainly located in the jejunum, ileum and colon. GLP-1 (7-36) amide inhibits food intake and decreases body weight in rats and human subjects (Meier *et al.* 2002). Previous observations have demonstrated that the peripheral administration of cannabinoid-related molecules such as SR141716A and OEA reduces food intake, and that this effect appears quite rapidly after their administration (Kirkham & Williams, 2001; Rodriguez de Fonseca *et al.* 2001). The aim of the present study is to investigate if the ability of the two compounds to inhibit food consumption is related to the modulation of plasma ghrelin and GLP-1 (7-36) amide levels before food presentation.

Materials and methods

Animals

Male Wistar HAN rats (*n* 18; Harlan, The Netherlands) weighing 280–320 g were housed in a temperature- and humidity-controlled room with a 12 h light–dark cycle. Animals were housed in individual bottomed cages. Rats were given free access to a powdered A04 standard diet (A04; UAR, Villemoisson-sur-Orge, France; energy value 13·844 kJ (3·312 kcal)/g) and water. All rats received care in compliance with the institution's guidelines from the National Academy of Sciences (NIH publication no. 86–23; http://www.nih.gov). All animal experiments were approved by the local committee and the housing conditions were as specified by the Belgian Law of 14 November 1993 on the protection of laboratory animals (agreement no. LA 1230315).

Chemicals

SR141716A was a gift from Sanofi-Synthelabo Recherche (Montpellier, France). OEA was synthesised as previously described (Lambert *et al.* 1999).

Treatments

Drugs were administered by intraperitoneal (1 ml/kg) injection 15 min before food presentation. Drugs were dissolved in sterile saline with 70 % dimethylsulfoxide and some drops of Tween 80.

Food-intake studies

The effects of the drugs on feeding behaviour were analysed in animals habituated to handling after 24 h food deprivation for the 'fasted study' and in animals fed *ad libitum* in the 'non-fasted study' (Rodriguez de Fonseca *et al.* 2001). The drugs or vehicle were administered intraperitoneally; blood was sampled and, thereafter (meaning after completion of blood sampling; 45 min), powdered food was presented to the rats. The feeding dishes were weighed at 20, 40, 60, 80, 100, 120, 180, 240, and 300 min after the beginning of food presentation.

Western blot of the cannabinoid CB_1 receptor

Brain and stomach of the fed rats were rapidly removed and placed on an ice-cold plate. Cells from the mucosae were scraped from the entire organ; then fundus was separated from the rest of the stomach. Samples were treated according to Casu *et al.* (2003). Sodium dodecyl sulfate stop solution was added to samples of tissue preparation containing 40 μg proteins. Incubations were done with 1:100 dilutions of CB_1 rabbit polyclonal antibody (Biosource International, Camarillo, CA, USA) followed by peroxidase-labelled anti-rabbit antibody (1:10000) (Amersham Biosciences Benelux, Roosendaal, The Netherlands). Immunoreactivity was visualised as enhanced chemiluminescence.

Glycaemia

Glucose plasma concentrations were measured using a kit, coupling enzymic reactions and spectrophotometric detection of reaction endproducts (Glucose PAP; Elitech Diagnostics, Brussels, Belgium).

Peptide analysis

In preliminary studies, it has been observed that food intake was reduced within the first 45 min following either SR141716A or OEA injections. Concentrations of plasma ghrelin were measured using a RIA kit designed to measure acylated ghrelin, namely active ghrelin (active ghrelin RIA kit; Linco Research, St Charles, MO, USA). For GLP-1 (7-36) amide measurements, animals were anaesthetised 20 min after the administration of the drugs (5 mg OEA or SR141716A/kg) using an intraperitoneal injection of sodium pentobarbital solution (using 60 mg Nembutal®/kg body weight; Sanofi Santé Animale, Benelux, Brussels, Belgium). Portal vein blood samples were collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) containing dipeptidyl peptidase IV inhibitor (Linco Research, St Charles, MO, USA); after centrifugation, plasma was stored at −80°C. GLP-1 (7-36)

amide concentrations were measured using an ELISA kit (GLP-1 active ELISA kit; Linco Research, St Charles, MO, USA).

Statistics

Results are given as mean values and standard errors of the mean. Statistical differences between groups were evaluated by one-way ANOVA followed by Dunnet's *post hoc* test or ANOVA for repeated measures to compare time, treatment × time and treatment effects using SPSS 9.0.0 for Windows (SPSS, Chicago, IL, USA). $P<0.05$ was considered as significant.

Results

Food-intake measurements

The intraperitoneal administration of the selective CB_1 cannabinoid receptor antagonist SR141716A (5 mg/kg) significantly reduced food intake in the animals previously deprived of food for 24 h. The inhibition was already observed 20 min after food administration and was maintained for several hours (Fig. 1 (A)). OEA (5 mg/kg intraperitoneally) produced a rapid inhibition of food intake in fasted animals, lasting for 3 h. At 4 h after the OEA injection, food consumption was lower than in the control rats, but this difference was not significant (Fig. 1 (B)).

Ghrelin, glucagon-like peptide-1 (7-36) amide and glucose levels

In fasted rats. Ghrelin plasma levels were measured at 0, 15 and 45 min after the administration of SR141716A or OEA to the animals previously fasted for 24 h. Table 1 shows a time-dependent increase in ghrelin levels in the control animals after vehicle administration. The increase in ghrelin levels was abolished in the animals receiving 5 mg OEA/kg; the administration of 5 mg SR141716A/kg resulted in a significant decrease of ghrelin levels, compared with at time 0 and with ghrelin levels measured at time 15 and 45 min in the control rats.

On the contrary, no statistical differences of GLP-1 (7-36) amide levels measured in the portal vein taken at 20 min were observed between the groups, neither after SR141716A nor after OEA administrations in fasted animals. GLP-1 (7-36) amide levels were 9·04 (SEM 0·68) pM for the control rats, 7·80 (SEM 0·48) pM for the OEA-treated rats and 8·59 (SEM 0·81) pM for the SR141716A-treated rats.

In the same experiment, glucose levels were determined after collection in the portal vein and in the cava vein plasma; no significant differences were observed between the groups. Portal vein glycaemia was 4·42 (SEM 0·22) mmol/l for the control rats, 4·64 (SEM 0·16) mmol/l for the SR141716A-treated rats and 5·16 (SEM 0·220) mmol/l for the OEA-treated rats. Cava vein glycaemia was 4·88 (SEM 0·29) mmol/l for the control rats, 4·81 (SEM 0·10) mmol/l for the SR141716A-treated rats and 4·90 (SEM 0·29) mmol/l for the OEA-treated rats.

In fed rats. Ghrelin plasma levels were measured at 0, 15 and 45 min after the administration of either SR141716A or

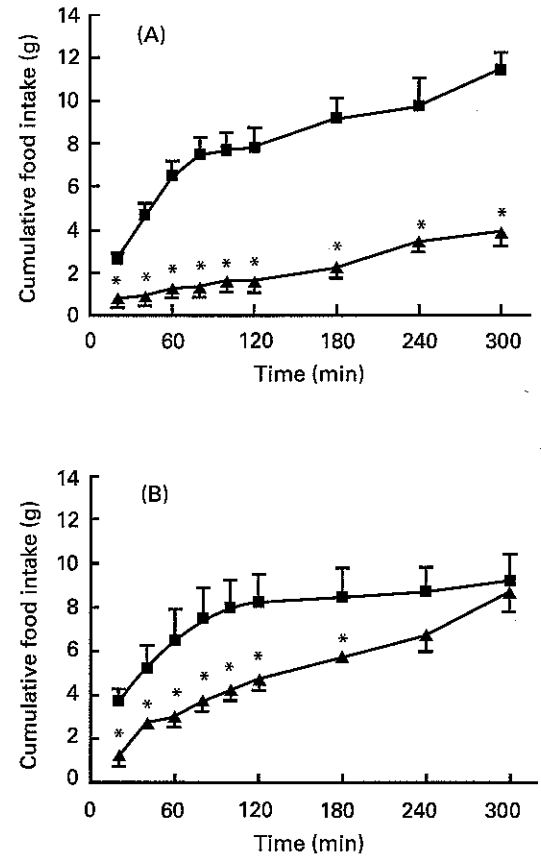

Fig. 1. Effects of *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide (rimonabant; SR141716A) and oleoylethanolamide (OEA) (5 mg/kg intraperitoneally) on food intake after 24 h of food deprivation. (A) Acute intraperitoneal injection of SR141716A (–▲–) compared with injection of vehicle (–■–). (B) Acute intraperitoneal injection of OEA (–▲–) compared with injection of vehicle (–■–). Results are mean values for four to six rats per group, with standard errors of the mean represented by vertical bars. *Mean value was significantly different to that of the vehicle-treated group ($P<0.05$).

Table 1. Plasma ghrelin levels (pg/ml) after administration of *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide (rimonabant; SR141716A) or oleoylethanolamide (OEA) (5 mg/kg intraperitoneally) in fasted and fed animals (four to six animals per group)†

(Mean values and standard errors of the mean)

	0 min		15 min		45 min	
	Mean	SEM	Mean	SEM	Mean	SEM
Fasted						
Vehicle	499·0	55·2	573·9	42·7	833·0	102·2
OEA	496·2	58·3	599·8	44·8	562·1*	61·0
SR141716A	495·6	61·2	423·0	38·1	362·6*	49·1
Fed						
Vehicle	11·5	1·3	37·7	10·8	34·7	8·9
OEA	12·0	3·7	20·6	5·7	23·5	3·5
SR141716A	10·9	1·6	32·6	5·4	22·8	6·2

Statistical analysis was performed using ANOVA with repeated measures for fasted and fed experiments separately. Fasted animals: *time, $P=0.304$; time × treatment, $P=0.014$; treatment, $P=0.006$. Fed animals: time, $P=0.006$; time × treatment, $P=0.136$, treatment, $P=0.327$.

† For details of procedures, see p. 758.

OEA to animals fed *ad libitum*. Table 1 shows that before the injection of the vehicle or drugs, plasma ghrelin levels were approximately 45-fold lower than those observed in fasted rats. Plasma ghrelin levels increased by about 3·5-fold in the control rats between 0 and 45 min; this increase was less pronounced (by 2-fold) in the OEA- and SR141716A-treated rats than in the control rats but the differences were not significant (Table 1).

Western blot of CB_1 cannabinoid receptor

Brain was used as a positive control to verify the conditions allowing the detection of typical fragments of CB_1-receptor protein. A band of approximately 63 kDa, corresponding to the active glycosylated form of CB_1 receptor, was found in the scraped mucosal tissue of the stomach. Bands of 29 and 6 kDa, which also correspond to fragments of the CB_1-receptor protein, were found in the fundus and the stomach after mucosal scraping (data not shown).

Discussion

Several data suggest an intimate relationship between the cannabinoid system and the peptides involved in food-intake and appetite regulation (Harrold & Williams, 2003). Some authors have suggested that the increase in food intake observed after leptin administration could be linked to the modulation of hypothalamic endocannabinoid levels (Di Marzo *et al.* 2001). During food deprivation, when leptin levels rapidly decline, circulating ghrelin levels increase, suggesting that leptin and ghrelin regulate hypothalamic peptidergic systems in opposite ways (Tschop *et al.* 2000). Nevertheless, recent data do not support a role for the regulation of circulating ghrelin by leptin levels independently of changes in adiposity and suggest that the leptin and ghrelin systems for energy homeostasis function independently in healthy human subjects (Chan *et al.* 2004). Some authors support the hypothesis that an increased ghrelin release in the peripheral serum is a hunger signal devoted to prevent energy deficit and to activate meal initiation. In the present study, we postulated that cannabinoid-related compounds involved in satiety could modulate ghrelin. Plasma ghrelin concentration increases during fasting and rapidly falls during meal consumption (Cummings *et al.* 2001). Therefore, we measured ghrelin levels after 24 h of fasting. In that condition, the intestinal cannabinoid system seems to be particularly activated: AEA levels are increased 7-fold in the small intestine (Gomez *et al.* 2002), whereas OEA synthesis and contents in the small intestine are decreased during fasting as compared with the fed state (Rodriguez de Fonseca *et al.* 2001). Ghrelin levels continued to progressively increase after intraperitoneal injection of the vehicle in fasted rats. However, this increase was abolished through the intraperitoneal injection of SR141716A given at a dose able to exert a satietogenic effect through a selective antagonist effect on the CB_1 cannabinoid receptor. OEA, a monounsaturated analogue of AEA devoid of significant affinity for cannabinoid receptors (Lambert *et al.* 1999, Jonsson *et al.* 2001; Lambert *et al.* 2002) with a less prolonged effect on food intake, also decreased ghrelin levels. OEA effects on the modification of meal pattern (Gaetani *et al.* 2003) and on the diminution of appetite (Rodriguez de Fonseca *et al.* 2001) have been recently shown to be linked to the activation of nuclear peroxisome proliferator-activated receptor α (Fu *et al.* 2003). OEA decreases food intake in food-deprived rats via a mechanism that requires intact sensory fibres (Rodriguez de Fonseca *et al.* 2001). We have performed a similar experiment in fed *ad libitum* animals. As expected, ghrelin levels were lower (45-fold) than in fasted rats. Nevertheless, 45 min after intraperitoneal injection of OEA or SR141716A, plasma ghrelin levels remained 35 % lower in the OEA- and SR141716A-treated rats as compared with in the anorexigenic effect of CB_1 receptor antagonist and, at a lesser extent, to the satietogenic effect of OEA. The localisation of CB_1 receptors in the mucosal tissue of stomach fundus, confirmed in the present study, on the one hand, and ghrelin synthesis occurring in stomach fundus on the other hand, are in favour of a physiological interaction between both systems. Events occurring in the lower part of the intestinal tract might also be involved. Recent data suggest a key role played by GLP-1 (7-36) amide in the control of food intake and body weight (Meier *et al.* 2002). Interestingly, GLP-1 (7-36) amide secretion by L-cells through nutrient intake leads to an important increase of endogenous peptide concentration in the portal vein, with no or minor modification of its levels in the peripheral serum (Thorens & Waeber, 1993). Therefore, we have measured GLP-1 (7-36) amide level in this compartment, 20 min after the administration of the drugs, at the moment where food intake was reduced at maximum. However, neither OEA nor SR141716A modified GLP-1 (7-36) amide portal levels at that time.

In conclusion, there is now increased evidence for a peripheral role of the cannabinoid system in food-consumption behaviour; however, the molecular mechanisms allowing the different cannabinoid-related molecules to act on food intake through their action on the gastrointestinal tract remain to be fully elucidated. Although interactions between cannabinoid and ghrelin signalling pathways have been recently suggested in the regulation of food intake by the brain–gut axis (Konturek *et al.* 2004), it is too early to anticipate with the present data the precise molecular basis of such a potential interaction. We have shown that the peripheral administration of SR141716 maintains ghrelin plasma levels at a lower level, which is in 'concordance' with the rapid decrease of food intake observed after its peripheral administration. The peripheral administration of OEA shows also a tendency to decrease this level compared with the vehicle, confirming the role of this analogue of AEA as an anorexigenic endogenous mediator. The cannabinoid system interacts with several peptides involved in the complex regulation of energy homeostasis such as leptin (Di Marzo *et al.* 2001), Acrp 30 (Bensaid *et al.* 2003) and orexin (Cota *et al.* 2003*b*; Hilairet *et al.* 2003). The present study suggests that the modulation of the secretion of gastrointestinal orexigenic peptides, such as ghrelin, by the cannabinoid system, mainly expressed in the upper part of the gastrointestinal tract, may participate in food regulation.

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