RESEARCH PAPER

The role of endocannabinoids in the regulation of gastric emptying: alterations in mice fed a high-fat diet

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Background and purpose: Endocannabinoids (via cannabinoid CB_1 receptor activation) are physiological regulators of intestinal motility and food intake. However, their role in the regulation of gastric emptying is largely unexplored. The purpose of the present study was to investigate the involvement of the endocannabinoid system in the regulation of gastric emptying in mice fed either a standard diet (STD) or a high-fat diet (HFD) for 14 weeks.
Experimental approach: Gastric emptying was evaluated by measuring the amount of phenol red recovered in the stomach after oral challenge; CB_1 expression was analysed by quantitative reverse transcription-PCR; endocannabinoid (anandamide and 2-arachidonoyl glycerol) levels were measured by liquid chromatography-mass spectrometry.
Key results: Gastric emptying was reduced by anandamide, an effect counteracted by the CB_1 receptor antagonist rimonabant, but not by the CB_2 receptor antagonist SR144528 or by the transient receptor potential vanilloid type 1 (TRPV1) antagonist 5′-iodoresiniferatoxin. The fatty acid amide hydrolase (FAAH) inhibitor *N*-arachidonoyl-5-hydroxytryptamine (but not the anandamide uptake inhibitor OMDM-2) reduced gastric emptying in a way partly reduced by rimonabant. Compared to STD mice, HFD mice exhibited significantly higher body weight and fasting glucose levels, delayed gastric emptying and lower anandamide and CB_1 mRNA levels. *N*-arachidonoylserotonin (but not rimonabant) affected gastric emptying more efficaciously in HFD than STD mice.
Conclusions and implications: Gastric emptying is physiologically regulated by the endocannabinoid system, which is downregulated following a HFD leading to overweight.
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Introduction

A number of reports have suggested that the endocannabinoid system may regulate food intake and energy balance through cannabinoid CB_1 receptors in brain and in periphery (Di Marzo and Matias, 2005; Sharkey and Pittman, 2005; Osei-Hyiaman *et al.*, 2006). Consistent with this hypothesis, Phase III clinical trials have shown that a CB_1 receptor antagonist reduces body weight and waist circumference to a significantly greater extent than placebo in obese patients (Despres *et al.*, 2005; Van Gaal *et al.*, 2005; Pi-Sunyer *et al.*, 2006; Scheen *et al.*, 2006).

Apart from cannabinoid (CB_1 and CB_2) receptors, the endocannabinoid system includes endogenous ligands that activate them (that is, the endocannabinoid anandamide (AEA) and 2-arachidonoyl glycerol (2-AG)) and mechanisms for endocannabinoid biosynthesis and inactivation (Bradshaw and Walker, 2005; Di Marzo and Matias, 2005; Mackie, 2006). The latter occurs through cellular reuptake, which might be facilitated by a putative membrane transporter, and enzymatic degradation by hydrolytic enzymes including fatty acid amide hydrolase (FAAH) (Di Marzo and Matias, 2005). CB_1 receptors have been identified on enteric nerves, and their activation results in inhibition of excitatory transmission *in vitro* (Pertwee *et al.*, 1996; Izzo *et al.*, 1998; Guagnini *et al.*, 2006; Hinds *et al.*, 2006) and intestinal propulsion *in vivo* (Pinto *et al.*, 2002; Carai *et al.*, 2006). Furthermore, there is substantial evidence that the endocannabinoid system is involved in the physiological control of intestinal motility, both in the small and in the large intestine (Coutts and Izzo, 2004; Hornby and Prouty, 2004; Duncan *et al.*, 2005; Massa *et al.*, 2005). Such evidence comes mainly from pharmacological studies in rodents showing prokinetic effects of the CB_1 receptor antagonist rimonabant and reduction of intestinal motility by inhibitors of endocannabinoid inactivation (Pinto *et al.*, 2002; Capasso *et al.*, 2005). The involvement of endocannabinoids in the physiological regulation of intestinal motility is consistent with data from Phase III clinical trials, which highlighted diarrhoea as one of the initial adverse events associated with administration of the antiobesity drug rimonabant (Van Gaal *et al.*, 2005). However, the possible involvement of endocannabinoids as physiological modulators of gastric motility has been hardly explored in physiological states and never evaluated in obese animals. Previous studies in which the effect of the CB_1 receptor antagonist rimonabant on gastric motility was investigated *in vivo* in control rats yielded inconclusive results (Izzo *et al.*, 1999; Krowicki *et al*, 1999; Landi *et al.*, 2002). In addition, the effect on gastric motility of drugs that inhibit endocannabinoid degradation has not been evaluated to date.

The aim of the present study was to evaluate the role of the endocannabinoid system in the regulation of gastric emptying in the mouse *in vivo*. For this purpose, we used synthetic AEA, the selective CB_1 receptor antagonist rimonabant, the selective CB_2 receptor antagonist SR144528, the AEA cellular reuptake inhibitor OMDM-2 and the FAAH inhibitor *N*-arachidonoyl-5-hydroxytryptamine (AA-5-HT). In addition, because obesity may affect gastric motility (Park and Camilleri, 2005) and the CB_1 receptor antagonist rimonabant is clinically used in obese patients, additional experiments were performed in mice fed a high-fat diet (HFD) for 14 weeks.

Methods

Animals and diet
Male ICR mice (22–24 g) were generally used. Mice were fed *ad libitum* with standard mouse food, except for the 12 h period immediately preceding the measurement of gastric emptying. In another set of experiments (that is, HFD-fed

mice), male 7-week-old C57Bl/6J mice were used. After 1 week of acclimatization, C57Bl/6J mice were fed a diet containing 25.5% fat (49% of calories), 22% protein and 38.4% carbohydrate (TD97366; Harlan Italy, Correzzana, Milan, Italy) for 14 weeks. Control C57B1/6J mice received a standard diet (STD) containing 5.7% fat (10.9% of calories), 18.9% protein and 57.3% carbohydrate (2018, Harlan Teklad global diet (18% of proteins); Harlan Italy). Mice were fed *ad libitum*, except for the 12 h period immediately preceding the experiments. Animals were purchased from Harlan Italy. Fasting plasma glucose levels were determined in animals fasted for 12 h by using the glucose test kit with an automatic analyzer (Accu-Chek Active; Roche, Mannheim, Germany) in blood samples obtained from tail vein (Darmani *et al.*, 2005).

Drug administration
Anandamide ($1–15 \, mg \, kg^{-1}$), AA-5-HT ($10 \, mg \, kg^{-1}$), OMDM-2 ($10 \, mg \, kg^{-1}$) or vehicles were injected intraperitoneally 30 min before the administration of the marker (phenol red solution) used to evaluate gastric emptying. In some experiments, rimonabant ($0.1 \, mg \, kg^{-1}$), 5′-iodoresiniferatoxin (I-RTX, $0.75 \, mg \, kg^{-1}$) or SR144528 ($1 \, mg \, kg^{-1}$) were injected intraperitoneally 10 min before AEA ($7.5 \, mg \, kg^{-1}$) or AA-5-HT ($10 \, mg \, kg^{-1}$). I-RTX and SR144528 doses were selected on the basis of the previous work (Capasso *et al.*, 2005). In some experiments, the effect of either rimonabant ($0.1–3 \, mg \, kg^{-1}$, i.p.), AA-5-HT ($2.5–15 \, mg \, kg^{-1}$, i.p.) or OMDM-2 ($10 \, mg \, kg^{-1}$, i.p.) was evaluated in HFD-fed mice and, for comparison, in aged-matched STD-fed mice.

Gastric emptying
Gastric emptying in both control and HFD-fed mice (14 weeks of dietary treatment) was performed as described previously (El-Salhy, 2001). Briefly, after an overnight fast, the animals received by gavage 0.2 ml of a solution of 50 mg phenol red in 100 ml 1.5% carboxymethylcellulose, which was constantly stirred and held at 37 °C. After 20 min, mice were killed stomach was quickly ligated at the lower oesophageal sphincter and pyloric region and removed. The stomach was opened and its contents were poured into a test tube and washed with 4 ml distilled water. At the end of the experiment, 2 ml 1 M NaOH was added to each tube to develop the maximum intensity of colour. The solutions were assayed with spectrometer at 560 nm. The percentage of gastric emptying was calculated according to the following formula:

$100 \times (1 -$ amount of phenol red recovered after 20 min/ amount of phenol red recovered after 0 min).

Identification and quantification of endocannabinoids (AEA and 2-AG)
Full-thickness stomachs from HFD-fed mice and aged-matched STD-fed mice (8 and 14 weeks of dietary treatment) were removed and tissue specimens were immediately weighed, immersed into liquid nitrogen and stored at $-70 \, ^\circ C$ until chromatographic separation of endocannabinoids. Tissues were extracted with chloroform/methanol

(2:1 (v/v)) containing 100 pmol d$_8$-AEA, synthesized as described previously (Bisogno *et al.*, 1999), and 100 pmol d$_5$-2-AG provided by Cayman Chemicals (Ann Arbor, MI, USA). The lipid extracts were purified by silica column chromatography as described previously (Bisogno *et al.*, 1999; Maione *et al.*, 2007), and the fractions containing AEA and 2-AG were analysed by isotope dilution LC-APCI-MS (liquid chromatography-atmospheric pressure-chemical ionization mass spectrometry) and carried out in the selected monitoring mode as described in detail elsewhere (Maione *et al.*, 2007). Results are expressed as pmol or nmol per g wet tissue. As during tissue extraction/purification, both d$_8$-AEA and native 2-AG were partly transformed into the 1(3)-isomers, and only a limited amount of arachidonic acid was present on the sn-1(3) position of (phospho)glycerides, the amounts of 2-AG shown here represent the combined mono-arachidonyl-glycerol peaks.

Quantitative real-time PCR
Stomach antrum and fundus from control and HFD-fed mice were homogenized separately in 1 ml of Trizol (Invitrogen Carlsbad, CA, USA). Total RNA was extracted according to manufacturer's recommendations, dissolved in RNA storage solution (Ambion, Austin, TX, USA), ultraviolet quantified by a Bio-Photometer (Eppendorf, Hamburg, Germany) and stored at $-80\,^{\circ}$C. RNA aliquots (6 µg) were digested by RNAse-free DNAse I (Ambion DNA-free kit) in a 20 µl final volume reaction mixture to remove contaminating genomic DNA. After DNAse digestion, concentration and purity of RNA samples were evaluated by the RNA-6000-Nano microchip assay using a 2100 Bioanalyzer equipped with a 2100 Expert Software (Agilent, Santa Clara, CA, USA), following manufacturer's instructions. For all samples tested, the RNA integrity number (RIN) was greater than 6 (relatively to a 0–10 scale); 3 µg of total RNA, as evaluated by the 2100 Bioanalyzer, was reverse transcribed in a 25 µl reaction mixture containing as follows: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl$_2$, 10 mM dithiothreitol, 1 mM dNTPs, 20 U RNAse inhibitor (Invitrogen), 0.125 A_{260} units of hexanucleotide mixture (Invitrogen) for random priming and 200 U MoMuLV (Moloney murine leukemia virus) Superscript III reverse transcriptase (Invitrogen). The reaction mixture was incubated in a termocycler iCycler-iQ for a 5 min at 55 $^{\circ}$C step, followed by a rapid chilling for 2 min at 4 $^{\circ}$C. The protocol was stopped at this step and the MoMuLV reverse transcriptase was added to the samples, except the negative controls ($-$RT). The incubation was resumed by two thermal steps: 10 min at 20 $^{\circ}$C followed by 90 min at 50 $^{\circ}$C. Finally, the reaction was terminated by heating at 95 $^{\circ}$C for 10 min. Quantitative real-time PCR was performed by an iCycler-iQ in a 25 µl reaction mixture containing: 1$\times$ iQ-SYBR-Green-Supermix (Bio-Rad, Hercules, CA, USA), 20 ng cDNA (calculated on the basis of the retro-transcribed RNA) and 330 nM for each primer. The amplification profile consisted of an initial denaturation of 2 min at 94 $^{\circ}$C and 40 cycles of 30 s at 94 $^{\circ}$C, annealing for 30 s at TaOpt (optimum annealing temperature—see below) and elongation for 45 s at 68 $^{\circ}$C. Fluorescence data were collected during the elongation step. A final extension of 7 min was carried out at 72 $^{\circ}$C, followed by melt-curve data analysis. Optimized primers for SYBR Green analysis (and relative TaOpt) were designed by the Beacon Designer software 6.0 version (Biosoft International, Palo Alto, CA, USA) and were synthesized (HPLC purification grade) by MWG Biotech AG (Ebersberg, Germany) (CB$_1$: forward primer CTGATGTTCTG GATCGGAGTC, reverse primer TCTGAGGTGTGAATGATGA TGC; β-actin: forward primer CCAGGCATTGCTGACAGG, reverse primer TGGAAGGTGGACAGTGAGG; GADPH: forward primer GCCTTCCGTGTTCCTACC, reverse primer CCTGGTCCTCAGTGTAGC). Assays were performed in quadruplicate (maximum ΔC_t of replicate samples <0.5), and a standard curve from consecutive fivefold dilutions (100–0.16 ng) of a cDNA pool representative of all samples was included for PCR efficiency determination. Relative expression analysis, correct for PCR efficiency and normalized with respect to reference genes β-actin and GADPH was performed by GENEX software (Bio-Rad) for groupwise comparison and statistical analysis.

Drugs
Anandamide and I-RTX were purchased from Tocris Cookson (Bristol, UK). AA-5-HT and OMDM-2 ((S)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine) were synthesized as described previously (Ortar *et al.*, 2003). Rimonabant (5-(*p*-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-piperidinopyrazole-3-carboxamide hydrochloride) and SR144528 (*N*-[-1*S*-endo-1,3, 3-trimethyl-bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methyl-phenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) were a kind gift from Drs Madaleine Mossè and Francis Barth (SANOFI Recherche, Montpellier, France).

N-arachidonoyl-5-hydroxytryptamine was dissolved in dimethyl sulphoxide (DMSO)/Tween 80 (1:4), OMDM-2 and I-RTX in DMSO, AEA in Tocrisolve (soya oil/water (1:4 emulsion)). The drug vehicles (10 µl 10 g^{-1} DMSO/Tween 80, 2 µl 10 g^{-1} DMSO, 20 µl 10 mg^{-1} Tocrisolve) had no significant effect on gastric emptying.

Statistics
Data are expressed as the mean ± s.e.mean of experiments in *n* mice. To determine statistical significance, Student's *t*-test was used for comparing a single treatment mean with a control mean, and ANOVA followed by a Tukey–Kramer multiple comparisons test was used for analysis of multiple treatment means (or Bonferroni's for endocannabinoid levels). *P*-values <0.05 were considered significant.

Results

Gastric emptying in control mice
Intraperitoneal administration of AEA (1–15 mg kg^{-1}) produced a dose-dependent inhibition of gastric emptying (Figure 1), with a significant effect starting from the 5 mg kg^{-1} dose. The inhibitory effect of AEA (7.5 mg kg^{-1}) was counteracted by a non-effective dose *per se* of the CB$_1$ receptor antagonist rimonabant (0.1 mg kg^{-1}), but not by the CB$_2$ receptor antagonist SR144528 (1 mg kg^{-1}) or the TRPV1 (transient receptor potential vanilloid type 1) antagonist
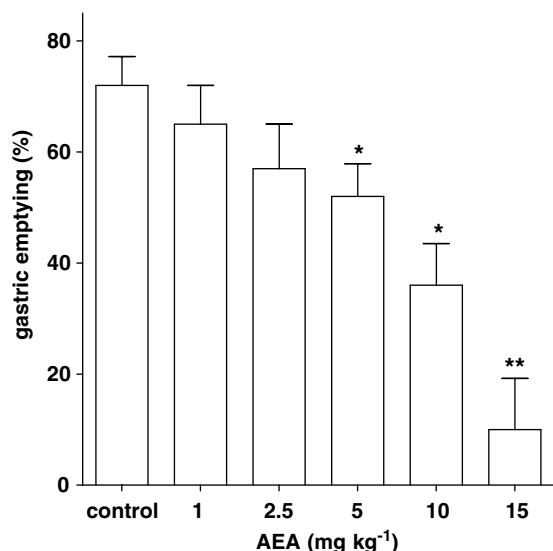
Figure 1 Effect of anandamide (AEA), injected intraperitoneally, on gastric emptying in ICR control mice. Columns represent the mean ± s.e.mean of 9–11 animals for each experimental group. *$P < 0.05$ and **$P < 0.01$ vs corresponding control.
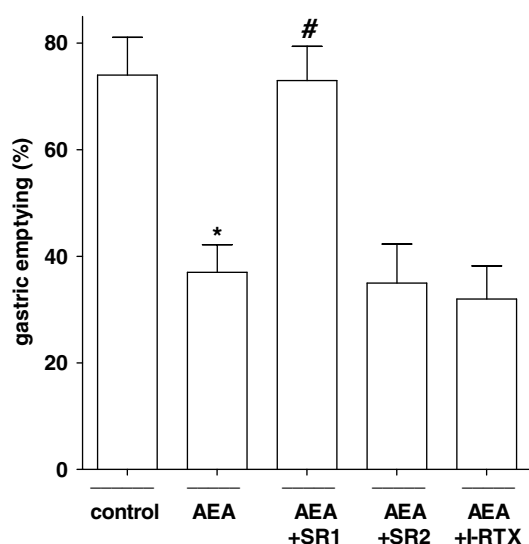

Figure 2 Effect of anandamide (AEA, 7.5 mg kg^{-1}, i.p.) alone or in the presence of the CB$_1$ receptor antagonist rimonabant (SR1, 0.1 mg kg^{-1}, i.p.), or the CB$_2$ receptor antagonist SR144528 (SR2, 1 mg kg^{-1}, i.p.) or the TRPV1 antagonist 5'-iodoresiniferatoxin (I-RTX; 0.75 mg kg^{-1}, i.p.) on gastric emptying in ICR control mice. Columns represent the mean ± s.e.mean of 7–10 mice for each experimental group. *$P < 0.05$ vs corresponding control and #$P < 0.05$ vs AEA alone. AEA, anandamide.

I-RTX (0.75 mg kg^{-1}) (Figure 2). Figure 3 shows that the FAAH inhibitor AA-5-HT (10 mg kg^{-1}) decreased gastric emptying, whereas the CB$_1$ receptor antagonist rimonabant (1 mg kg^{-1}) increased gastric emptying. These results are extrapolated from dose–response curves (data not shown). By contrast, the endocannabinoid reuptake inhibitor OMDM-2 (10 mg kg^{-1}) did not affect gastric emptying significantly (Figure 3). A non-effective dose *per se* of the CB$_1$ receptor antagonist rimonabant (0.1 mg kg^{-1}), but not of the CB$_2$ receptor antagonist SR144528 (1 mg kg^{-1}) or the
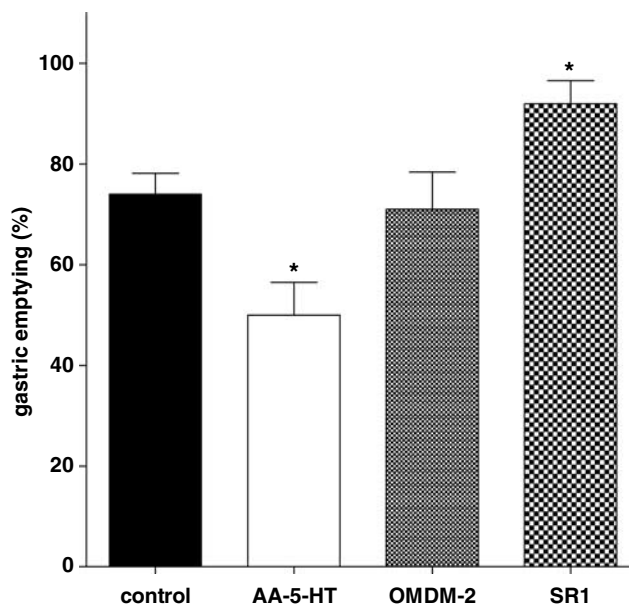

Figure 3 Effect of the FAAH inhibitor *N*-arachidonoyl-5-hydroxy-tryptamine (AA-5-HT, 10 mg kg^{-1}, i.p.), the anandamide cellular reuptake inhibitor OMDM-2 (10 mg kg^{-1}) or the CB$_1$ receptor antagonist rimonabant (SR1, 1 mg kg^{-1}) on gastric emptying in ICR control mice. Columns represent the mean ± s.e.mean of 8–10 mice for each experimental group. *$P < 0.05$ vs control. FAAH, fatty acid amide hydrolase.

TRPV1 antagonist I-RTX (0.75 mg kg^{-1}) significantly reduced the inhibitory effect of AA-5-HT (10 mg kg^{-1}) on gastric emptying (Figure 4). However, AA-5-HT, in the presence of rimonabant, still exerted a significant inhibitory effect (Figure 4).

Given alone (that is, in absence of any agonist), the CB$_2$ antagonist SR144528 (1 mg kg^{-1} i.p.) and the TRPV1 antagonist I-RTX (0.75 mg kg^{-1} i.p.) did not modify significantly gastric emptying (percentage of gastric emptying: control 70.4 ± 6.2, SR144528 68.4 ± 5.2; control 72.0 ± 4.9, I-RTX 64.2 ± 6.7; $n = 8$–10 for each experimental group).

Gastric emptying in HFD-fed mice

Compared to aged-matched STD-fed animals, HFD-fed mice showed significantly increased body weight (STD 29.6 ± 0.4 g; HFD 41.5 ± 0.7 g, $n = 38$ for each experimental group, $P < 0.001$), higher blood glucose (STD 121.1 ± 5.8; HFD 167.5 ± 4.8 mg per 100 ml, $n = 7$–8 for each experimental group, $P < 0.05$) and delayed gastric emptying (percentage gastric emptying: STD 79.3 ± 4.9; HFD 65.0 ± 3.5, $n = 9$ for each experimental group, $P < 0.05$). Figure 5 shows the effect of rimonabant on gastric emptying in control and HFD-fed mice. Rimonabant increased gastric emptying both in control and HFD-fed mice (Figure 5a). No statistical difference was observed between the curves representing the percentage of increase in control and HFD-fed mice (Figure 5b). By contrast, the FAAH inhibitor AA-5-HT was significantly more effective in reducing gastric emptying in HFD-fed animals compared to control mice (Figure 6). Finally, the endocannabinoid reuptake inhibitor OMDM-2 (10 mg kg^{-1}) did not affect gastric emptying (data not shown).
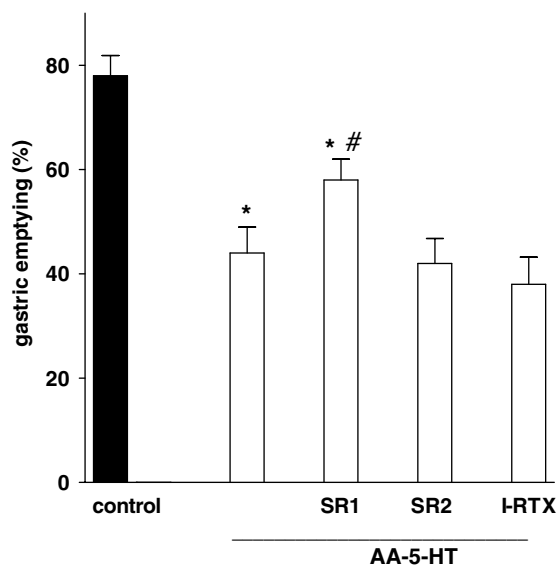
Figure 4 Effect of intraperitoneally injected N-arachidonoyl-5-hydroxytryptamine (AA-5-HT, 10 mg kg^{-1}) on gastric emptying in ICR control mice pretreated (i.p.) with the CB$_1$ receptor antagonist rimonabant (SR1, 0.1 mg kg^{-1}), or the CB$_2$ receptor antagonist SR144528 (SR2, 1 mg kg^{-1}) or the TRPV1 (transient receptor potential vanilloid type 1) antagonist 5'-iodoresiniferatoxin (I-RTX, 0.75 mg kg^{-1}). Columns represent the mean ± s.e.mean of 7–9 mice for each experimental group. *$P < 0.05$ vs control and #$P < 0.05$ vs AA-5-HT alone.

Levels of endocannabinoids

Gastric AEA and 2-AG levels were measured after the periods of 8 and 14 weeks of a HFD or a STD. As shown in Table 1, after 14 weeks of HFD, the levels of AEA were significantly reduced by 25.2%, but no significant changes were observed after 8 weeks. No significant changes were observed for 2-AG levels at both time points.

Expression of cannabinoid CB$_1$ receptors

Results are presented in Table 2. HFD was accompanied by a 12.5-fold decrease in the levels of CB$_1$ receptor mRNA ($P < 0.05$) in the antrum. CB$_1$ expression in the fundus also appeared to be decreased, but these changes did not achieve statistical significance.

Discussion

The possible involvement of endocannabinoids in regulating gastric functions, including gastric motility, is largely unexplored. Immunohistochemical studies have revealed the presence of CB$_1$ receptors on vagal afferents (Burdyga et al., 2004) and myenteric cholinergic nerves innervating rat stomach smooth muscles (Van Sickle et al., 2001; Adami et al., 2002). Previous pharmacological studies have shown that cannabinoid receptor agonists (via CB$_1$) exert gastric antimotility effects in the rat in vivo (Izzo et al., 1999; Krowicki et al., 1999; Landi et al., 2002). In these studies, the CB$_1$ receptor antagonist rimonabant, administered alone, was without effect (Izzo et al., 1999; Landi et al., 2002) or even produced an effect in the same direction as that

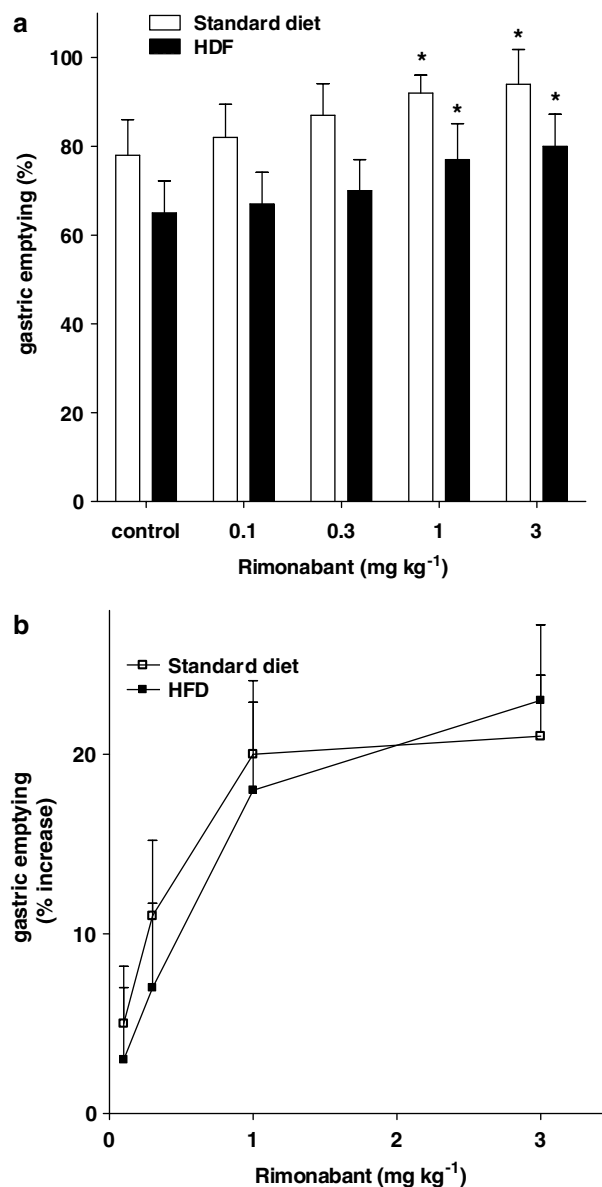**Figure 5** Effect of rimonabant (0.1–3 mg kg^{-1}, i.p.) on gastric emptying in C57Bl/6J mice fed for 14 weeks a standard diet or a high-fat diet (HFD). Results (mean ± s.e.mean of 4–6 mice for each experimental group) are expressed as a percentage of gastric emptying (**a**) or a percentage of increase of corresponding control values (**b**). (**a**) *$P < 0.05$ vs corresponding control; (**b**) no statistical differences were observed between the two dose–response curves.

of cannabinoid agonists (Krowicki et al., 1999), thus preventing the investigators from concluding that endocannabinoids endogenously control gastric motility. Furthermore, the effect on gastric motility of drugs that inhibit endocannabinoid inactivation has never been investigated to date. It is also not known whether endocannabinoids and CB$_1$ receptors undergo any gastric emptying-related adaptive change during HFD-induced overweight. This is an important issue in view of the recently acquired evidence that the endocannabinoid system is overactivated in several peripheral tissues and blood of mice with diet-induced obesity and in obese and hyperglycaemic patients
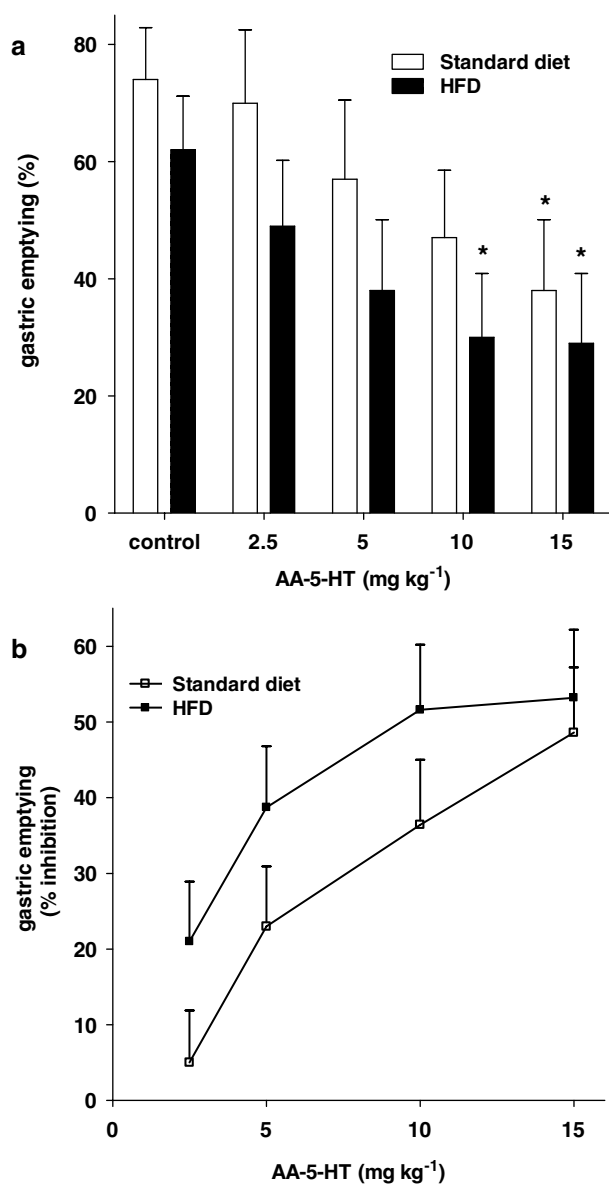
Figure 6 Effect of *N*-arachidonoyl-5-hydroxytryptamine (AA-5-HT, 2.5–15 mg kg^{-1}, i.p.) on gastric emptying in C57Bl/6J mice fed for 14 weeks a standard diet or a high-fat diet (HFD). Results (mean ± s.e.mean of 3–5 mice for each experimental group) are expressed as a percentage of gastric emptying (**a**) or as a percentage of inhibition of corresponding control values (**b**). (**a**) *$P < 0.05$ vs corresponding control. (**b**) A significant difference ($P < 0.05$) was observed between the two dose–response curves.

Table 1 AEA and 2-AG levels in the stomach of mice fed for up to 8 or 14 weeks with STD or HFD

Diet	8 weeks		14 weeks	
	AEA (pmol g^{-1})	*2-AG (nmol g^{-1})*	*AEA (pmol g^{-1})*	*2-AG (nmol g^{-1})*
STD	72.9 ± 3.1	6.2 ± 1.4	91.4 ± 4.2	10.3 ± 1.9
HFD	85.5 ± 9.2	10.4 ± 2.5	68.3 ± 4.6*	8.4 ± 0.4

Abbreviations: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; HFD, high-fat diet; STD, standard diet.
Data are mean ± s.e.mean of $n = 4$ animals. *$P < 0.05$ compared to corresponding STD samples.

Table 2 Changes in CB$_1$ mRNA expression in the antrum and fundus of the stomach of mice fed a HFD for 14 weeks

Receptor	Antrum	Fundus
CB$_1$	0.084 ± 0.043*	0.484 ± 0.170

Abbreviations: HFD, high-fat diet; STD, standard diet.
Data are mean ± s.e.mean of $n = 3$ determinations from three independent experiments and are expressed as fold changes of mRNA expression in the antrum and fundus of the stomach of mice fed HFD vs STD (control, considered as 1). Refer to Results for expression fold data. *$P < 0.05$ vs control.

Izzo, 2007; Sanger, 2007). With regard to gastric functions, previous experimental studies have shown that cannabinoids exert, via CB$_1$ activation, gastric antisecretory (Adami *et al.*, 2002) and antiulcer (Germano *et al.*, 2001; Dembinski *et al.*, 2006; Rutkowska and Fereniec-Goltbiewska, 2006) actions in rats and also inhibit transient lower oesophageal sphincter relaxations in dogs and ferrets (Lehmann *et al.*, 2002; Partosoedarso *et al.*, 2003). Moreover, clinical studies performed in healthy volunteers have demonstrated that oral Δ^9-tetrahydrocannabinol significantly retards gastric emptying in humans (McCallum *et al.*, 1999; Esfandyari *et al.*, 2006). Here, we have shown that the endogenous cannabinoid AEA, which exhibits no marked selectivity for CB$_1$ or CB$_2$ receptors (Pertwee, 2006), reduces gastric emptying dose-dependently. This effect is uniquely mediated by CB$_1$ receptors, as the action of AEA was counteracted by the selective CB$_1$ receptor antagonist rimonabant, but not by the CB$_2$ receptor antagonist SR144528; others have shown that AEA reduces cholinergic-mediated twitch contractions in rodents *in vitro* (Storr *et al.*, 2002; Mulé *et al.*, 2007), an effect that could involve both CB$_1$ and CB$_2$ receptors (Mulé *et al.*, 2007). Anandamide is also an endogenous ligand of the TRPV1 receptor, a non-selective cation channel that belongs to the large family of transient receptor potential ion channels, and is activated by the pungent ingredient of hot chilli peppers, capsaicin (Ross, 2003; Van der Stelt and Di Marzo, 2004). TRPV1 is present in the gastrointestinal tract where it is primarily associated with axons of spinal afferent neurons and, to some extent, vagal afferents (Holzer, 2004). There is evidence that TRPV1 activation by AEA induces ileitis in rats (McVey *et al.*, 2003), increases the release of γ-aminobutyric acid from guinea pig myenteric nerves (Begg *et al.*, 2002) and facilitates spontaneous acetylcholine release from the myenteric plexus of the guinea pig ileum (Mang *et al.*, 2001). However, we have shown here that the

(Matias *et al.*, 2007). This overactivation could also occur in the gastrointestinal tract and might affect the levels of gastrointestinal hormones that control food intake, such as ghrelin (Cani *et al.*, 2004). In the present study, we provide evidence that AEA and CB$_1$ receptors are indeed involved in the inhibition of gastric emptying, both in lean and overweight mice, and that their gastric levels undergo downregulation after a prolonged HFD.

The actions of cannabinoids in the digestive tract include inhibition of motility, secretion, inflammation, visceral pain and cell proliferation (reviewed in Duncan *et al.*, 2005; Massa *et al.*, 2005; Di Marzo and Izzo, 2006; Izzo and Capasso, 2006;

inhibitory effect of AEA on gastric motility was not mediated by activation of TRPV1, as the selective and ultrapotent TRPV1 antagonist I-RTX failed to affect endocannabinoid-induced changes in motility. These data are consistent with previous results that showed the lack of involvement of TRPV1 receptors in AEA-induced reduction of small intestinal motility (Izzo et al., 2001). Furthermore, Bartho et al. (2002) found no evidence for AEA activation of TRPV1 receptors in the human-isolated sigmoid colon.

The first step of AEA inactivation is cellular reuptake, which seems to be facilitated by a putative membrane transporter (Ho and Hillard, 2005). Previous studies have shown that an inhibitor of the AEA transporter may reduce motility in the colon (Pinto et al., 2002), but not in the small intestine (Calignano et al., 1997; Mascolo et al., 2002). However, in pathophysiological states, the AEA uptake inhibitor VDM11 was shown to reduce small intestinal motility in the experimental model of ileus induced by acetic acid (Mascolo et al., 2002), to exert anti-inflammatory effects in experimental colitis (D'Argenio et al., 2006) and to reduce cholera toxin-induced hypersecretion (Izzo et al., 2003). In the present study, we have shown that the AEA uptake inhibitor OMDM-2 (de Lago et al., 2004) does not modify gastric emptying in control animals, thus contesting the importance of such a mechanism in the physiological control of gastric motility in vivo. The dose of OMDM-2 used in the present study was previously shown to potentiate the motor inhibitory effects of AEA in vivo (de Lago et al., 2004).

The second step of endocannabinoid inactivation is enzymatic degradation via hydrolytic enzymes, among which the best characterized is FAAH (Jhaveri et al., 2007). In the periphery, the FAAH presence has been found along the small and large intestine (Capasso et al., 2005). In the digestive tract, FAAH inhibition results in anti-inflammatory effects (Massa et al., 2004; D'argenio et al., 2006), inhibition of motility in the small intestine (Capasso et al., 2005) and anticancer effects (Izzo et al., 2007). In the present study, we have shown that the FAAH inhibitor AA-5-HT, at a dose previously shown to increase intestinal endocannabinoid levels (Capasso et al., 2005) and not to activate CB_1 receptors, reduces gastric emptying; this effect was attenuated by the CB_1 receptor antagonist rimonabant (but not by the TRPV1 antagonist I-RTX), although even in the presence of rimonabant, AA-5-HT exerted a significant inhibitory effect on gastric motility. These results suggest that AA-5-HT, through an increase of endocannabinod levels, could indirectly activate CB_1 and hence could reduce gastric emptying. However, a CB_1-independent mechanism in AA-5-HT-induced delay in gastric motility also exists. A possible candidate for this CB_1-independent mechanism is oleoylethanolamide, an FAAH substrate with the ability to reduce gastric emptying (AA Izzo and V Di Marzo, unpublished observations) and intestinal motility (Capasso et al., 2005), and whose mouse gastric levels, like those of AEA, are increased after the AA-5-HT treatment (AA Izzo and V Di Marzo, unpublished observations).

An ever increasing number of studies have suggested that the endocannabinoid system regulates food intake and energy balance in control and obese animals via CB_1

receptors in brain and in periphery (Di Marzo and Matias, 2005; Sharkey and Pittman, 2005; Osei-Hyiaman et al., 2006). These studies have led to the development of the antiobesity drug rimonabant. Phase III clinical trials have shown that this antagonist reduces weight and waist circumference to a significantly greater extent than placebo in obese patients (Despres et al., 2005; Van Gaal et al., 2005; Pi-Sunyer et al., 2006; Scheen et al., 2006). Here, we found that a 14-week HFD increased body weight and glucose blood levels as well as reduced gastric emptying. Our data are in agreement with those of previous investigators who reported delayed gastric emptying in ob/ob mice, a genetic model of obesity and diabetes (Asakawa et al., 2003). The reduced gastric emptying in HFD-fed animals was associated with a modest (30%) but significant decrease of AEA gastric content and of CB_1 receptor expression, with no changes in 2-AG levels. These findings indicate that, in contrast to other peripheral organs of mice with high-fat-induced obesity, that is, epididymal adipose tissue, pancreas and liver, where an upregulation was observed (Osei-Hyiaman et al., 2006; Matias et al., 2007), the endocannabinoid system is down-regulated in the stomach of HFD-fed mice. This might have consequences on food intake, as it has been suggested that CB_1 activation tonically stimulates gastric ghrelin secretion (Cani et al., 2004), and ghrelin in turn disinhibits cholecys-tokinin-induced suppression of CB_1 receptor expression in the nodose ganglion (Burdyga et al., 2006). Thus, suppression of gastric endocannabinoid signalling during HFD-induced obesity might represent an adaptive response aimed at reducing food intake in synergy with reduced gastric emptying. However, it is very unlikely that the decreased AEA and CB_1 levels contribute to the reduction of gastric emptying observed in HFD-fed mice, as this signalling system was shown to reduce gastric motility in both lean and obese mice (see above).

To elucidate the role of the endocannabinoid system also in HFD-treated mice, we investigated the effect of a drug that antagonizes the effect of endocannabinoids at CB_1 receptors (that is, the CB_1 receptor antagonist rimonabant) and the effect of drugs that inhibit endocannabinoid inactivation (that is, the FAAH inhibitor AA-5-HT and the AEA cellular reuptake inhibitor OMDM-2) on gastric emptying. We found that rimonabant exerted a prokinetic effect in HFD-treated animals, although with the same potency observed in control mice. This confirms that HFD does not cause any increase in endocannabinoid 'tone' in the stomach, as an antagonist would be more efficacious in the presence of a higher endogenous tone. Indeed, our present data indicate that, if anything, gastric endocannabinoids levels and, particularly, CB_1 receptor mRNA expression are reduced after a HFD. The similar efficacy of rimonabant in enhancing gastric motility in lean and obese mice also suggests that the weight loss-inducing effects of this compound, which are observed preferentially in obese animals (Vickers et al., 2003), are unlikely to be effected at the level of gastric emptying. Nevertheless, the prokinetic effect of rimonabant in HFD-fed animals with hyperglycaemia may have some clinical relevance in the light of the observation that diabetic gastroparesis is a well-recognized disturbance in obese patients (Park and Camilleri, 2006) and that rimonabant

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can produce a clinically meaningful reduction in body weight and improve a number of cardiovascular and metabolic risk factors in overweight or obese patients with type 2 diabetes (Scheen *et al.*, 2006). We also found that, unlike rimonabant, the FAAH inhibitor AA-5-HT was significantly more potent in inhibiting gastric motility in HFD-fed compared to STD-fed mice. The different potency of AA-5-HT could be explained by the observation that FAAH mRNA expression is reduced in the stomach of HFD-fed mice (Aviello *et al.*, 2008) and by the fact that FAAH also catalyzes the hydrolysis of the anti-prokinetic compound oleoyle-thanolamide, whose gastric levels are increased in overweight animals (Aviello *et al.*, 2008).

Finally, we showed here that the AEA cellular reuptake inhibitor OMDM-2 failed to affect gastric emptying in HFD-fed mice, thus arguing against the importance of this inactivating mechanism in regulating gastric motility in obese animals.

Conclusion

In the present study, we have shown for the first time that the endocannabinoid system is physiologically involved in the regulation of gastric emptying *in vivo*, both in control and in overweight animals. This conclusion is mainly based on the findings of (a) high amount of endocannabinoids in gastric tissues, (b) a prokinetic effect of the cannabinoid receptor antagonist rimonabant and (c) an inhibitory action on gastric emptying of the FAAH inhibitor AA-5-HT as well as of AEA. We also observed that the endocannabinoid system undergoes adaptive changes after a HFD, as revealed by the changes in AEA and CB_1 mRNA levels and by the different potency of the FAAH inhibitor AA-5-HT at delaying gastric emptying after this dietary regime. Targeting the endo-cannabinoid system might thus offer novel therapeutic strategies in the treatment of gastric motility diseases and has potential for the treatment of diabetic obese patients with dyspepsia.

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Conflict of interest

The authors state no conflict of interest.

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