ACUTE ACTIVATION OF CANNABINOID RECEPTORS BY ANANDAMIDE REDUCES GASTRO-INTESTINAL MOTILITY AND IMPROVES POSTPRANDIAL GLYCEMIA IN MICE

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Running title: Anandamide improves postprandial glycemia in mice

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Endocannabinoid system (ECS) is associated with an alteration of glucose homeostasis dependent on cannabinoid receptor-1 (CB1R) activation. However, very little information is available concerning the consequences of ECS activation on intestinal glucose absorption.

Mice were injected intraperitoneally with anandamide, an endocannabinoid binding both CB1R and CB2R. We measured plasma glucose and xylose appearance after oral loading, gastrointestinal motility and glucose transepithelial transport using the everted sac method.

Anandamide improved hyperglycemia after oral glucose charge whereas glucose clearance and insulin sensitivity were impaired pointing out some gastro-intestinal events. Plasma xylose appearance was delayed in association with a strong decrease in gastro-intestinal transit while anandamide did not alter transporter-mediated glucose absorption. Interestingly, transit was nearly normalized by co-injection of SR141716 and AM630 (CB1R and CB2R antagonist respectively) and AM630 also reduced the delay of plasma glucose appearance induced by anandamide. When gastric emptying was by-passed by direct glucose administration in the duodenum, anandamide still reduced plasma glucose appearance in wild-type but not in CB1R<sup>−/−</sup> mice.

In conclusion, our findings demonstrated that acute activation of intestinal ECS reduced postprandial glycemia independently on intestinal glucose transport but rather inhibiting gastric emptying and small intestine motility and strongly suggest the involvement of both CB1R and CB2R.
INTRODUCTION

The endocannabinoid system (ECS) consists of cannabinoid receptors (CBR), endocannabinoids and enzymes involved in their synthesis and degradation. The two main receptors, CB1R and CB2R, are both G protein-coupled receptors differing structurally and by their tissue localization. Whereas CB2R are mainly expressed in the immune system, CB1R are abundantly present in the central nervous system and to a lower extent in peripheral tissues (1). Arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG) are the two best characterized endogenous cannabinoids produced in the brain and peripheral tissues that bind CB1R and CB2R with no marked selectivity (2) to activate or inactivate metabolic pathways similarly to ∆9-THC (Tetrahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzopyran-1-ol), the major active constituent of marijuana (1; 3).

It is now well established that the ECS is involved in many biological functions including the regulation of energy balance and the pathogenesis of obesity. Obesity is associated with central ECS overactivation and since activation of CB1R in the brain stimulates food intake, ECS appears to be located at the center of a vicious circle inducing weight gain and related disorders (4; 5). However, even if the CB1R-dependent stimulation of food intake may be the main initial cause of various metabolic deregulations associated with obesity, several data collected from animal and human studies using the specific CB1R antagonist SR141716 (Rimonabant) suggested that peripheral ECS might also directly regulate energy metabolism (6-8). It was recently reported that activation of peripheral CB1R signaling in key tissues relevant to insulin action is associated with alteration of glucose homeostasis and contributes to diet-induced insulin resistance in mice (9-11). Nevertheless, very little information is available concerning the impact of peripheral ECS activation on intestinal glucose absorption.
that is however the major determinant of how quickly glucose appears in the circulation during the fed state.

The presence of a functional ECS in the gastro-intestinal tract has been pointed out in many species (12-14) and available evidence suggests that cannabinoids reduce gastric and intestinal motility (15; 16) but the consequences on plasma glucose appearance were poorly studied. However, consistent data indicate the existence of a positive relationship between gastro-intestinal motility and plasma glucose appearance in healthy and diabetic patients (17-19). We therefore investigated the impact of acute activation of CBR by anandamide on postprandial glycemia, recognized as an important component of the overall glycemic control (20; 21). Our data showed that activation of gastro-intestinal ECS improved postprandial glycemia and further indicated this was associated with neither an increase in glucose clearance nor a decrease in intestinal transport but rather with a reduction in gastro-intestinal motility involving both CB1R and CB2R.

METHODOLOGY

Animals and diets

Official French regulations (n°87848) for the use and care of laboratory animals were followed throughout the experimental period. The experimental protocol was approved by the local ethic committee for animal experimentation (n°BX0622). Eleven week-old C57BL/6J male mice (JanvierLabs, Le Genest Saint Isle, France) and global CB1R<sup>-/-</sup> mice (generous gift from Dr. James Pickel, NIMH, Bethesda, USA) were housed individually on a 12:12h light:dark schedule at 22–23°C with <i>ad libitum</i> access to water and food (Standard diet AO4; UAR, Epinay-sur-Orge, France). A group of mice was subject to a high fat diet (30% lard)
manufactured by SSNIF (Soest, Germany). After 16 weeks of diet, only animals with a weight gain < +10 g compared to controls were excluded from the study. Diet-induced obesity (DIO) mice (39.1 ± 1.1 vs 27.3 ± 0.9 g DIO vs control) were glucose intolerant and insulin resistant (Sup Figure 2A and 2B). On the day of each experiment, food was removed from the cages for 6 h (from 8:00 a.m. to 2:00 p.m.).

**Drugs**

Anandamide, AM630, AM6545 and exendin 9-39 were purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France) and SR141716 provided by Sanofi Aventis (Paris, France). Anandamide and CBR antagonists were prepared in 4% dimethyl sulfoxide (DMSO)/1% Tween 80 and administered intraperitoneally (i.p.) at 10 mg/kg. In control experiments, animals were injected with vehicle (4% DMSO/1% Tween 80).

**Glucose, xylose and insulin tolerance tests**

For glucose tolerance test (GTT) and insulin tolerance test (ITT), mice respectively received a glucose load (2g/kg) or insulin i.p. injection (0.5 UI/kg; Actrapid®, Novo Nordisk, France). For GTT, a D-glucose solution (20% w/v) was either injected intraperitoneally (i.p.GTT) or given orally (OGTT). Oral D-xylose loading test (OXT) consisted in the administration of a D-xylose solution (10% w/v; 1 g/kg body weight). Drugs were administered i.p. to 6-hour fasting mice 10 minutes before glucose, xylose or insulin. Each treatment was tested with a one-week interval on the same series of mice in the same conditions. Glycemia was measured directly in blood sampled from the tail with a My Life Pura™ glucose meter (Ypsomed, Paris, France). In some OGTt experiments, blood (25 µl) from tail vein was collected in chilled tubes containing EGTA and DPPIV inhibitor (Millipore S.A.S., Molsheim, France) for determination of insulin, GIP and GLP-1 concentration with a Bio-Plex Mouse Diabetes
Assay (Bio-Rad, Marnes-la-Coquette, France). In OXT, some control experiments included oral administration of phlorizin (Sigma) as a SGLT-1 inhibitor, 15 min before the first injection. Plasma xylose concentrations were measured with a spectrophotometric kit (Megazyme, Wicklow, Ireland).

**Intestinal glucose transport**

Wistar rats (JanvierLabs) weighting 250-300 g were anesthetized by i.p. injection of 50 mg/kg sodium pentobarbital. Two 5-cm proximal jejunum segments (5 cm from the ligament of Treitz) were taken from each animal and prepared as previously described (Wagner, 2003 #162). Briefly, sleeves were flushed, everted and mounted in a 15-ml centrifuge tube. The installation allowed to introduce buffer into the sac (serosal compartment) and to collect samples periodically. Sacs were filled with 200 µl of buffer consisting of 150 mM NaCl, 1 mM KH2PO4, 4 mM K2HPO4 and 5 mM fructose, pH 7.4 and immersed in 14 ml of the same buffer (mucosal compartment) added with 0.1 µM or 30 mM D-glucose. 14C-D-glucose (3.2 µCi/µmole, PerkinElmer, Courtaboeuf, France) was used as a marker to quantify transporter-mediated uptake from the mucosal to the serosal compartment. Fragments were incubated for 40 min at 37°C in the presence of anandamide (5 µM) or vehicle added both in the serosal and mucosal compartment. A 30 µl-sample was collected from the serosal compartment every 10 min and replaced with equivalent volume of glucose-free buffer. Samples were mixed with 4.5 ml of Ultima Gold XR liquid scintillation cocktail (PerkinElmer) and radiolabeled glucose counted by a Packard Tricarb 2000 counter (PerkinElmer).

**Gastro-intestinal transit**
Transit through the stomach and small intestine was measured by administering a non-absorbed marker (10% charcoal suspension in 5% gum Arabic) as previously described (22). Briefly, fasting mice were injected i.p. with anandamide and CBR antagonists respectively 10 and 30 min before gavage with 0.2 ml of the charcoal suspension. Animals were killed by cervical dislocation 25 minutes later. The entire small intestine was removed and the distance from the pylorus to the front of the charcoal bolus was measured. Transit rate was calculated as \[
\frac{\text{distance to charcoal front}}{\text{total length from pylorus to caecum}} \times 100\%\].

**RT-PCR**

Total mRNAs were extracted with Tri-Reagent (Euromedex, Souffelweyersheim, France) and 1 µg RNA was reverse-transcripted using the Iscript cDNA kit (Bio-Rad). Real-time PCR was performed as described previously (23) using a StepOnePlus™ real-time PCR system (Life Technologies, Saint-Aubin, France). Primer sequences used for amplification were: forward cgcacaagatigatecaag, reverse aaccccccagtttgac for CB1R; forward cgcacaagatgtcccaag, reverse aaccccccagtttgac for CB2R and forward cgcacaagatgtcccaag, reverse aaccccccagtttgac for TATA Box binding protein. For each gene, a standard curve was established from four cDNA dilutions (1/10 to 1/10000) and used to determine the relative gene expression variation after normalization with TATA box binding protein expression.

**Statistical analysis.**

Results are expressed as means ± SEM. Data were analyzed statistically with GraphPad InStat software (GraphPad Software, Inc. La Jolla, CA, USA) using 2-way ANOVA followed by the Tukey post-hoc test, or using Student t-test. Differences were considered significant at P<0.05.
RESULTS

**Anandamide prevents hyperglycemia induced by oral glucose loading**

To evaluate the impact of CBR activation on whole body glucose homeostasis, we first assessed glucose tolerance after a single i.p. anandamide injection (10 mg/kg). In order to respect the natural route of nutrients administration, we performed oral glucose tolerance test (OGTT, 2g/kg). Interestingly, the increase in glycemia in response to glucose ingestion was considerably smaller in mice treated with anandamide compared to control and this was associated with an improvement of glucose tolerance as illustrated by the AUC\textsubscript{0-2h} calculations (Figure 1).

**Anandamide alters blood glucose clearance and insulin sensitivity**

To determine whether anandamide affected tissue glucose utilization, we measured glucose clearance after i.p. administration (i.p.GTT, 2g/kg). In these conditions excluding any involvement of the gastro-intestinal tract, results indicated that anandamide induced glucose intolerance (Figure 2A) and prevents insulin (i.p. injection, 0.5 UI/kg) to rapidly decrease plasma glucose compared to controls (Figure 2B). Figure 2C also showed that anandamide did not decrease fasting glycemia, rather drug injection even induced a rapid elevation of t\textsubscript{0-15 min} plasma glucose levels (+29.69±2.68 % for anandamide vs 11.3±2.87% for vehicle).

**Anandamide-induced improvement of postprandial glycemia is not dependent on a reduction of intestinal glucose transport.**
To explore the possibility of an alteration of intestinal glucose absorption in anandamide-injected mice, we first carried out an oral D-xylose loading test (OXT, 1g/kg), an investigative tool to study small intestinal function (24). To verify that OXT accurately reflected the efficiency of glucose absorption, OGTT and OXT were also performed in the presence of phlorizin a potent inhibitor of the sodium-dependent glucose transporter 1 (SGLT-1) (25). Results confirmed that phlorizin suppressed both plasma appearance of glucose (data not shown) and xylose in response to oral glucose and xylose load respectively (Figure 3A). Using this method, we interestingly observed that plasma xylose appearance was strongly delayed in response to anandamide injection compared to vehicle while the maximum plasma levels were not different (Figure 3A) suggesting that anandamide did not alter trans-epithelial transport. To further investigate this notion, the direct effect of anandamide on intestinal glucose transport was evaluated using jejunal everted sacs exposed either to low glucose (10 µM) or high glucose (30 mM) concentration to study respectively SGLT-1 and GLUT-2-mediated transport. As expected, glucose transport was strongly inhibited by phlorizin in 10 µM glucose experiments (Figure 3B) and by the combination of both phlorizin and phloretin in 30 mM glucose experiments (Figure 3C). On the other hand, we did not observe any effect of anandamide on transporter-mediated uptake of glucose in comparison with vehicle whatever the concentration tested (Figure 3B and C).

**Anandamide-induced delay in plasma glucose appearance is associated with a decrease in gastro-intestinal motility**

The effect of acute anandamide treatment on gastro-intestinal motility was evaluated using the charcoal meal test. The distance traveled by the non-metabolisable charcoal solution in the small intestine 25 min after gavage was considerably reduced after administration of anandamide compared with vehicle (Figure 4A) suggesting that the drug decreased either
gastric emptying or small intestine motility or both. To determine whether the delay in plasma glucose appearance induced by anandamide was exclusively due to a slower gastric emptying, we next performed a glucose tolerance test in response to a direct duodenal load. In these conditions, plasma glucose levels were significantly lower at t=15 min after infusion in anandamide-treated mice compared to control mice (Figure 4B). Furthermore, we noticed both in duodenal GTT and OXT that anandamide delayed absorption without affecting maximal blood glucose and xylose levels suggesting that anandamide could reduce small intestine motility. We also tested the possibility that the inhibitory action of the drug could be limited due to its rapid degradation in cells and tissues (26). To verify this hypothesis, two successive doses of anandamide (at t-10 and t0 min) were administered during duodenal GTT. Results showed a prolonged effect of the drug as it further delayed maximal blood glucose associated with a glucose intolerance as indicated by AUC<sub>0-120</sub> (Figure 4B) suggesting that a steady-state activation of CBR would profoundly alter glucose absorption and plasma clearance. Remarkably, glucose absorption was no more altered by anandamide when duodenal GTT were performed in CB1R<sup>−/−</sup> mice (Figure 4C).

**Anandamide-induced improvement of hyperglycemia is not mediated by incretins**

Glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) are rapidly released from enteroendocrine cells of the small intestine in response to food ingestion. Both stimulate insulin secretion in a glucose-dependent manner in order to tightly control post-prandial glycemia (27). As we showed that anandamide prevents hyperglycemia when glucose was given orally, we investigated whether anandamide’s effect on blood glucose was mediated by incretin-dependent stimulation of insulin production. We showed that plasma GIP, GLP-1 and insulin levels were strongly lower in anandamide-injected animals during OGTT (Figure 5A-C). In addition, when glucose clearance was measured in
the presence of the GLP-1R antagonist exendin 9-39 (to block the action of GLP-1 on pancreas β-cells) the effect of anandamide on glycemia was not prevented (Figure 5D).

**Anandamide-induced reduction in gastro-intestinal transit and plasma glucose appearance involves both CB1R and CB2R.**

The role of CB1R and CB2R in controlling gastro-intestinal motility and postprandial glycemia was approached using different CBR antagonists. The inhibitory effect of anandamide on transit was partially abrogated when mice were pretreated with specific CB1R antagonist SR141716 or AM6545, the action of the latter being restricted to the periphery (Figure 6A). While the CB2R antagonist AM630 also slightly limited the effect of anandamide, the co-injection of AM630 and SR141716 nearly normalized gastro-intestinal transit (Figure 6A). It was concomitantly observed that the delay of plasma glucose appearance induced by anandamide during OGTT was minored in the presence of AM630 (Figure 6B). Besides, in the stomach of these animals, molecular analysis revealed the presence of much higher levels of CB2R mRNA compared to CB1R mRNA (Figure 6C).

**Anandamide causes CB2R-mediated reduction in plasma glucose appearance in CB1R⁻/⁻ mice**

To further investigate the contribution of CB2R on postprandial glycemia, OGTT and OXT were performed in CB1R⁻/⁻ mice. Plasma glucose and xylose levels were also altered by anandamide during carbohydrate challenge in CB1R⁻/⁻ mice (Figure 7A and 7B). However plasma xylose appearance was far more reduced by anandamide in wild type mice than in CB1R⁻/⁻ (Figure 7B) suggesting that the action of the drug was largely mediated by CB1R but also by CB1R-independent mechanisms. In line with this hypothesis, we showed that AM630 partially counteracted the action of anandamide on plasma glucose levels during OGTT.
performed in CB1R^{−/−} mice (Figure 7A). Interestingly, we also observed that CB1R^{−/−} mice exhibited a much slower gastro-intestinal motility compared to wild type and that AM630 strongly improved transit (Figure 7C). Glucose absorption being no longer altered by anandamide during duodenal GTT in CB1R^{−/−} mice (Figure 4C), it could be advanced that the delay in glucose absorption in CB1R^{−/−} mice observed during OGTT was consecutive to a slowing down of gastric emptying induced by stomach CB2R activation.

Anandamide-induced inhibition of glucose appearance is amplified in obese mice

The impact of anandamide on whole body glucose homeostasis was also assessed in high-fat diet-induced obesity (DIO) mice. These mice were both glucose intolerant and insulin resistant. Injection of anandamide to DIO mice reduced blood glucose appearance during OGTT (Figure 8A). Xylose appearance was also strongly inhibited by anandamide in the 0-45 min range of OXT (Figure 8B). It should be pointed out that the administration of the CB1R antagonist SR141716 alone before OXT did not induce any effect on xylose absorption (Figure 8B) suggesting that intestinal CB1R were likely not activated by endogenous cannabinoids in DIO mice. Thus, evidence comes from these findings that the effect of anandamide on glucose appearance was maintained in obese mice. Considering that anandamide was more potent in reducing xylose appearance in DIO versus lean mice (Figure 8C), we explored the possibility that intestinal ECS activation by anandamide was accentuated in obese mice due to the presence of high levels of CB1R. This assumption was supported by the expression levels of CB1R mRNA in the small intestine mucosa that were 3-fold higher in DIO than in lean mice (Figure 8D).

DISCUSSION
In a recent study showing that hepatic CB1R contribute to diet-induced hepatic insulin resistance by inhibiting both insulin signaling and clearance, Liu et al. (10) provided evidence that acute peripheral administration of anandamide causes CB1R-mediated glucose intolerance in mice. While we drew the same conclusions from the present findings obtained after i.p. glucose load, we also intriguingly observed that the same dose of anandamide improved postprandial glycemia during an oral glucose tolerance test (OGTT). The discrepancies between the results of i.p.GTT experiments ((Liu, 2012 #82) and present study) and OGTT experiments reported here are unquestionably due to the impact of anandamide on events related to intestinal glucose absorption that are bypassed when glucose load is administered i.p.. Since concordant findings from this study and literature (10) showed that peripheral CB1R activation by anandamide induced insulin resistance and endogenous glucose production, it could be excluded that the drug prevented hyperglycemia after oral load increasing blood glucose clearance, insulin sensitivity and/or insulin production. Rather, we presented evidence that anandamide reduced postprandial glycemia delaying intestinal glucose absorption. This was first demonstrated using oral xylose test (OXT) as a clinical marker for intestinal absorption. Since xylose metabolism is not affected by either insulin secretion or insulin resistance, OXT directly informed about the impact of anandamide on gastro-intestinal events without being hindered by the peripheral effects of the drug on glucose clearance. Therefore, it should be considered that two main factors might prevent hyperglycemia after oral glucose loading following anandamide injection: 1) reduction of gastro-intestinal motility and/or 2) inhibition of small intestine glucose transport.

The presence of a functional ECS has been identified in the gut. CB1R are expressed in the gastrointestinal tract of many species (12; 14). Immunohistochemical studies indicate that the enteric nervous system is the main site of CB1R expression and could be the main site of
action for cannabinoids in the gastrointestinal tract (13). Myenteric CB1R are physiologically involved in the regulation of gastric and intestinal motility and may constitute a physiological “brake” along the gastro-intestinal tract in vivo (15; 28; 29). Interestingly, CB1R and CB2R are expressed in the epithelium of human colonic tissue (30) and our molecular analysis also revealed CB1R and CB2R gene expression in gastric and small intestine mucosa.

It is generally accepted that there is a positive relationship between gastrointestinal motility and glucose absorption. Notably, even minor perturbations in gastric emptying may have a substantial impact on postprandial glycemia in healthy subjects and patients with diabetes. Indeed, more rapid gastric emptying results in a greater initial glycemic response (17-19). The reduction of gastric and intestinal motility by cannabinoids has been well documented in both human and animal studies (15; 16) while the consequences on glycemic control are poorly described. In our study, reduction of maximal plasma glucose and delay in xylose appearance is consistent with the strong reduction of gastrointestinal motility induced by anandamide. Available evidence indicates that CBR agonists reduce smooth muscle contractility in different regions of the gastro-intestinal tract in animals and humans (Izzo, 2008 #164). In line with this, distinct effects of anandamide on gastric emptying and small intestine motility could explain why blood glucose appearance was also reduced in duodenal GTT experiments in which the influence of gastric emptying was insubstantial.

Monosaccharides are absorbed through the intestine by a transepithelial transport system. Two types of hexose transporters have been identified in human and murine small intestine: sodium-dependent glucose transporters (SGLT) and sodium-independent glucose transporters (GLUT) (31). When the lumen of the bowel is exposed to high concentrations of glucose, GLUT-2 appears to account for the absorption from the enterocytes after a rapid dynamic
process of translocation to the apical membrane. Recent studies suggest that the presence of GLUT-2 at the apical membrane is dependent on the activity of SGLT-1 (32-34). The effect of cannabinoids on intestinal absorption of 3H-2-deoxy-D-glucose (2-DG) has previously been investigated in Caco-2 cells (35). From this study, authors hypothesized that cannabinoids do not interfere with the intestinal GLUT-2-mediated apical uptake of glucose. In accordance with these previous data, our findings obtained from the everted sac method further indicated that activation of intestinal CBR by anandamide does not alter SGLT-1 nor GLUT-2-mediated glucose transport. Finally, this strongly suggests that improvement of postprandial glycemia induced by anandamide was not due to a direct inhibition of the intestine mucosa capacity to absorb glucose.

GIP release is triggered by the arrival of nutrients in the upper small intestine, where it is produced by enteroendocrine K cells located predominantly in the duodenal epithelium (36) and it is well recognized that exogenous GLP-1 and GIP inhibit glucose absorption by slowing gastric emptying (37; 38) or reducing intestinal motility (39). However, it is very unlikely that incretin variations were responsible for alteration of glucose absorption in our study since GIP and GLP-1 plasma levels were reduced after anandamide injection and blockade of GLP-1 receptors by exendine 9-39 did not modify glycemic profile. Rather, it is very likely that hormone secretion was reduced due to the slow delivery of glucose into the duodenum.

To date, available evidence suggests a role for CB2R in the regulation of gastro-intestinal motility. CB2R has been showed to inhibit defecation in mice (40) and to reduce the gastro-intestinal transit in a model of intestinal inflammation (41; 42). It has also been demonstrated that CB2R were expressed in the stomach and that activation of the receptor with exogenous
agonists reduces gastric motility (43; 44). In the present study, we first observed that CB2R gene expression was significant in the stomach. Then, we showed that part of the effects of anandamide on glycemia during glucose challenge observed in wild type mice persisted in CB1R<sup>j/j</sup> and were partially counteracted by the CB2R antagonist AM630. In addition, the action of anandamide on gastro-intestinal transit was abrogated only by the combined injection of SR141716 and AM630, suggesting that anandamide could reduce transit, and thereby plasma glucose appearance, activating both receptors. Nevertheless, the problem of the respective contribution of CB1R and CB2R in regulating gastric emptying and small intestine motility was not resolved. The case of CB1R<sup>j/j</sup> mice is also of particular interest since these animals exhibited a naturally low transit rate that could be strongly improved by AM630 suggesting the existence of a CB2R-mediated inhibitory tone. However, in these conditions, AM630 could also have exerted a CB2R inverse agonist effect as already described in CHO cells (45). Finally, our data suggested that the regulation of gastro-intestinal motility by CB2R deserves to be further studied especially in pathological conditions since an enhanced expression of these receptors has been reported during obesity and endotoxic inflammation (30).

In our study, the inhibitory effect of anandamide on glucose appearance was amplified in DIO mice and associated with high mRNA CB1R expression in the intestine of these animals. Nevertheless, recent data revealed that obesity was associated with a decrease in anandamide content in the stomach of obese mice and the intestine of zucker rats (15; 46) suggesting that ECS activity is downregulated in the gastro-intestinal tract of obese animals (47) contrary to other peripheral organs (5; 48). In line with this, we also showed that SR141716 administered alone to DIO mice before OXT did not modify xylose appearance while it was strongly inhibited with exogenous anandamide. This suggests that even though CB1R expression was
increased, it is very unlikely that CB1R were tonically activated by endocannabinoids in DIO mice.

In summary, our findings demonstrated that acute activation of intestinal ECS reduced postprandial glycemia. Reduction of plasma glucose appearance was not due to an alteration of intestinal glucose transport but rather to the inhibition of gastro-intestinal transit consecutive both to CB1R and CB2R activation. Identifying precisely the respective role of CB1R and CB2R in each part of the gastro-intestinal tract could help to develop new pharmacological tools to improve postprandial glycemia, an important component of the overall glycemic burden (20; 21). Further studies are also necessary to determine whether the endocannabinoid tone is modified in the gastro-intestinal tract of DIO mice and the consequences on postprandial intestinal glucose delivery.

Acknowledgments

SF, LD, JG, and PD designed, performed and analysed the experiments and wrote the manuscript; TM performed some experiments; BV analysed the data and reviewed the written draft. This work was supported by funds from the Regional Council of Burgundy and European Union (FEDER). Dr. Pascal Degrace is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors declare no conflicts of interest.
REFERENCES


FIGURE LEGEND

**Figure 1.** Anandamide reduces hyperglycemia induced by oral glucose loading. Mice were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg) or vehicle. Blood samples were collected from the tail tip vein at the indicated time points after glucose loading. Data represent % variation from basal glycemia during OGTT expressed as means ± SEM (n=20) and corresponding AUC<sub>0-2h</sub> calculations. Glycemia values expressed as mg/dl are presented in Supplementary figure 1. *P<0.05 vs corresponding value in vehicle-treated mice.

**Figure 2.** Effect of anandamide on blood glucose clearance and insulin sensitivity. (A) i.p. glucose tolerance test (i.p.GTT, 2 g/kg) and corresponding AUC<sub>0-2h</sub> calculations. (B) Insulin tolerance test (ITT, 0.5 UI/kg). (C) Fasting glycemia monitoring. For all experiments, blood samples were collected from the tail tip vein of wild-type mice at the indicated time points, 10 min after an i.p. administration of anandamide (10 mg/kg) or vehicle. Results are expressed as means ± SEM (n=10). *P<0.05 vs corresponding value in vehicle-treated mice.

**Figure 3.** Effect of anandamide on intestinal glucose transport. (A) Mice were subject to oral xylose loading test (OXT, 1 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg), or vehicle. A series of mice received an oral dose of phlorizin (200 mg/kg) 15 min before OXT. Plasma xylose appearance was determined in blood samples collected from the tail tip vein at the indicated time points after xylose loading. Values are expressed as means ± SEM (n=6 per group). *P<0.05 anandamide vs corresponding value in vehicle-treated mice. "P<0.05 phlorizin vs corresponding value in vehicle-treated mice. (B) SGLT-1-mediated glucose transport measured in rat everted jejunal fragments incubated with 0.1 µM 14C-D-glucose in the presence of anandamide (5 µM) or vehicle. In some experiments, phlorizin (0.2
mM) was added into the medium to inhibit SGLT-1 transport. (C) GLUT-2-mediated glucose transport measured in rat everted jejunal fragments incubated with 30 mM $^{14}$C-D-glucose in the presence of anandamide (5 µM) or vehicle. In some experiments, phlorizin (0.2 mM) and phloretin (1 mM) were added into the medium to inhibit both SGLT-1 and GLUT-2 transport. For (B) and (C) Values are pico or nano moles glucose transported per mg of tissue expressed as means ± SEM (n=5 per group). *P<0.05 inhibitors vs corresponding value in vehicle-treated segments.

**Figure 4.** Effect of anandamide on gastrointestinal motility and on glycemia after duodenal glucose loading. (A) Wild-type mice received by gavage 0.2 ml of a non-metabolisable solution of charcoal 10 min after a single i.p. administration of anandamide (10 mg/kg) or vehicle. Mice were sacrificed 25 min later and distance from the pylorus to the front of the charcoal bolus was measured. Transit rate corresponds to [(distance to charcoal front)/(total length from pylorus to caecum)] × 100 (%) expressed as means ± SEM (n=6 per group). *P<0.05 anandamide vs vehicle-treated mice. (B) Wild-type mice were subject to intraduodenal glucose tolerance test (DGTT, 1 g/kg) after one or two consecutive i.p. injections of anandamide (10 mg/kg) or vehicle, the first 10 min before the beginning of DGTT and the second at t = 15 min (anandamide x 2) after glucose infusion. Results represent % variation from basal glycemia during glucose challenge and corresponding AUC$_{0-2h}$ calculations. (C) CB1R$^{-/-}$ mice were subject to DGTT (1 g/kg) 10 min after a single i.p. administration of anandamide (10 mg/kg) or vehicle. For experiments B and C, blood samples were collected from the tail tip vein at the indicated time points. Values are expressed as means ± SEM (n=7 per group). *P<0.05 anandamide and #P<0.05 anandamide x 2 vs corresponding value in vehicle-treated mice.
Figure 5. Effect of anandamide on plasma glucose appearance during OGTT is not mediated by incretins. (A) Mice were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg), or vehicle. Blood samples were collected from the tail tip vein at the indicated time points for GIP, GLP-1 and insulin assay. Values are expressed as means ± SEM (n=6). *P<0.05 anandamide vs corresponding value in vehicle-treated mice. (B) Mice were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg), exendin 9-39 (ex 9-39; 170 µg/kg), ex 9-39 in combination with anandamide, or vehicle. Results represent % variation from basal glycemia during OGTT. Values are expressed as means ± SEM (n=10). *P<0.05 ex 9-39 vs vehicle ; †P<0.05 anandamide and anandamide + ex 9-39 vs vehicle.

Figure 6. Role of CB1R and CB2R in anandamide-induced reduction in gastro-intestinal transit and plasma glucose appearance. (A) Wild-type mice were injected i.p. (10 mg/kg) with CB1R antagonists SR141716 and AM65456 or CB2R antagonist AM630, 20 min before a single i.p. administration of anandamide (10 mg/kg). Animals received 10 min later by gavage, 0.2 ml of a non-metabolisable solution of charcoal. Mice were sacrificed 25 min after gavage and distance from the pylorus to the front of the charcoal bolus was measured. Transit rate corresponds to [(distance to charcoal front)/(total length from pylorus to caecum)] × 100 (%) expressed as means ± SEM (n=6 per group). *P<0.05 vs vehicle, †P<0.05 vs anandamide, †† P<0.05 vs other treatments (B) Mice were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide alone (10 mg/kg), anandamide in combination with the specific CB2R antagonist AM630 (10 mg/kg) or vehicle. Blood samples were collected from the tail tip vein at the indicated time points after glucose loading. Results represent % variation from basal glycemia during OGTT and are expressed as means ± SEM (n=6). *P<0.05 anandamide vs vehicle and †P<0.05 anandamide+AM630 vs vehicle. (C) RT-
PCR analysis of stomach CB1R and CB2R mRNA expression in control mice. Results are expressed as means ± SEM (n=6). *P<0.05.

Figure 7. Effect of anandamide on plasma glucose appearance and gastro-intestinal motility in CB1R−/− mice. (A) CB1R−/− mice were injected i.p. with the specific CB2R antagonist AM630 (10 mg/kg) and/or with anandamide (10 mg/kg) respectively 20 min and 10 min before they were subject to an oral glucose tolerance test (OGTT, 2 g/kg). Blood samples were collected from the tail tip vein at the indicated time points after glucose loading. Results represent % variation from basal glycemia during OGTT. *P<0.05 anandamide vs vehicle and #P<0.05 anandamide+AM630 vs vehicle (n = 10 per group). (B) CB1R−/− mice were subject to oral xylose loading test (OXT, 1 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg) or vehicle. Anandamide effect on xylose absorption in wild-type mice was reported on the graph (dotted line). For all experiments, values are expressed as means ± SEM (n=10 per group). *P<0.05 anandamide vs vehicle-treated CB1R−/− mice and #P<0.05 anandamide-treated wild-type mice vs corresponding value in anandamide-treated CB1R−/−. (C) CB1R−/− mice received by gavage 0.2 ml of a non-metabolisable solution of charcoal 10 min after i.p. administration of anandamide (10 mg/kg) or 30 min after i.p. administration of AM630 alone. Mice were sacrificed 25 min later and distance from the pylorus to the front of the charcoal bolus was measured. Transit rate corresponds to [(distance to charcoal front)/(total length from pylorus to caecum)] × 100 (%) expressed as means ± SEM (n=6 per group). *P<0.05 AM630 vs vehicle-treated mice.
**Figure 8.** Effect of anandamide on plasma glucose and xylose appearance after oral loading in obese mice (DIO). (A) Obese mice (n=10) were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg) or vehicle. Results represent % variation from basal glycemia during OGTT. *P<0.05 anandamide vs vehicle. (B) Obese mice (n=7) were subjected to oral xylose loading test (OXT, 1 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg), or SR141716 (10 mg/kg) or vehicle. *P<0.05 anandamide vs vehicle and #P<0.05 SR141716 vs vehicle. (C) Comparison of anandamide effect on plasma xylose appearance during OXT in obese vs lean mice *P<0.05 DIO vs lean mice. For all experiments, blood samples were collected from the tail tip vein at the indicated time points after glucose or xylose loading. (D) RT-PCR analysis of small intestine CB1R mRNA expression in DIO vs lean mice (n=6 per group). *P<0.05 DIO vs lean mice.

**Supplementary figure 1.** Anandamide reduces hyperglycemia induced by oral glucose loading. Mice were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg) or vehicle. Blood samples were collected from the tail tip vein at the indicated time points after glucose loading. Data represent glycemia (mg/dl) during OGTT expressed as means ± SEM (n=20). *P<0.05 vs corresponding value in vehicle-treated mice.

**Supplementary figure 2.** Characterization of glucose intolerance and insulin resistance in obese mice. Obese (DIO) and lean mice (n=10 per group) were subject to (A) oral glucose tolerance test (OGTT, 2 g/kg) and (B) insulin tolerance test (ITT, 0.5 UI/kg). Blood samples were collected from the tail tip vein at the indicated time points. Results represent % variation from basal glycemia expressed as means ± SEM (n=7 per group). *P<0.05 DIO vs lean.
Anandamide reduces hyperglycemia induced by oral glucose loading. Mice were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg) or vehicle. Blood samples were collected from the tail tip vein at the indicated time points after glucose loading. Data represent % variation from basal glycemia during OGTT expressed as means ± SEM (n=20) and corresponding AUC0-2h calculations. Glycemia values expressed as mg/dl are presented in Supplementary figure 1. *P<0.05 vs corresponding value in vehicle-treated mice.
Effect of anandamide on blood glucose clearance and insulin sensitivity. (A) I.p. glucose tolerance test (i.p.GTT, 2 g/kg) and corresponding AUC0-2h calculations. (B) Insulin tolerance test (ITT, 0.5 UI/kg). (C) Fasting glycemia monitoring. For all experiments, blood samples were collected from the tail tip vein of wild-type mice at the indicated time points, 10 min after an i.p. administration of anandamide (10 mg/kg) or vehicle. Results are expressed as means ± SEM (n=10). *P<0.05 vs corresponding value in vehicle-treated mice.
Effect of anandamide on intestinal glucose transport. (A) Mice were subject to oral xylose loading test (OXT, 1 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg), or vehicle. A series of mice received an oral dose of phlorizin (200 mg/kg) 15 min before OXT. Plasma xylose appearance was determined in blood samples collected from the tail tip vein at the indicated time points after xylose loading. Values are expressed as means ± SEM (n=6 per group). *P<0.05 anandamide vs corresponding value in vehicle-treated mice. #P<0.05 phlorizin vs corresponding value in vehicle-treated mice. (B) SGLT1-mediated glucose transport measured in rat everted jejunal fragments incubated with 0.1 µM 14C-D-glucose in the presence of anandamide (5 µM) or vehicle. In some experiments, phlorizin (0.2 mM) was added into the medium to inhibit SGLT-1 transport. (C) GLUT2-mediated glucose transport measured in rat everted jejunal fragments incubated with 30 mM 14C-D-glucose in the presence of anandamide (5 µM) or vehicle. In some experiments, phlorizin (0.2 mM) and phloretin (1 mM) were added into the medium to inhibit both SGLT-1 and GLUT-2 transport. For (B) and (C) Values are pico or nano moles glucose transported per mg of tissue expressed as means ± SEM (n=5 per group). #P<0.05 inhibitors vs corresponding value in vehicle-treated
segments.
352x470mm (300 x 300 DPI)
Effect of anandamide on gastro-intestinal motility and on glycemia after duodenal glucose loading. (A) Wild-type mice received by gavage 0.2 ml of a non-metabolisable solution of charcoal 10 min after a single i.p. administration of anandamide (10 mg/kg) or vehicle. Mice were sacrificed 25 min later and distance from the pylorus to the front of the charcoal bolus was measured. Transit rate corresponds to \([(\text{distance to charcoal front})/(\text{total length from pylorus to caecum})] \times 100\ (%)\) expressed as means ± SEM (n=6 per group). *P<0.05 anandamide vs vehicle-treated mice.

(B) Wild-type mice were subject to intraduodenal glucose tolerance test (DGTT, 1 g/kg) after one or two consecutive i.p. injections of anandamide (10 mg/kg) or vehicle, the first 10 min before the beginning of DGTT and the second at t = 15 min (anandamide x 2) after glucose infusion. Results represent % variation from basal glycemia during glucose challenge and corresponding AUC0-2h calculations. *P<0.05 anandamide vs vehicle-treated mice. #P<0.05 anandamide x 2 vs corresponding value in vehicle-treated mice.

(C) CB1R/- mice were subject to DGTT (1 g/kg) 10 min after a single i.p. administration of anandamide (10 mg/kg) or vehicle. For experiments B and C, blood samples were collected from the tail tip vein at the indicated time points. Values are expressed as means ± SEM (n=7 per group). *P<0.05 anandamide and #P<0.05 anandamide x 2 vs corresponding value in vehicle-treated mice.
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Effect of anandamide on plasma glucose appearance during OGTT is not mediated by incretins. (A) Mice were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg), or vehicle. Blood samples were collected from the tail tip vein at the indicated time points for GIP, GLP-1 and insulin assay. Values are expressed as means ± SEM (n=6). *P<0.05 anandamide vs corresponding value in vehicle-treated mice. (B) Mice were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg), exendin 9-39 (ex 9-39; 170 µg/kg), ex 9-39 in combination with anandamide, or vehicle. Results represent % variation from basal glycemia during OGTT. Values are expressed as means ± SEM (n=10). *P<0.05 ex 9-39 vs vehicle ; #P<0.05 anandamide and anandamide + ex 9-39 vs vehicle.
Role of CB1R and CB2R in anandamide-induced reduction in gastro-intestinal transit and plasma glucose appearance. (A) Wild-type mice were injected i.p. (10 mg/kg) with CB1R antagonists SR141716 and AM65456 or CB2R antagonist AM630, 20 min before a single i.p. administration of anandamide (10 mg/kg). Animals received 10 min later by gavage, 0.2 ml of a non-metabolisable solution of charcoal. Mice were sacrificed 25 min after gavage and distance from the pylorus to the front of the charcoal bolus was measured. Transit rate corresponds to \[\frac{(\text{distance to charcoal front})}{(\text{total length from pylorus to caecum})} \times 100\%\] expressed as means ± SEM (n=6 per group). *P<0.05 vs vehicle, #P<0.05 vs anandamide, † P<0.05 vs other treatments (B) Mice were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide alone (10 mg/kg), anandamide in combination with the specific CB2R antagonist AM630 (10 mg/kg) or vehicle. Blood samples were collected from the tail tip vein at the indicated time points after glucose loading. Results represent % variation from basal glycemia during OGTT and are expressed as means ± SEM (n=6). *P<0.05 anandamide vs vehicle and #P<0.05 anandamide+AM630 vs vehicle. (C) RT-PCR analysis of stomach CB1R and CB2R mRNA expression in control mice. Results are
expressed as means ± SEM (n=6). *P<0.05.
352x470mm (300 x 300 DPI)
Effect of anandamide on plasma glucose appearance and gastro-intestinal motility in CB1R-/- mice. (A) CB1R-/- mice were injected i.p. with the specific CB2R antagonist AM630 (10 mg/kg) and/or with anandamide (10 mg/kg) respectively 20 min and 10 min before they were subject to an oral glucose tolerance test (OGTT, 2 g/kg). Blood samples were collected from the tail tip vein at the indicated time points after glucose loading. Results represent % variation from basal glycemia during OGTT. *P<0.05 anandamide vs vehicle and #P<0.05 anandamide+AM630 vs vehicle (n = 10 per group). (B) CB1R-/- mice were subject to oral xylose loading test (OXT, 1 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg) or vehicle. Anandamide effect on xylose absorption in wild-type mice was reported on the graph (dotted line). For all experiments, values are expressed as means ± SEM (n=10 per group). *P<0.05 anandamide vs vehicle-treated CB1R-/- mice and #P<0.05 anandamide-treated wild-type mice vs corresponding value in anandamide-treated CB1R-/- mice. (C) CB1R-/- mice received by gavage 0.2 ml of a non-metabolisable solution of charcoal 10 min after i.p. administration of anandamide (10 mg/kg) or 30 min after i.p. administration of AM630 alone. Mice were sacrificed 25 min later and distance from the pylorus to
the front of the charcoal bolus was measured. Transit rate corresponds to \([(\text{distance to charcoal front})/(\text{total length from pylorus to caecum})]\) \times 100\% expressed as means ± SEM (n=6 per group). *P<0.05 AM630 vs vehicle-treated mice.
Effect of anandamide on plasma glucose and xylose appearance after oral loading in obese mice (DIO). (A)
Obese mice (n=10) were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p.
administration of anandamide (10 mg/kg) or vehicle. Results represent % variation from basal glycemia
during OGTT. *P<0.05 anandamide vs vehicle. (B) Obese mice (n=7) were subjected to oral xylose loading
test (OXT, 1 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg), or SR141716 (10 mg/kg)
or vehicle. *P<0.05 anandamide vs vehicle and #P<0.05 SR141716 vs vehicle. (C) Comparison of
anandamide effect on plasma xylose appearance during OXT in obese vs lean mice *P<0.05 DIO vs lean
mice. For all experiments, blood samples were collected from the tail tip vein at the indicated time points
after glucose or xylose loading. (D) RT-PCR analysis of small intestine CB1R mRNA expression in DIO vs lean
mice (n=6 per group). *P<0.05 DIO vs lean mice.
Anandamide reduces hyperglycemia induced by oral glucose loading. Mice were subject to oral glucose tolerance test (OGTT, 2 g/kg) 10 min after an i.p. administration of anandamide (10 mg/kg) or vehicle. Blood samples were collected from the tail tip vein at the indicated time points after glucose loading. Data represent glycemia (mg/dl) during OGTT expressed as means ± SEM (n=20). *P<0.05 vs corresponding value in vehicle-treated mice.
Characterization of glucose intolerance and insulin resistance in obese mice. Obese (DIO) and lean mice (n=10 per group) were subject to (A) oral glucose tolerance test (OGTT, 2 g/kg) and (B) insulin tolerance test (ITT, 0.5 UI/kg). Blood samples were collected from the tail tip vein at the indicated time points. Results represent % variation from basal glycemia expressed as means ± SEM (n=7 per group). *P<0.05 DIO vs lean.