Tanya J. Little, Nada Cvijanovic, Nicholas V. DiPatrizio, Donovan A. Argueta, Christopher K. Rayner, X Christine Feinle-Bisset, and Richard L. Young

Plasma endocannabinoid levels in lean, overweight, and obese humans: relationships to intestinal permeability markers, inflammation, and incretin secretion

American Journal of Physiology: Endocrinology and Metabolism, 2018; 315(4):E489-E495

Published version at: http://dx.doi.org/10.1152/ajpendo.00355.2017

Plasma endocannabinoid levels in lean, overweight and obese humans: relationships with intestinal permeability markers, inflammation and incretin secretion

Tanya J. Little[1,3*], Nada Cvijanovic[1,2,3*], Nicholas V. DiPatrizio[5], Donovan A. Argueta[5], Christopher K. Rayner[1,3,4], Christine Feinle-Bisset[1,3], Richard L. Young[1,2,3]

Contributions of authors: TJL, CFB, RY designed research; TJL, NC, NVP, DA, CKR conducted the research, TJL analysed data and prepared manuscript. All authors read, edited and approved the final manuscript.

Affiliations: [1]University of Adelaide School of Medicine; [2]South Australian Health and Medical Research Institute; [3]NHMRC Centre of Research Excellence in Translating Nutritional Science to Good Health, University of Adelaide; [4]Gastroenterology and Hepatology, Royal Adelaide Hospital, Adelaide, Australia; [5]Division of Biomedical Sciences, School of Medicine, University of California, Riverside, Riverside, USA

*Shared first authorship: TJL and NC contributed equally to the work.

Running head: Endocannabinoids, intestinal permeability and incretin secretion

Address for correspondence: Dr Tanya J. Little, Adelaide Medical School, Adelaide Health and Medical Sciences Building, The University of Adelaide, Corner of North Terrace and George Street, Adelaide SA 5005, Australia. Phone: +61 8 8313 2999, E-mail: tanya.little@adelaide.edu.au.

ABSTRACT

Introduction/Aims: Intestinal production of endocannabinoid and oleoylethanolamide (OEA) is impaired in high-fat diet/obese rodents, leading to reduced satiety. Such diets also alter the intestinal microbiome in association with enhanced intestinal permeability and inflammation, however little is known of these effects in humans. This study aimed to: (i) evaluate effects of lipid on plasma anandamide (AEA), 2-arachidonyl-*sn*-glycerol (2-AG) and OEA in humans, and (ii) examine relationships with intestinal permeability, inflammation markers and incretin hormone secretion.

Methods: 20 lean, 18 overweight and 19 obese participants underwent intraduodenal Intralipid® infusion (2 kcal/min) with collection of endoscopic duodenal biopsies and blood. Plasma AEA, 2-AG, and OEA (HPLC/tandem mass spectrometry), tumour necrosis factor-α (TNF-α), glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (multiplex), and duodenal expression of occludin, zona-occludin-1 (ZO-1), intestinal-alkaline-phosphatase (IAP), and toll-like receptor-4 (TLR4) (RT-PCR), were assessed.

Results: Fasting plasma AEA was increased in obese, compared with lean and overweight ($P<0.05$), with no effect of BMI group or ID lipid infusion on plasma 2-AG or OEA. Duodenal expression of IAP and ZO-1 was reduced in obese, compared with lean ($P<0.05$), and these levels related negatively to plasma AEA ($P<0.05$). The iAUC for AEA was positively related to iAUC GIP ($r=0.384$, $P=0.005$).

Conclusions: Obese individuals have increased plasma AEA and decreased duodenal expression of ZO-1 and IAP, in comparison to lean and overweight. The relationships between plasma AEA with duodenal ZO-1 and IAP, and GIP, suggest that altered endocannabinoid signalling may

contribute to changes in intestinal permeability, inflammation and incretin release in human obesity.

Keywords: Intestinal fat sensors, anandamide, 2-arachidonylglycerol, n-acylethanolamines, tight junction proteins, inflammation.

INTRODUCTION

Obesity is associated with chronic low-grade inflammation and metabolic disturbances, including insulin resistance and type 2 diabetes. One of the major drivers of the obesity epidemic is the consumption of an energy-dense high-fat diet, which promotes hyperphagia secondary to attenuation of the gut-brain signalling mechanisms involved in the control of food intake (24). In rodents, high-fat diet-induced hyperphagia is driven by alterations in the composition of the gut microbiota with consequent development of low-grade inflammation in the gastrointestinal tract. This is evidenced by increased expression of toll-like receptor 4 (TLR-4, the receptor for the microbial toxin, lipopolysaccharide (LPS or endotoxin)), in the ileum (11), increased systemic levels of LPS and proinflammatory cytokines (3), and altered intestinal permeability characterised by decreased intestinal expression and localisation of tight junction proteins, including zona-occludin 1 (ZO-1) and occludin (4). Furthermore, the intestinal expression of intestinal alkaline phosphatase (IAP), an enzyme that inactivates LPS, is decreased by a high-fat diet in obesity-prone, but not obesity-resistant, rodents (11). Recent studies have also highlighted an important role of interactions between the gut microbiota and the endocannabinoid system in the regulation of energy homeostasis (29).

The endocannabinoids, 2-arachidonyl-*sn*-glycerol (2-AG) and anandamide (AEA), are lipid-derived mediators that are involved in the control of food intake (1, 13). 2-AG and AEA stimulate food intake by activating cannabinoid-1 (CB_1) receptors (16). In the small intestine of rodents, 2-AG and AEA concentrations increase with fasting (14, 21) and upon oral sensing of dietary fats (13), and both 2-AG and AEA decrease with refeeding (21). Intestinal levels of endocannabinoids are altered in obesity, for example, obese Zucker rats exhibit higher duodenal levels of 2-AG and

AEA than wild-type rats following both food deprivation and refeeding, while in high-fat diet induced obese rats, duodenal 2-AG is higher, but AEA lower, than in control rats fed a standard laboratory diet (21). In Western diet (high-fat, high-sucrose)-induced obese mice, both 2-AG and AEA are increased (1). While studies are inconsistent, human obesity is generally associated with increased concentrations of endocannabinoids in plasma and adipose tissues, and most commonly elevated 2-AG, but not AEA, correlates with BMI and waist circumference (2, 7, 12). While now withdrawn from the market due to adverse psychological effects, the CB_1 receptor antagonist Rimonabant promoted weight loss in obese humans (23). Interest in the endocannabinoid system as a target for obesity therapy has re-emerged since rodent studies demonstrated that obesity-associated changes in the gut microbiota activated the intestinal endocannabinoid system (via LPS) to increase gut permeability, which further enhanced plasma LPS levels and peripheral inflammation (5, 29). Studies examining the relationships between intestinal endocannabinoids, intestinal permeability and inflammation are lacking in humans, although a recent study using human colonic explants from patients with inflammatory bowel disease has reported that cannabidiol and palmitoylethanolamine exert anti-inflammatory effects (8).

Endocannabinoids may also determine the secretion of the incretin hormones, glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). CB_1 receptors are expressed on enteroendocrine K- and L-cells in rodents (19, 28), and activation of the CB_1 receptor by AEA prior to an oral glucose tolerance test inhibits GIP secretion in rodents (28). In contrast, in humans, acute administration of the CB_1 receptor agonist, nabilone, enhances fasting plasma GIP by ~80%, has no effect on glucose-mediated GIP release, but decreases the GLP-1 response to oral glucose

(6). Therefore, while it has not been directly evaluated, changes in incretin levels observed in obesity (35) may arise due to altered endocannabinoid levels.

Endocannabinoid-related molecules, such as the fatty acid-derived n-acylethanolamines, and in particular, OEA, have also been shown to play a role in energy homeostasis, where they reduce energy intake (by prolonging the inter-meal interval and reducing meal frequency) via activation of the nuclear receptor, peroxisome-proliferator-activated receptor-α (PPAR-α) (17, 25, 31). OEA is also an endogenous ligand for the fatty acid sensing receptor, GPR119, which stimulates GLP-1 secretion (22, 30). Whether OEA levels are altered in human obesity is currently unclear.

We recently characterised the effects of intraduodenal lipid infusion on the duodenal expression of fat sensing molecules in lean, overweight and obese humans. We reported that human obesity was associated with altered capacity for the sensing of dietary fat, including decreased duodenal expression of free fatty acid receptors (FFAR) 1 and 4, and increased expression of cluster of differentiation 36 (CD36) (9, 10). Using plasma samples and duodenal biopsies collected in these studies, the aim of the current investigation was to (i) evaluate effects of intraduodenal lipid infusion on plasma levels of AEA, 2-AG and OEA in humans, and (ii) examine relationships between plasma concentrations of endocannabinoid related molecules with BMI, intestinal permeability and inflammation markers, and incretin hormone secretion.

MATERIALS AND METHODS

Participants

20 lean (10 males, body mass index (BMI: 22±0.5 kg/m^2, age: 28±2 years), 18 overweight (12 males, BMI: 27±0.3 kg/m^2, age: 32±3 years) and 19 obese (12 males, BMI: 35±1 kg/m^2, age: 30±2 years) participants were enrolled in the study. Participant demographics have been published previously (9, 10). In brief, all participants were weight-stable for at least 3 months prior to inclusion in the study and reported no gastrointestinal (GI) symptoms, had no prior GI surgery, did not take medications or supplements known to affect GI motility or appetite (including fish oil), consumed <20 g of alcohol per week, and were non-smokers. The study protocol was approved by the Royal Adelaide Hospital Research Ethics Committee and carried out in accordance with the Declaration of Helsinki. Each participant provided written informed consent prior to inclusion in the study. The trial was registered with the Australia and New Zealand Clinical Trial Registry (www.anzctr.org.au trial number: ACTRN12612000376842).

Study design and protocol

Part A: Endoscopy and collection of duodenal mucosal biopsies

Participants attended the Department of Gastroenterology and Hepatology, Royal Adelaide Hospital, at 0830 h following a standardized evening meal (400 g beef lasagne (2479 kJ, 20 g fat, 20 g protein, 80 g carbohydrate), McCain Foods, Wendouree, Victoria, Australia) and an overnight fast of 12 h from solids and 10 h from liquids. The protocol for endoscopic collection of mucosal biopsies has been described previously (9). Briefly, a small diameter video endoscope was passed through the nose into the second part of the duodenum and once positioned, 2 duodenal biopsies were collected using standard endoscopic biopsy forceps and placed immediately in Allprotect®

Tissue Reagent (Qiagen, Australia). An intravenous cannula was inserted into a forearm vein and a baseline blood sample (10 ml) was collected (t = 0 min). Following this, an intraduodenal infusion of 10% Intralipid® (Fresenius Kabi AB, China; 2 kcal/min, 109 ml/hr) commenced via the endoscope infusion channel for 30 min. Blood samples, for analysis of plasma TNF-α concentrations, were collected at t = 10, 20 and 30 min, and placed in ice-chilled EDTA-treated and serum tubes, as described previously (9). After the infusion (t = 30 min), two additional duodenal biopsies were collected.

Part B: Effect of intraduodenal lipid infusion on plasma concentrations of endocannabinoids and incretins

Participants attended the Discipline of Medicine at 0830 h following a standardized evening meal and overnight fast, as described above. Anesthetic spray and gel was administered into the nasal cavity (as above) prior to insertion of a small-diameter (3.5 mm) catheter (Dentsleeve International, Mui Scientific), which was allowed to pass via peristalsis through the pylorus into the second part of the duodenum. Accurate positioning of the catheter across the pylorus was achieved by monitoring the transmucosal potential difference using a monitoring electrode (Red Dot, 3M Healthcare) placed on the forearm as a reference (20). Once positioned, an intravenous cannula was inserted into a forearm vein and a baseline blood sample (10 ml) was collected (t = 0 min). Intraduodenal infusion of 10% Intralipid® then commenced at a rate of 2 kcal/min for 120 min (t = 0 - 120 min), during which blood samples were collected every 15 min and placed in ice-chilled EDTA-treated and serum tubes, as described previously (9).

Measurements

Analysis of AEA, OEA and 2-AG levels in plasma

Plasma samples collected at t = 0, 30, 60 and 120 min were analysed. Lipid extraction and analysis were performed as previously described (1). Plasma (0.5 mL) was added to 1.0 mL of methanol solution containing the internal standards, [$^2H_5$]- 2-AG, [$^2H_4$]-AEA, and [$^2H_4$]-OEA (Cayman Chemical, Ann Arbor, MI, USA). Lipids were extracted with chloroform (2 mL) and washed with 0.9 % saline (0.5 mL). Organic phases were collected and separated by open-bed silica gel column chromatography. The eluate was gently dried under a N_2 stream (99.998% pure) and resuspended in 0.1 mL of methanol:chloroform (9:1), with 1 µL injected for ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) analysis.

Data were collected using an Acquity I Class UPLC system coupled to a Xevo TQ-S Mass Spectrometer (Waters, Milford, MA, USA) with accompanying electrospray ionization (ESI) interface. Lipids were separated on an Acquity UPLC BEH C_{18} column (2.1 x 50 mm i.d., 1.7µm, Waters) with inline Acquity guard column (UPLC BEH C_{18} VanGuard Pre-column; 2.1 x 5 mm i.d., 1.7µm, Waters), and eluted by a gradient of methanol in water (0.25% acetic acid, 5mM ammonium acetate) according to the following gradient at a flow rate of 0.4 mL per min: 80% methanol 0.5 min, 80% - 100% methanol 0.5-2.5 min, 100% methanol 2.5-3 min, 100% - 80% methanol 3-3.1 min). Column temperature was maintained at 40 °C, and samples were maintained in the sample manager at 10 °C. Argon (99.998%) was used as collision gas. MS detection was in positive ion mode and capillary voltage set at 0.1 kV. Cone voltage and collision energy as follows, respectively: 2-AG = 30v, 12v; [$^2H_5$] 2-= 25v, 44v; AEA = 30v, 14v; [$^2H_4$] AEA = 26v, 16v; OEA = 28v, 16v; [$^2H_4$]- OEA = 48v. 14v. Lipids were quantified using a stable isotope dilution method

detecting protonated adducts of the molecular ions $[M+H]^+$ in the multiple reaction monitoring (MRM) mode. Acyl migration from 2-arachidonylglycerol to 1-arachidonylglycerol is known to occur (32), thus all reported values for 2-AG represent the sum of 2-AG and 1-AG. Tissue processing and LCMS analysis from an individual experiment occurred independently of other experiments. Extracted ion chromatograms were used to quantify 2-AG (*m/z* = 379.3>287.3), AEA (*m/z* = 348.3>62.0), and OEA (*m/z* = 326.4 > 62.1), and $[^2H_5]$2-AG (*m/z* = 384.3 > 93.4), $[^2H_4]$-AEA (*m/z* = 352.3>66.1), and $[^2H_4]$-OEA (*m/z* = 330.4 > 66.0), which were used as internal standards.

RNA extraction

Frozen duodenal biopsies were disrupted using a bead-based tissue homogeniser (TissueLyser LT, Qiagen) and homogenised through Qiashredder columns (Qiagen). Total cellular RNA was isolated using the PureLink™ MicroKit (Invitrogen, Thermo Fisher Scientific), which included an on-column DNase digestion, as per manufacturer's instructions. RNA quantity was determined using a Nanodrop™ Lite Spectrophotometer (Thermo Fisher Scientific) and purity assessed using A_{260}/A_{280} ratio.

Quantification of duodenal gene expression by relative RT-PCR

Real-time RT-PCR was performed using the 7500 fast Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific). Taqman® primers (Life Technologies, Thermo Fisher Scientific) were used to determine the expression of TLR4 (Hs00152939_m1), IAP (alkaline phosphatase-1) (ALP1, Hs00357579_g1), occludin (OCLN, Hs00170162_m1), and ZO-1 (tight junction protein-1) (TJP1, Hs01551861_m1) relative to expression of the housekeeper beta-2

microglobulin (β2M) (Hs00984230_m1). All targets were assessed in triplicate, according to manufacturer's instructions.

Plasma tumour necrosis factor-α (TNF-α) and incretin hormone concentrations

Multiplex assays were used to determine plasma TNF-α concentrations (pg/mL) (t = 0, 10, 20, 30 min) (Milliplex® $_{MAP}$ Human Cytokine/Chemokine Magnetic Bead Panel, HCYTOMAG-60K), and total GLP-1 and GIP concentrations (both pg/ml) (t = 0, 15, 30, 60, 90, 120 min) (Milliplex MAP Human Metabolic Hormone Magnetic Bead Panel, HMHEMAG-34K; Millipore Corporation, Temecula, CA, USA) using the Bio-plex® MAGPIX™ Multiplex Reader (Luminex®, Millipore Corporation) and xPONENT® software (Luminex®, Millipore Corporation, version 4.2) according to manufacturer's instructions. There was negligible antibody cross-reactivity. Intra-assay coefficients of variation (CVs) were ≤ 10%, and inter-assay CVs were ≤ 15% for all analytes. The minimum detection limits were for TNF-α: 0.7 pg/mL**,** GLP-1: 2.5 pg/ml, GIP: 0.6 pg/ml.

Data and statistical analyses

Statistical analysis was performed using SPSS® software (SPSS Inc, IBM®, version 24), and all graphs were generated using GraphPad Prism 7 (GraphPad Software Inc). One-way ANOVA with post-hoc Bonferroni testing was used to compare values for all variables across BMI group (i.e. lean, overweight, obese) and time. Post-hoc paired comparisons, corrected for multiple comparisons using Bonferroni's correction, were performed if ANOVAs revealed significant effects. Relationships between variables were determined by correlation, with Pearson's r values presented. Incretin hormone, AEA, 2-AG and OEA data were expressed as incremental area under the curve (iAUC) (calculated using the trapezoidal rule from t = 0 min to 120 min (or t = 0 – 30

min for TNF-α)). This iAUC value was divided by the time of last measurement to obtain a final weighted average (AUC) to account for rare times when samples could not be collected (e.g. bathroom breaks) or data could not be obtained from the assays (10). Data are expressed as mean ± standard error of the mean (SEM), with statistical significance accepted at $P < 0.05$.

RESULTS

Plasma 2-AG, AEA and OEA concentrations

There was no significant difference between BMI groups, or effect of lipid infusion, on plasma 2-AG concentrations (**Figure 1A**). There was a significant effect of BMI group (P=0.003), but not lipid infusion, on plasma AEA concentrations (**Figure 1B**). Plasma AEA was higher in obese compared with both lean (P=0.005) and overweight (P=0.017), with no difference between lean and overweight groups. There was no significant difference between BMI groups, or effect of lipid infusion, on plasma OEA concentrations (**Figure 1C**).

Duodenal expression of TLR4, IAP, occludin, and ZO-1

Baseline duodenal expression of IAP (P=0.018) and ZO-1 (P=0.002) was significantly different across BMI groups, with a trend for difference evident for TLR-4 (P=0.08) and occludin (P=0.08) (**Figure 2**). Baseline IAP expression was significantly lower in obese compared with lean (P<0.05), with no difference between lean and overweight, or obese and overweight groups. Baseline ZO-1 expression was significantly lower in obese compared with lean (P=0.002), and in overweight compared with lean (P=0.029), with no difference between overweight and obese groups. There was no effect of intraduodenal lipid on duodenal expression of TLR-4, IAP, occludin or ZO-1 (data not shown).

Plasma TNF-α concentrations

There was no significant difference between BMI groups (P=0.7), and no effect of lipid infusion (P=0.2) on plasma concentrations of TNF-α (**Figure 3**).

Plasma incretin hormone concentrations

Data are available in the publication from Cvijanovic et al (10).

Relationships between variables

Plasma AEA concentrations at baseline were positively related to BMI (r=0.34, P=0.014). Baseline expression of IAP (r=-0.329, P=0.014), occludin (r=-0.28, P=0.038) and ZO-1 (r=-0.395, P=0.003) was negatively related to BMI. Intralipid-evoked AEA concentrations (plasma iAUC) were negatively related to baseline TLR4 (r=-0.283, P=0.04), IAP (r=-0.305, P=0.032), occludin (r=-0.398, P=0.001) and ZO-1 expression (r=-0.445, P=0.001). Plasma TNF-α concentrations at baseline related positively to expression of IAP (r=0.401, P=0.002) and ZO-1 at baseline (r=0.256, P=0.05). Finally, Intralipid-evoked AEA was positively related to evoked GIP (iAUC, r=0.384, P=0.005), but not to evoked GLP-1.

DISCUSSION

We have demonstrated that fasting plasma levels of the endocannabinoid, AEA, but not 2-AG or the endocannabinoid-related OEA, were higher in obese, when compared with lean and overweight individuals. Obese individuals also had lower duodenal expression of ZO-1 and IAP, and a trend towards lower duodenal occludin and TLR4 expression at baseline. These changes were negatively related to plasma AEA concentrations, suggesting that altered endocannabinoid signalling may contribute to changes in intestinal permeability and inflammation in human obesity. Plasma AEA levels were also positively related to plasma GIP, adding support that the endocannabinoid system plays a significant role in mediating GIP secretion in humans.

Our finding of increased plasma AEA in obesity is consistent with reports in animals and humans that document increased peripheral endocannabinoid levels in obesity (1, 12, 21, 26). While many studies report strong correlations between plasma 2-AG with body fat (2), we were not able to replicate these findings. Although mean levels of 2-AG were higher in obese, when compared with both lean and overweight individuals, the values were far more variable than those for AEA, and for this reason we believe our study may have been underpowered to detect such a correlation. Rodent studies indicate that changes in peripheral endocannabinoid concentrations are induced by a "Western" style high-fat diet (1), and that elevated endocannabinoid levels in plasma and jejunal mucosa act via peripheral CB_1 receptors to promote hyperphagia (1). Mammals have an innate preference for dietary fats, and in sham fed rats, where food is tasted but does not enter the small intestine, corn oil, or emulsions containing oleic or linoleic acid, rapidly and markedly increase jejunal levels of 2-AG and AEA (13, 15). Moreover, CB_1 receptor blockade abolishes effects of oral fat on sham feeding and preference for fat (15). Accordingly, changes in peripheral

endocannabinoid signalling in obesity may be important mediators of both fat preference and gut-to-brain signalling mechanisms involved in the regulation of energy intake. Acute intraduodenal infusion of lipid had no effect on plasma endocannabinoid levels in the current study. It is possible that the duration and/or load of lipid exposure was insufficient, or that oral stimulation of fatty-acid sensing receptors (13), such as CD36 and GPR40 and 120, is required to elicit changes in peripheral endocannabinoid levels, and further studies are needed to address this question in humans.

Duodenal expression of IAP and the tight-junction protein ZO-1 was reduced in obese compared to lean individuals, and there was a trend for reduced expression of TLR4 and occludin. These molecules are important markers of increased intestinal permeability and inflammation, that have been shown in animal studies to be altered secondary to high-fat diet and obesity-induced changes in the intestinal microbiome (11), associated with increased systemic levels of LPS, termed "metabolic endotoxemia" (2, 3). These data suggest that the observed changes in duodenal expression of IAP and ZO-1 in obese, compared to lean, are surrogate markers of an altered intestinal microbiome and enhanced intestinal permeability, however, this requires further direct investigation. Furthermore, the potential role of dietary fat intake remains to be elucidated. In humans, acute ingestion of fat has been associated with an increase in circulating LPS (18), and increased intestinal permeability (assessed by lactulose/mannitol tests) in obese individuals (33). Not all studies show changes in intestinal permeability, despite evidence of increased local and systemic inflammation in obese individuals (34), which may reflect the relative insensitivity of the intestinal permeability tests based on detection of urinary markers in humans. In the present study, we identified a relationship between the duodenal expression of IAP and ZO-1 with plasma levels

of the pro-inflammatory cytokine, TNF-α, suggesting an important role for these molecules in mediating the chronic low-grade inflammation associated with obesity.

We also found that lipid-evoked plasma AEA levels were negatively related to baseline duodenal expression of TLR4, IAP, occludin and ZO-1. Rodent studies have provided evidence that the obesity-associated changes in the gut microbiota leads to activation of the endocannabinoid system (via LPS). Administration of the CB_1 receptor agonist, HU-210, to wild-type mice increased intestinal permeability, and decreased ZO-1 and occludin expression *in vitro*, in Caco-2 cells (29). Conversely, in obese mice, pharmacological blockade of the CB_1 receptor restored the distribution and localisation of the tight-junction proteins ZO-1 and occludin, and decreased plasma LPS levels (5, 29). Accordingly, in human obesity, altered plasma AEA may be an important mediator of the effects of obesity on intestinal permeability and inflammation. In rodents, alterations in the composition of the gut microbiota with consequent development of low-grade inflammation in the gastrointestinal tract, have been shown to be responsible for the development of hyperphagia and obesity (11). Further studies in humans are now required to understand the relationships between these changes in endocannabinoid system activity, intestinal permeability and food intake.

Plasma AEA concentrations related positively to plasma GIP, but not GLP-1, concentrations. Recent studies have suggested a role of the endocannabinoid system in mediating the secretion of incretin hormones (6). As discussed, CB_1 receptors are expressed on enteroendocrine K- and L-cells in rodents (19, 28), and AEA inhibits the GIP response to oral glucose in rodents (28). In contrast, our data, and a recent study using acute administration of the CB_1 receptor agonist, nabilone (6), provide evidence that AEA enhances GIP secretion in humans. The reasons for these

discrepancies are unclear, however, the endocannabinoid system is likely to play an influential role in mediating the GIP response to fat in humans.

Plasma OEA concentrations did not differ between lean, overweight and obese humans during fasting, and did not change in response to duodenal fat infusion. In rodents, the presence of fat in the duodenum stimulates the small intestinal conversion of oleic acid, found commonly in the diet, to OEA (31). When administered exogenously to rodents, OEA reduces overall energy intake by prolonging the time interval between meals (31). Our infused lipid emulsion comprised ~19-30% oleic acid, which may have been insufficient to stimulate OEA mobilisation in the current study. Previous studies in humans have demonstrated a significant increase in plasma OEA concentrations in proportion to the oleic content of the ingested oil (30 g), which was associated with reduced subsequent energy intake (27).

In conclusion, we have demonstrated increased plasma levels of AEA, but not 2-AG or OEA, in obese, compared to lean and overweight individuals. Obese individuals also displayed decreased fasting duodenal expression of ZO-1 and IAP. Plasma AEA concentrations were negatively related to duodenal expression of ZO-1 and IAP, and positively related to plasma GIP, suggesting that altered endocannabinoid signalling may contribute to changes in intestinal permeability, inflammation and GIP secretion in human obesity.

ACKNOWLEDGEMENTS

We thank the participants who volunteered their time for this study, and the nursing staff of the Department of Gastroenterology and Hepatology at the Royal Adelaide Hospital for assisting with endoscopy. We would like to acknowledge biostatistician, Kylie Lange, for statistical support, and Nicole Isaacs and Penelope Fitzgerald for their assistance with participant recruitment and infusion studies, and Jaspreet Kaur for technical assistance with lipid analysis.

GRANTS

TJL was supported by an NHMRC Career Development Fellowship (grant 1022706), CFB by an NHMRC Senior Research Fellowship (grant 1103020, 2016-21). The study was supported by a Royal Adelaide Hospital Research Committee Clinical Project Grant (to TJL, 2015-16), and National Institutes of Health Grant DA034009 to NVD.

DISCLOSURES

None of the authors have any conflicts of interest, financial or otherwise.

REFERENCES

1. **Argueta DA, and DiPatrizio NV**. Peripheral endocannabinoid signaling controls hyperphagia in western diet-induced obesity. *Physiology & behavior* 171: 32-39, 2017.
2. **Bluher M, Engeli S, Kloting N, Berndt J, Fasshauer M, Batkai S, Pacher P, Schon MR, Jordan J, and Stumvoll M**. Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. *Diabetes* 55: 3053-3060, 2006.
3. **Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, and Burcelin R**. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56: 1761-1772, 2007.
4. **Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, and Burcelin R**. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 57: 1470-1481, 2008.
5. **Cani PD, Plovier H, Van Hul M, Geurts L, Delzenne NM, Druart C, and Everard A**. Endocannabinoids--at the crossroads between the gut microbiota and host metabolism. *Nature reviews Endocrinology* 12: 133-143, 2016.
6. **Chia CW, Carlson OD, Liu DD, Gonzalez-Mariscal I, Santa-Cruz Calvo S, and Egan JM**. Incretin Secretion in Humans is under the Influence of Cannabinoid Receptors. *American journal of physiology Endocrinology and metabolism* ajpendo 00080 02017, 2017.
7. **Cote M, Matias I, Lemieux I, Petrosino S, Almeras N, Despres JP, and Di Marzo V**. Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. *Int J Obes (Lond)* 31: 692-699, 2007.
8. **Couch DG, Tasker C, Theophilidou E, Lund JN, and O'Sullivan SE**. Cannabidiol and palmitoylethanolamide are anti-inflammatory in the acutely inflamed human colon. *Clinical science* 131: 2611-2626, 2017.
9. **Cvijanovic N, Isaacs NJ, Rayner CK, Feinle-Bisset C, Young RL, and Little TJ**. Duodenal fatty acid sensor and transporter expression following acute fat exposure in healthy lean humans. *Clinical nutrition* 36: 564-569, 2017.
10. **Cvijanovic N, Isaacs NJ, Rayner CK, Feinle-Bisset C, Young RL, and Little TJ**. Lipid stimulation of fatty acid sensors in the human duodenum: relationship with gastrointestinal hormones, BMI and diet. *Int J Obes (Lond)* 41: 233-239, 2017.
11. **de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, and Raybould HE**. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. *American journal of physiology Gastrointestinal and liver physiology* 299: G440-448, 2010.
12. **Di Marzo V**. The endocannabinoid system in obesity and type 2 diabetes. *Diabetologia* 51: 1356-1367, 2008.
13. **DiPatrizio NV, Astarita G, Schwartz G, Li X, and Piomelli D**. Endocannabinoid signal in the gut controls dietary fat intake. *Proceedings of the National Academy of Sciences of the United States of America* 108: 12904-12908, 2011.
14. **DiPatrizio NV, Igarashi M, Narayanaswami V, Murray C, Gancayco J, Russell A, Jung KM, and Piomelli D**. Fasting stimulates 2-AG biosynthesis in the small intestine: role of cholinergic pathways. *American journal of physiology Regulatory, integrative and comparative physiology* 309: R805-813, 2015.
15. **DiPatrizio NV, Joslin A, Jung KM, and Piomelli D**. Endocannabinoid signaling in the gut mediates preference for dietary unsaturated fats. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 27: 2513-2520, 2013.
16. **DiPatrizio NV, and Piomelli D**. Intestinal lipid-derived signals that sense dietary fat. *The Journal of clinical investigation* 125: 891-898, 2015.

17. **Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G, and Piomelli D**. Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature* 425: 90-93, 2003.
18. **Ghanim H, Abuaysheh S, Sia CL, Korzeniewski K, Chaudhuri A, Fernandez-Real JM, and Dandona P**. Increase in plasma endotoxin concentrations and the expression of Toll-like receptors and suppressor of cytokine signaling-3 in mononuclear cells after a high-fat, high-carbohydrate meal: implications for insulin resistance. *Diabetes care* 32: 2281-2287, 2009.
19. **Gonzalez-Mariscal I, Krzysik-Walker SM, Kim W, Rouse M, and Egan JM**. Blockade of cannabinoid 1 receptor improves GLP-1R mediated insulin secretion in mice. *Molecular and cellular endocrinology* 423: 1-10, 2016.
20. **Heddle R, Collins PJ, Dent J, Horowitz M, Read NW, Chatterton B, and Houghton LA**. Motor mechanisms associated with slowing of the gastric emptying of a solid meal by an intraduodenal lipid infusion. *J Gastroen Hepatol* 4: 437-447, 1989.
21. **Izzo AA, Piscitelli F, Capasso R, Aviello G, Romano B, Borrelli F, Petrosino S, and Di Marzo V**. Peripheral endocannabinoid dysregulation in obesity: relation to intestinal motility and energy processing induced by food deprivation and re-feeding. *British journal of pharmacology* 158: 451-461, 2009.
22. **Lauffer LM, Iakoubov R, and Brubaker PL**. GPR119 is essential for oleoylethanolamide-induced glucagon-like peptide-1 secretion from the intestinal enteroendocrine L-cell. *Diabetes* 58: 1058-1066, 2009.
23. **Leite CE, Mocelin CA, Petersen GO, Leal MB, and Thiesen FV**. Rimonabant: an antagonist drug of the endocannabinoid system for the treatment of obesity. *Pharmacol Rep* 61: 217-224, 2009.
24. **Little TJ, and Feinle-Bisset C**. Oral and gastrointestinal sensing of dietary fat and appetite regulation in humans: modification by diet and obesity. *Frontiers in neuroscience* 4: 178, 2010.
25. **Lobo MV, Huerta L, Ruiz-Velasco N, Teixeiro E, de la Cueva P, Celdran A, Martin-Hidalgo A, Vega MA, and Bragado R**. Localization of the lipid receptors CD36 and CLA-1/SR-BI in the human gastrointestinal tract: towards the identification of receptors mediating the intestinal absorption of dietary lipids. *J Histochem Cytochem* 49: 1253-1260, 2001.
26. **Matias I, Petrosino S, Racioppi A, Capasso R, Izzo AA, and Di Marzo V**. Dysregulation of peripheral endocannabinoid levels in hyperglycernia and obesity: Effect of high fat diets. *Molecular and cellular endocrinology* 286: S66-S78, 2008.
27. **Mennella I, Savarese M, Ferracane R, Sacchi R, and Vitaglione P**. Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy intake in humans. *Food & function* 6: 204-210, 2015.
28. **Moss CE, Marsh WJ, Parker HE, Ogunnowo-Bada E, Riches CH, Habib AM, Evans ML, Gribble FM, and Reimann F**. Somatostatin receptor 5 and cannabinoid receptor 1 activation inhibit secretion of glucose-dependent insulinotropic polypeptide from intestinal K cells in rodents. *Diabetologia* 55: 3094-3103, 2012.
29. **Muccioli GG, Naslain D, Backhed F, Reigstad CS, Lambert DM, Delzenne NM, and Cani PD**. The endocannabinoid system links gut microbiota to adipogenesis. *Mol Syst Biol* 6: 392, 2010.
30. **Overton HA, Babbs AJ, Doel SM, Fyfe MC, Gardner LS, Griffin G, Jackson HC, Procter MJ, Rasamison CM, Tang-Christensen M, Widdowson PS, Williams GM, and Reynet C**. Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. *Cell metabolism* 3: 167-175, 2006.
31. **Schwartz GJ, Fu J, Astarita G, Li X, Gaetani S, Campolongo P, Cuomo V, and Piomelli D**. The lipid messenger OEA links dietary fat intake to satiety. *Cell metabolism* 8: 281-288, 2008.
32. **Stella N, Schweitzer P, and Piomelli D**. A second endogenous cannabinoid that modulates long-term potentiation. *Nature* 388: 773-778, 1997.

33. **Teixeira TF, Souza NC, Chiarello PG, Franceschini SC, Bressan J, Ferreira CL, and Peluzio Mdo C**. Intestinal permeability parameters in obese patients are correlated with metabolic syndrome risk factors. *Clinical nutrition* 31: 735-740, 2012.

34. **Verdam FJ, Fuentes S, de Jonge C, Zoetendal EG, Erbil R, Greve JW, Buurman WA, de Vos WM, and Rensen SS**. Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. *Obesity* 2013.

35. **Verdich C, Toubro S, Buemann B, Lysgard Madsen J, Juul Holst J, and Astrup A**. The role of postprandial releases of insulin and incretin hormones in meal-induced satiety--effect of obesity and weight reduction. *Int J Obes Relat Metab Disord* 25: 1206-1214, 2001.

FIGURE LEGENDS

Figure 1: Plasma 2-arachidonylglycerol (2-AG) (A), anandamide (AEA) (B), and oleoylethanolamide (OEA) (C) concentrations at fasting (0 min) and during intraduodenal infusion of Intralipid (2 kcal/min for 120 min) in lean (n= 20), overweight (n=18) and obese (n=19) individuals. There was no significant difference between BMI groups, or effect of lipid infusion, on plasma 2-AG (A) or OEA (C) concentrations. Plasma AEA was higher in obese compared with both lean (P=0.005) and overweight (P=0.017), with no difference between lean and overweight groups. *P<0.05 vs. lean and overweight.

Figure 2: Baseline duodenal expression of toll-like receptor-4 (TLR4) (A), intestinal alkaline phosphatase (IAP) (B), occludin (C), and zona-occludin 1 (ZO-1) (D) in lean (n=20), overweight (n=18) and obese (n=19) individuals. Baseline IAP expression (B) was significantly lower in obese compared with lean (P<0.05), with no difference between lean and overweight, or obese and overweight groups. Baseline ZO-1 expression (D) was significantly lower in obese compared with lean (P=0.002), and in overweight compared with lean (P=0.029), with no difference between overweight and obese groups. *P<0.05 obese vs. lean, #P<0.05 overweight vs. lean.

Figure 3: Plasma tumour-necrosis factor-α (TNF-α) concentrations at fasting (0 min) and during intraduodenal infusion of Intralipid (2 kcal/min for 120 min) in lean (n=19), overweight (n=16) and obese individuals (n=17). There was no significant difference between BMI groups (P=0.7), and no effect of lipid infusion (P=0.2) on plasma concentrations of TNF-α. Sufficient plasma was not available for 1 lean, and 2 overweight and 2 obese individuals.