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Blockade of cannabinoid 1 receptor improves GLP-1R mediated insulin secretion in mice

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ABSTRACT

The cannabinoid 1 receptor (CB1) is an important regulator of energy metabolism. Reports of *in vivo* and *in vitro* studies give conflicting results regarding its role in insulin secretion, possibly due to circulatory factors, such as incretins. We hypothesized that this receptor may be a regulator of the entero-insular axis. We found that despite lower food consumption and lower body weight postprandial GLP-1 plasma concentrations were increased in $CB1^{-/-}$ mice compared to $CB1^{+/+}$ mice administered a standard diet or high fat/sugar diet. Upon exogenous GLP-1 treatment, $CB1^{-/-}$ mice had increased glucose-stimulated insulin secretion. In mouse insulinoma cells, cannabinoids reduced GLP-1R-mediated intracellular cAMP accumulation and subsequent insulin secretion. Importantly, such effects were also evident in human islets, and were prevented by pharmacologic blockade of CB1. Collectively, these findings suggest a novel mechanism in which endocannabinoids are negative modulators of incretin-mediated insulin secretion.

1. Introduction

In the past two decades, the endogenous cannabinoid system (ECS) has emerged as an important regulator of energy metabolism (Silvestri and Di Marzo, 2013). Up-regulation of this system, which includes endocannabinoids (ECs), their synthesizing/degrading enzymes and their receptors, is associated with dyslipidemia, obesity and type 2 diabetes mellitus (Engeli, 2008; Matias and Di Marzo, 2007). Many of the metabolic regulatory effects associated with the ECS are modulated through the cannabinoid 1 receptor (CB1), a $G_{i/o}$-protein coupled receptor found in brain and peripheral tissues. Central CB1 stimulation leads to increase food intake (Di Marzo et al., 2001; Silvestri and Di Marzo, 2013), and CB1 blockade induces weight loss (Van Gaal et al., 2005). Conversely, peripheral CB1s play an important role in glucose homeostasis by modulating lipogenesis in liver and adipose tissues (Cota et al., 2003; Matias et al., 2006; Osei-Hyiaman et al., 2005), glucose uptake in skeletal muscle (Esposito et al., 2008) and motility of the gastrointestinal tract (Izzo and Sharkey, 2010; Troy-Fioramonti et al., 2014). While most researchers agree that pancreatic beta (β) cells also contain CB1 (Bermúdez-Silva et al., 2008; Kim et al., 2011; Starowicz et al., 2008), its exact role in insulin secretion remains controversial.

Insulin secretion is a tightly regulated process such that blood glucose concentrations are maintained within a narrow range at all times. Secretion of insulin is stimulated by both glucose and incretins, which include glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). Incretins are hormones secreted from enteroendocrine cells in response to food intake (Montrose-Rafizadeh et al., 1994). Once in circulation, GIP and GLP-1 bind to their specific G_s-protein coupled receptors (GIPR and GLP-1R, respectively) on β cells to activate adenylyl cyclase (AC) (Drucker et al., 1987; Thorens, 1992). The subsequent rise in intracellular cAMP is critical to incretin-mediated insulin secretion and accounts for approximately 50% of the total insulin secreted following an oral glucose challenge (Kim and Egan, 2008).

Abbreviations: AC, Adenylyl cyclase; ACEA, arachidonoyl 2′-chloroethylamide; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; EC, endocannabinoid; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; DPP-4, Dipeptidyl peptidase-4; Ex-4, exendin-4; IPGTT, Intraperitoneal glucose tolerance test; SD, standard diet; HFS, high fat/high sugar diet; Glut2, glucose transporter 2; PKA, protein kinase A; NAPE-PLD, N-Acyl phosphatidylethanolamine-specific phospholipase D; DAGL, Diacylglycerol lipase.

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Activation of CB1 stimulates $G_{i/o}$, which inhibits AC activity and cAMP synthesis (Turu and Hunyady, 2010). In mouse, enteroendocrine cells express CB1 receptor (Sykaras et al., 2012), and its activation inhibits GIP secretion (Moss et al., 2012; Troy-Fioramonti et al., 2014), which suggests that blockade of CB1 would at least indirectly stimulate insulin secretion through GIPR. Several recent studies have suggested a potential interaction between incretin receptors and CB1, particularly with regard to food intake (Bojanowska and Radziszewska, 2011; Patel et al., 2014; Radziszewska et al., 2014). However, there is a lack of data as to how CB1 would influence the entero-insular axis if both incretin and insulin secretion are impacted by the same receptor system.

In the present study, we investigated the role of CB1 on both GLP-1 secretion and subsequent receptor-mediated insulin secretion. We demonstrate herein that mice deficient in CB1 exhibit higher postprandial incretin secretion and greater GLP-1 sensitivity than control mice. Using both genetic and pharmacological manipulations, we further show that activation of CB1 down-regulates GLP-1R signaling and, in turn, insulin secretion, in mouse insulinoma cells as well as human islets.

2. Materials and methods

2.1. Reagents

Anandamide (AEA), 2-arachidonoylglycerol (2-AG), arachidonyl-2-chloroethylamide (ACEA), AM251 and WIN55,212-2 were obtained from Cayman Chemical (Ann Arbor, MI). JD-5037 was provided by Jenrin Discovery, Inc. (Wilmington, DE). Exendin-4 (Ex-4), GLP-1 and GIP were obtained from Bachem (Torrance, CA). Dipeptidyl peptidase-4 (DPP-4) inhibitor was purchased from Millipore (Billerica, MA). Aprotinin was obtained from Fisher-Scientific (Middletown, VA). Intralipid 20% was purchased from Fresenius-Kabi (Uppsala, Sweden). The human *Cnr1* (CB1 encoding gene) cDNA was amplified by RT-PCR from a human pancreas RNA (Stratagene, Agilent Technologies, Santa Clara, CA), and cloned into the mCerulean-N1 vector (Rizzo and Piston, 2005).

2.2. Animal models and experimental diets

Global CB1 receptor knockout ($CB1^{-/-}$) mice and their wild-type littermates ($CB1^{+/+}$) backcrossed to a C57Bl/6J background were bred as previously described (Zimmer et al., 1999). Mice (male, 2–3 months old) were provided with water and feed *ad libitum* on either a standard chow diet (SD; 16.7% kcal fat and 12.4% kcal sugar) or a high fat/high sugar diet (HFS; 49.2% kcal fat and 21.1% kcal sugar; Dyets Inc., Bethlehem, PA) for 15 weeks. At the end of the study, body weight was measured and animals were placed in metabolic cages in order to obtain precise measurements of food intake. All animal care and experimental procedures followed US National Institutes of Health guidelines and were approved by the National Institute on Aging Animal Care and Use Committee.

2.3. Intraperitoneal glucose tolerance tests

Mice were fasted overnight and given free access to water. Intraperitoneal glucose tolerance tests (IPGTT) were carried out as we previously described (Wang et al., 1997). After 36 h of GLP-1 (1.5 pmol/kg·min) treatment *via* subcutaneously-implanted Alzet microosmotic pumps (Cupertino, CA) (n = 6 per genotype), a bolus of glucose (1 g/kg body weight) was administered intraperitoneally. Tail-vein blood samples were collected at 0, 15, 30, 60, and 90 min.

2.4. Mouse circulating hormone and glucose quantification

Blood glucose concentrations were determined using a glucometer (Elite, Bayer Inc.) from fresh tail-vein blood. In order to determine active levels of GLP-1, mice were orally administered a single dose of Intralipid (20%) containing D-glucose (30%) *via* oral gavage and blood collected 20 min post-dose (Althage et al., 2008; Lu et al., 2007) into pre-chilled tubes containing EDTA, aprotinin and DPP-4 inhibitor. Plasma insulin was measured with a mouse insulin ELISA (Crystal Chem Inc., Downers Grove, IL) and active GLP-1 determined with the GLP-1 (Active 7–36) ELISA (ALPCO, Salem, NH). Plasma GIP and leptin were analyzed in 100 μl of plasma (final bleed) using a MILLIPLEX Mouse Gut Hormone Magnetic Bead Panel (Millipore, Billerica, MA). HOMA-IR, a measure of liver insulin sensitivity, was quantified by: fasting insulin (μU/mL) x fasting glucose (mg/dL)/405 (Haffner et al., 1997).

2.5. Cell culture and insulin secretion and cAMP assays from cell lines

MIN6 and βTC6 insulinoma cells were maintained in DMEM medium with 10% FBS (Life Technologies, Grand Island, NY). CHO-GLP-1R (CHO-K1 cells stably transfected with GLP-1R) (Montrose-Rafizadeh, 1997) were maintained in DMEM/F-12 medium with 10% FBS. For insulin secretion and cAMP assays, cells were plated in 12-well plates, one or three days before transfection, respectively. Cells were washed three times in PBS and were pre-incubated for 2 h in the Krebs buffer containing 4 mM glucose at 37 °C. Subsequently, CB1 agonists or inverse agonists were pre-treated for 15 min before the subsequent addition of glucose (25 mM) or Ex-4 (10 or 25 nM) for a further 20 min. At the end of the experiment, the buffer was collected, centrifuged to remove cellular debris and saved for quantification of insulin. The cells were lysed with 0.1 M HCl and were centrifuged to remove cellular debris. The supernatant were collected for determination of cAMP and protein concentrations. cAMP was measured using a cAMP ELISA kit according to the manufacturer's instructions. The data were normalized to protein concentration, and estimated from three independent experiments, each performed in at least triplicate. Transfections of the expression vectors and siRNA (Santa Cruz, Dallas, Texas) for *Cnr1* were carried out 24 or 48 h before adding CB1 agonist using Lipofectamine 2000 and RNAiMAX (Life Technologies), respectively. Scramble siRNA (Silencer Negative Control #1; Life Technologies) or empty vector was transfected as negative control.

2.6. Insulin secretion and cAMP accumulation in isolated human islets

Human pancreatic islets were provided by the NIDDK-funded Integrated Islet Distribution Program (IIDP) at City of Hope and incubated in insulin secretion assay buffer (Montrose-Rafizadeh et al., 1994) containing 2 mM glucose for a total of 2 h at 37 °C, with media being refreshed after 1 h. Islets were then pre-treated for 15 min with 7.5 mM glucose (postprandial levels), IBMX (25 μM) and increasing concentrations of ACEA before stimulation with Ex-4 (0.33 nM) for an additional 20 min at 37 °C. Media were collected for measuring insulin secretion (Mercodia, Uppsala, Sweden). Islets were processed for cAMP *via* an ELISA (Enzo Life Sciences, Farmingdale, NY) and total protein concentration quantified determined by a Bicinchoninic Acid Protein Assay (Pierce, Rockford, IL).

2.7. Immunoblotting

Protein samples extracted from cells using RIPA buffer (50 mM