Chronic activation of cannabinoid receptors in vitro does not compromise mouse islet function

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Abstract
We have demonstrated previously that mouse and human islets express ECS (endocannabinoid system) elements, and that short-term activation of islet cannabinoid CB1r and CB2r (cannabinoid type 1 and 2 receptors respectively) stimulates insulin secretion in vitro. There is evidence that the ECS is overactive in Type 2 diabetes, impairing glucose homeostasis, but little is known about whether it is implicated in islet dysfunction. Therefore the aim of the present study was to investigate the effect of chronic exposure of isolated mouse islets to cannabinoid receptor agonists on islet gene expression and function. Quantitative RT–PCR (reverse transcription–PCR) indicated that mRNAs encoding synthesis [NAPE-PLD (N-acyl-phosphatidyl ethanolamidase-hydrolysing phospholipase D)] and degradation [FAAH (fatty acid amide hydrolase)] of the endocannabinoid AEA (anandamide) were the most abundant ECS elements in mouse islets, with much lower levels of CB1r, CB2r, DAGL (diacylglycerol lipase) and MAGL (monoacylglycerol lipase) mRNAs. Maintenance of islets for up to 7 days in the presence of the CB1r agonist ACEA [N-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide] or the CB2r agonist JWH015 [(2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone] did not compromise islet viability, as assessed by islet morphology and caspase activities, but there were some changes in mRNAs encoding ECS components. Neither glucose-stimulated insulin secretion nor acute insulin secretory responses to ACEA or JWH015 at 16 mM glucose were substantially modified by a 48 h or 7 day pre-exposure to these cannabinoid receptor agonists, but the stimulation of secretion at 3 mM glucose by 100 nM ACEA was significantly reduced after prolonged treatment with ACEA. Despite JWH015-induced reductions in islet glucagon content at 48 h and 7 days, there were no reductions in arginine-induced glucagon secretion from islets pre-exposed to JWH015 or ACEA. These data indicate that treatment of islets with agonists of CB1r and CB2r for up to 7 days does not have any major impact on islet function, suggesting that the impairments in glucose homeostasis observed following overactivation of the ECS should be sought in relation to insulin resistance rather than β-cell dysfunction.

Key words: apoptosis, cannabinoid receptor, gene expression, insulin secretion, mouse islet

INTRODUCTION

It is now acknowledged that β-cell dysfunction, through decreases in β-cell mass and/or insulin secretory output, plays a key role in the development of Type 2 diabetes. It is therefore important to understand what signals are responsible for β-cell failure that leads to the onset of diabetes, as a starting point for the development of novel therapies. In 2006, an anti-obesity agent named rimonabant, which antagonizes the CB1r (cannabinoid type 1 receptor), was approved for clinical use in the U.K. Despite being withdrawn because of its adverse psychological effects, improvements in metabolic parameters were observed following its administration [1]. The weight loss induced by rimonabant was originally thought to be solely a consequence of its anorexigenic effects, but its effects on appetite and energy expenditure are not limited to its central role of CB1r antagonism [2,3]. Thus it is now known that peripheral cannabinoid receptors are also involved in gastric emptying [4], lipogenesis [5] and glucose uptake [6] and...
their overactivation in obesity and diabetes has been implicated in dysregulation of glucose homeostasis [7].

In recent years it has become apparent that mouse [8–14], rat [8,15–18] and human [14,19–21] islets contain a local ECS (endocannabinoid system), responsible for the generation of the endocannabinoids AEA (anandamide) and 2-AG (2-arachidonoyl glycerol), which may act in an autocrine or paracrine manner at cannabinoid receptors to regulate islet function. Thus, in addition to CB1r and CB2r (cannabinoid type 2 receptor), islets also express the enzymes involved in the synthesis and degradation of their agonists: NAPE-PLD (N-acyl-phosphatidyl ethanolamide-hydrorylising phospholipid D) and FAAH (fatty acid amide hydrolase) regulate AEA levels, and DAGL (diacylglycerol lipase) and MAGL (monoacylglycerol lipase) control 2-AG production and hydrolysis [9–21]. Although islets produce AEA and 2-AG [8,14,19] and several recent studies have investigated the acute effects of these agents on insulin secretion [9,12–14,16,18,19,21–23], no studies have been performed to examine the direct effects of chronic exposure to cannabinoids on islet function in vitro.

Polymorphisms in CB1r and FAAH, which regulates the hydrolysis of the endocannabinoid AEA, have been associated with obesity [24,25]. In addition AEA and the other major endocannabinoid 2-AG are produced by mature adipocytes [26] and their levels are elevated in human obesity [27] and in Type 2 diabetes [8]. The chronic elevation in endogenous cannabinoid levels has detrimental effects in several organs. Thus in vivo studies as well as in vitro experiments with mouse adipocytes and hepatocytes have indicated that pathological overactivation of the ECS is associated with impaired insulin sensitivity, glucose intolerance and dyslipidaemia [8,28]. It has also been reported that acute and chronic exposure to cannabinoid agonists increases the expression of lipogenic genes and lipid content in hepatocytes [29–31], and there is some evidence that prolonged exposure to cannabinoids promotes apoptosis in sebocytes [32].

The present study was therefore designed to identify whether prolonged treatment of isolated mouse islets with cannabinoid agonists regulates expression of genes coding for elements of the ECS, and/or affects islet function.

**MATERIALS AND METHODS**

**Materials**

DMEM (Dulbecco’s modified Eagle’s medium; 5.5 mM glucose), penicillin/streptomycin, l-glutamine, collagenase (Type XI), histopaque-1077 and DNA primers were purchased from Sigma–Aldrich. ACEA [N-(2-chloroethyl)-5Z,8Z,11Z, 14Z-eicosatetraenamide] and JWH015 [[2-(methyl-1-propyl-1-H-indol-3-yl)-1-naphthalenylmethanone] were from Tocris Biosciences. RNeasy mini kits were obtained from Qiagen, and FBS (fetal bovine serum) and RT (reverse transcription) reagents from Invitrogen. Real-time PCR master mix and plates were purchased from Roche Diagnostics. Apoptosis anti-active caspase 3/7 glo kits were obtained from Promega.

**Mouse islet isolation and culture**

Islets were isolated from male ICR mice, under local animal care and ethical use protocols. Pancreata were digested by collagenase (1 mg/ml) and islets were separated from exocrine tissue using a histopaque gradient and by handpicking as reported previously [33]. After overnight culture in DMEM (5.5 mM glucose) containing 10% FBS, 2 mM l-glutamine and 100 units/ml penicillin/100 μg/ml streptomycin, islets were treated for 48 h or 7 days with DMEM (5.5 mM glucose) alone or with DMEM supplemented with 100 nM of either ACEA, a CB1r agonist, or JWH015, a CB2r agonist. For the 7-day incubation period, the medium was renewed every 2 days.

**PCR**

Total RNA was isolated from 50 mouse islets, previously treated for 48 h or 7 days, with or without cannabinoid agonists, using RNeasy mini kits. All RNA samples were adjusted to obtain 20 ng of cDNA/μl by RT reactions as reported previously [18]. Real-time PCR was performed with a LightCycler 480 multiwell-plate system, using the primers and conditions described in Table 1. The relative expression of mRNAs was determined after normalization against 18S rRNA as an internal reference, and calculated by the 2ΔΔCt method [34].

**Caspase 3/7 activities**

Apoptosis of islets pre-treated with cannabinoid agonists for 48 h and 7 days, was determined by detection of caspase 3/7 activities with a luminometer following cleavage of a proluminescent substrate [Z-DEVD (benzoyl oxy carbonyl-Asp-Glu-Val-Asp-aminoluciferin)]. Apoptosis was induced in these experiments by exposing a batch of islets from each group to a cytokine cocktail [0.5 unit/μl IL-1β (interleukin-1β), 5 units/μl TNFα (tumour necrosis factor α) and 5 units/μl IFNγ (interferon γ)] for the final 20 h of incubation.

**Dynamic insulin secretion by perfusion**

The chambers and the perfusion equipment established in a 37°C temperature-controlled environment have been described previously [35]. A total of 40 islets from each experimental condition were perfused at 0.5 ml/min in individual chambers containing 1 μm pore-size nylon filters, with a bicarbonate-buffered physiological salt solution [36] containing 3 mM glucose. Perifuse fractions were collected every 2 min, the first 10 min to identify basal insulin secretion at 3 mM glucose, and then islets were exposed to 3 mM or 16 mM glucose, with or without 100 nM ACEA or 100 nM JWH015. Samples were stored at –75°C until the insulin contents were determined by RIA, in duplicate [35]. Averages of the areas below the curve were calculated for each condition as a measure of total insulin secretion for statistical analysis.

**Static glucagon secretion**

After treatment for 48 h or 7 days in control conditions, or with 100 nM ACEA or 100 nM JWH015, batches of 12 islets were pre-incubated for 1 h at 37°C in 400 μl of buffer [36] containing 2 mM glucose. The effects of chronic exposure to cannabinoid agonists on 10 mM arginine-induced glucagon secretion were evaluated by incubation of islets for 1 h at 37°C, followed by RIA in duplicate [35].
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Table 1 Primer sequences and annealing temperatures used in real-time PCR
F, forward; R, reverse.

<table>
<thead>
<tr>
<th>Gene name for Mus musculus</th>
<th>Primer sequences</th>
<th>Annealing temperature (°C)</th>
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<tbody>
<tr>
<td>CB1r</td>
<td>F, 5′-GGGCAAATTTCCTTGTAGCA-3′&lt;br&gt;R, 5′-CAGGCTCAACGTAGCTCCT-3′</td>
<td>60</td>
</tr>
<tr>
<td>CB2r</td>
<td>F, 5′-GACAAGGCTACACAGACC-3′&lt;br&gt;R, 5′-CTCCCTCTATGCCGTTGACTC-3′</td>
<td>60</td>
</tr>
<tr>
<td>DAGLα</td>
<td>F, 5′-TCGGTACCTTTCCTACTTAC-3′&lt;br&gt;R, 5′-ATCCGCACTCATATTTT-3′</td>
<td>60</td>
</tr>
<tr>
<td>MAGL</td>
<td>F, 5′-GAGAAGGCGCACAATCTTT-T-3′&lt;br&gt;R, 5′-ATGCGACCCAGGGCTATTT-3′</td>
<td>60</td>
</tr>
<tr>
<td>FAAH</td>
<td>F, 5′-GCTGTCCTTTTCACTTCTG-3′&lt;br&gt;R, 5′-GAAGAATTCCTTGAGGCTCAC-3′</td>
<td>60</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>F, 5′-TGCGCCAGGGCTTTCGCC-3′&lt;br&gt;R, 5′-AGGCTATCCTGAGGCTAC-3′</td>
<td>62</td>
</tr>
<tr>
<td>PPI I and II</td>
<td>F, 5′-ACACACCCAGGTCTTTCGTA-3′&lt;br&gt;R, 5′-TGTGGGTCTCCACTACTCA-3′</td>
<td>60</td>
</tr>
<tr>
<td>PPG</td>
<td>F, 5′-GGCTCTTCTTCTGACAGATGAGACAC-3′&lt;br&gt;R, 5′-CTGCCAGAGATGTGGGAATGGG-3′</td>
<td>58</td>
</tr>
<tr>
<td>GLP-1r</td>
<td>F, 5′-CAGACCCACACAGCCTCGC-3′&lt;br&gt;R, 5′-CCAGAGTGTTGCGCCTCTGG-3′</td>
<td>62</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>F, 5′-GGGAGCCTGAGAAGCGG-3′&lt;br&gt;R, 5′-GGTCCGAGTGGTGATT-3′</td>
<td>60</td>
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Insulin and glucagon content in islets
Acidified ethanol (100 μl) was added to groups of 5 mouse islets which were sonicated and stored at −20 °C until RIAs were performed to measure insulin and glucagon contents.

Data analysis
Data are expressed as means ± S.E.M. of four to eight individual experiments. Student’s t tests and two-way ANOVA with Bonferroni’s test were used for analysis. Differences between treatments were considered statistically significant at P < 0.05.

RESULTS
Expression of endocannabinoid signalling element mRNAs by mouse islets
Figure 1 shows the mRNA levels of CB1r and CB2r, and of the enzymes responsible for endocannabinoid synthesis and degradation in mouse islets. PPI (preproinsulin) and PPG (preproglucagon) mRNAs were abundant, as expected, and mRNAs encoding the degradation (FAAH) and generation (NAPE-PLD) of the endocannabinoid AEA were relatively highly expressed. There was much lower expression of DAGL and MAGL mRNAs, which code for enzymes that synthesize and hydrolyse 2-AG, and cannabinoid receptor (CB1r and CB2r) mRNA levels were also very low (all <1% of FAAH levels). GLP-1r (glucagon-like peptide-1 receptor) mRNA was also amplified in the same islet samples used for quantification of CB1r and CB2r mRNAs, and this indicated that cannabinoid receptor expression levels were of the same order of magnitude as GLP-1r (52.8 ± 2.5% and 32.0 ± 1.4% of GLP-1r mRNA expression for CB1r and CB2r respectively, n = 11).

Effects of prolonged exposure of islets to ACEA and JWH015 on islet morphology and apoptosis
Having confirmed the presence of ECS components in mouse islets we determined whether prolonged exposure to CB1r (ACEA) or CB2r (JWH015) agonists, at 48 h and 7 days, modified islet morphology or the extent of apoptosis that occurred under basal conditions and in the presence of a cytokine cocktail. The micrographs in Figure 2 indicate that maintenance of islets for 48 h (upper panels) and 7 days (lower panels) in the absence (left-hand panels) and presence of 100 nM ACEA (middle panels) or JWH015 (right-hand panels) did not have any overt deleterious effects on islet integrity, although there was some evidence of central necrosis in all groups of islets after 7 days in culture. Furthermore, as shown in Figure 3, whereas control islets (open bars) maintained in a cytokine cocktail for the last 20 h of the 48 h and 7 day incubation periods showed significantly increased caspase 3/7 activities, as expected, exposure for 48 h or 7 days to ACEA (grey bars) or JWH015 (black bars) was not associated with increased islet apoptosis (Figure 3). In fact, cytokine treatment no longer promoted apoptosis in islets that had been pre-exposed to 100 nM ACEA or JWH015 for 7 days, suggesting a protective effect of long-term cannabinoid receptor activation.

Effects of prolonged exposure of islets to ACEA and JWH015 on gene expression
The effects of 48 h and 7 day treatment with ACEA and JWH015 on islet gene expression of ECS elements, and of PPI and PPG mRNAs, were evaluated by quantitative RT–PCR. It can be seen...
from Figure 4 that stimulation of CB1r with ACEA (grey bars) induced small increases in CB1r and MAGL mRNA levels at 48 h, but these changes were not statistically significant. However, there were significant (P < 0.05) increases in FAAH mRNA expression at 48 h (by 49 ± 11%) and 7 days (by 36 ± 5%), and exposure of islets to ACEA for 7 days also resulted in a significant reduction in CB2r mRNA (by 40 ± 26%). On the other hand, prolonged incubation of islets with 100 nM JWH015 (black bars) had more marked effects on islet gene expression. At both 48 h and 7 days we observed reductions in mRNAs encoding CB2r (by 54 ± 3% and 67 ± 18%; P < 0.05), DAGL (by 59 ± 13% and 45 ± 42%; P < 0.05) and PPG (by 70 ± 80% and 36 ± 8%; P < 0.05). NAPE-PLD and PPI mRNA abundance decreased after 48 h of CB2r stimulation by JWH015 (by 46 ± 15% and 52 ± 41%; P < 0.05), but not after a 7 day incubation period. Conversely, FAAH and MAGL expression only significantly decreased following exposure to JWH015 for 7 days (by 42 ± 32% and 37 ± 17%; P < 0.05), although there was a trend towards reduced MAGL mRNA levels after 48 h exposure to JWH015.

**Effects of prolonged exposure of islets to cannabinoid receptor agonists on insulin secretion**

We analysed the dynamic insulin secretion of perifused islets, treated previously either with the CB1r agonist ACEA or the CB2r agonist JWH015, at 48 h (Figure 6) and 7 days (Figure 7). As expected, increasing the glucose concentration from 3 to 16 mM induced rapid, sustained elevations in insulin secretion from control islets that had been maintained for 48 h and 7 days in DMEM (5.5 mM glucose) in the absence of cannabinoid receptor agonists (Figures 6A and 7A, open circles). Islets that had been exposed to ACEA or JWH015 for 48 h and 7 days also showed significant increases in insulin secretion in response to 16 mM glucose, although there was a small reduction in glucose-stimulated insulin secretion from islets treated with ACEA for 48 h (Figure 6A, grey circles) and exposure of islets to JWH015 for 7 days enhanced acute insulin secretion in response to 16 mM glucose (Figure 7A, black circles).

Acute exposure of control islets that had been in culture for 48 h to 100 nM ACEA induced a rapid, reversible increase in insulin secretion at 3 mM glucose (Figure 6B, open circles). However, in islets that had been pre-exposed to 100 nM ACEA for 48 h there was only a small delayed secretory response to an acute challenge with ACEA at 3 mM glucose that was slower in onset and significantly less (P < 0.01) than that obtained from control islets (Figure 6B, grey circles). In contrast, ACEA potentiated glucose-induced insulin secretion from the 48 h control and ACEA-treated islets with similar secretory profiles (Figure 6C). JWH015 (100 nM) did not have a clear stimulatory effect on insulin secretion at 3 mM glucose from islets that had been incubated for 48 h in the absence or presence of this CB2r agonist (Figure 6D), but both groups of islets responded significantly to JWH015 in the presence of 16 mM glucose (Figure 6E, P < 0.01). However, the response of the islets cultured for 48 h with JWH015 (black circles) was delayed in onset, and significantly less than the response to acute challenge with JWH015 seen in the control islets (open circles).

In experiments where islets were maintained for 7 days in the absence or presence of 100 nM ACEA, acute exposure to ACEA at 3 mM glucose resulted in a much reduced secretory response compared with that seen after a 48 h incubation period, and 7 day ACEA-treated islets failed to respond to a subsequent ACEA challenge (Figure 7B). However, control and ACEA-treated islets showed similar secretory profiles in response to exposure to ACEA at 16 mM glucose, with a slow potentiation of insulin secretion (Figure 7C). Insulin secretion was significantly
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Figure 2  Micrographs of islets maintained in culture for 48 h (upper panels) or 7 days (lower panels) in DMEM alone (left-hand panels) or in DMEM supplemented with 100 nM ACEA (middle panels) or 100 nM JWH015 (right-hand panels).

Micrographs are representative of islets in six separate experiments.

Figure 3  Caspase 3/7 activities in mouse islets after maintenance for 48 h (A) and 7 days (B) in DMEM (5.5 mM glucose) alone (open bars) or supplemented with 100 nM ACEA (grey bars) or 100 nM JWH015 (black bars).

Apoptosis was induced in the cytokines groups by adding a cytokine cocktail (0.5 unit/ml IL-1β, 5 units/ml TNFα and 5 units/ml IFNγ) for the final 20 h of incubation. Data are expressed in luminescence units as means ± S.E.M., n = 6.

*P < 0.05 with respect to the appropriate basal level.

increased from 7 day control and JWH015-treated groups of islets following exposure to 100 nM JWH015 at 3 mM glucose (Figure 7D), whereas glucose-induced insulin secretion was not modified by the acute treatment with the CB2r agonist in islets that had been in culture for 7 days (Figure 7E).

Effects of prolonged exposure of mouse islets to cannabinoid receptor agonists on glucagon secretion

We also evaluated whether chronic exposure of islets to cannabinoid agonists affected acute glucagon secretion. For these experiments arginine-induced glucagon secretion was quantified in islets that had been maintained in culture in the absence or presence of 100 nM ACEA or JWH015 for 48 h (Figure 8A) and 7 days (Figure 8B). As expected, 10 mM arginine stimulated glucagon secretion from islets that had been incubated in the absence of cannabinoid agonists (open bars), but the magnitude of the secretory response was reduced after islets had been in culture for 7 days. Arginine also induced significant increases in glucagon secretion after prolonged exposure of islets to 100 nM ACEA (grey bars) or 100 nM JWH015 (black bars), with similar responses to those obtained from control islets. Measurements of islet PPG mRNA levels (Figure 4) and glucagon content (Figure 5) had indicated that JWH015 treatment caused
Figure 4  Quantification of mRNAs encoding elements of ECS and PPI and PPG in mouse islets after maintenance for 48 h (A) and 7 days (B) in DMEM (5.5 mM glucose) alone (open bars) or supplemented with 100 nM ACEA (grey bars) or 100 nM JWH015 (black bars)

Data are expressed relative to the levels present in control islets in the absence of cannabinoid receptor agonists and normalized to 18S rRNA levels in the same samples. Values are means ± S.E.M., n = 4 experiments. *P < 0.05 and **P < 0.01 compared with the appropriate control.

Figure 5  Insulin (A) and glucagon (B) contents of islets treated for 48 h (left) or 7 days (right) in the absence of cannabinoid receptor agonists (open bars) or with 100 nM ACEA (grey bars) or 100 nM JWH015 (black bars)

Data are expressed as ng/islet as means ± S.E.M., n = 4. *P < 0.05 and **P < 0.01 compared with the appropriate control.
significant reductions in glucagon. However, in these short-term static incubation experiments basal and stimulated glucagon secretion from JWH015-treated islets was not significantly lower than from control islets, suggesting that the JWH015-induced decrease in glucagon content was not sufficient to impact on acute glucagon secretion. Exposure of islets for 1 h to the CB1r and CB2r agonists after 48 h and 7 day incubations had little effect on glucagon secretion, although arginine-induced glucagon release from islets that had been treated for 48 h with ACEA was potentiated by acute exposure to this agonist.

**DISCUSSION**

Several earlier studies have identified the presence of a local ECS in murine and human islets, with demonstrations of islet cell expression of cannabinoid receptors and the enzymes responsible for endocannabinoid synthesis and degradation [8,28,9–15,18–21,37]. Our quantitative RT–PCR experiments indicate that mouse islets have a relatively high expression of FAAH and NAPE-PLD mRNAs, which is consistent with previous reports of their detection, mainly in β-cells from rat and...
mouse [8,11,15,19,20], and suggest an important role for the synthesis and degradation of AEA in islets. We also detected mRNAs encoding the lipases responsible for regulating 2-AG levels in islets (DAGL and MAGL), as has been reported earlier [8,11,19,20,37] and our quantification of these mRNAs indicate that they are expressed in mouse islets at considerably lower levels than those of FAAH and NAPE-PLD. These observations, together with previous reports of 2-AG having lower affinity for CB1r than AEA [38], suggest that endogenously generated AEA may be the major endocannabinoid in islets.

Islet CB1r and CB2r may be activated by circulating endocannabinoids as well as by AEA and 2-AG synthesized within islets. In lean individuals the concentrations of cannabinoids in the blood are relatively low, but serum 2-AG and AEA levels increase significantly in obesity and hyperglycaemic Type 2 diabetes, in excess of 1 pmol/ml of serum [8], and they also increase in the pancreas under conditions of diet-induced obesity [8]. The increase in endocannabinoid synthesis in obesity and diabetes is associated with metabolic dysfunction, at least in part through CB1r-mediated reductions in adiponectin, an adipocyte-derived peptide that improves insulin sensitivity [39,40]. In addition,
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Figure 8 Glucagon secretion from mouse islets after maintenance for 48 h (A) or 7 days (B) in DMEM (5.5 mM glucose) alone (open bars) or supplemented with 100 nM ACEA (grey bars) or 100 nM JWH015 (black bars). Glucagon secretion was induced by incubation of all groups of islets for 1 h with 10 mM Arg (arginine) in the absence or presence of 100 nM ACEA or 100 nM JWH015. Data are expressed as pg of glucagon/islet per h as means ± S.E.M., n = 4. *P < 0.05 and **P < 0.01 compared with the appropriate basal control.

Exposure to cannabinoids for up to 48 h induces apoptosis in a number of different cell types [32,41–43], and 2-AG and AEA are reported to promote β-cell death [23]. In contrast, there is evidence that supports a protective role of endocannabinoids in cell survival [44,45]. Given the association of cannabinoid up-regulation with metabolic dysfunction and the possibility of enhanced β-cell apoptosis the current study was therefore designed to evaluate whether exposure to pharmacological cannabinoid agonists for up 7 days affects islet viability and function.

In contrast to the recent report of reduced viability and enhanced apoptosis of MIN6 β-cells and primary islet cells following exposure to cannabinoid receptor agonists for 48 h [23], we did not detect any increases in apoptosis of islets that had been maintained in the presence of 100 nM ACEA or JWH015 for 48 h, or even after a 7 day incubation period. ACEA and JWH015 were selected for these experiments since they are well-documented to be high-affinity selective agonists at CB1r and CB2r in the nanomolar range (Kᵢ = 1.4 and 13.8 nM respectively). In a previous study, the reduction in β-cell viability was only apparent following exposure to 1 μM 2-AG or AEA, which is 1000-fold higher than endocannabinoid concentrations detected in diabetes [8], and maximal inhibition of viability was observed at 25 μM of these cannabinoids [23]. It is therefore possible that induction of cell death at these high concentrations may have been a consequence of unintended activation of non-cannabinoid receptors such as TRPV1 (transient receptor potential vanilloid 1) (Kᵢ = 5.8 μM for AEA) that are expressed by β-cells [46] and are known to induce cell death [47].

Cellular expression of components of the ECS is a dynamic process that is modified in response to different stimuli [48] and chronic ECS activation induces cellular and molecular adaptations [30,31,49,50]. Previous studies have indicated that high fat diet and high glucose concentrations increase AEA and 2-AG synthesis in mouse and human islets, as well as inducing changes in gene expression of ECS enzymes and receptors [8,12,15,18,51]. Continuous exposure to cannabinoids is reported to lead to pharmacological tolerance, desensitization and attenuation of the drug effect, and, like other models of GPCRs (G-protein-coupled receptors), long-term exposure can modify receptor gene expression and number [49,52]. Similar feedback regulation on the synthesis and degradation of endocannabinoids has been reported in striatal neurons [53]. In the present study, although prolonged ACEA treatment did not affect CB1r mRNA levels, it up-regulated islet FAAH mRNA. This suggests that there is increased degradation of AEA after ACEA stimulation of CB1r, which may be a negative-feedback mechanism to limit accumulation of the endogenous CB1r agonist in the continued presence of an exogenous agonist and thus prevent overstimulation of the receptor. In parallel experiments, maintenance of mouse islets with JWH015 led to reductions in mRNAs encoding CB2r, DAGL, NAPE-PDL, MAGL and FAAH, consistent with sustained activation of CB2r leading to decreased CB2r synthesis and depletion of the enzymes responsible for endogenous regulation of 2-AG and AEA levels. These changes in the ECS mRNAs with chronic exposure to the CB1r and CB2r agonists support a model of dynamic regulation of islet endocannabinoids to maintain their ‘on-demand’ synthesis in response to physiological and pathological stimuli.

Prolonged exposure of islets to 100 nM JWH015 also resulted in significant reductions in prepro-insulin and glucagon mRNAs. Lower islet glucagon content was also observed after prolonged incubation with JWH015, consistent with reductions in PPG mRNA resulting in reduced glucagon biosynthesis. However, although there was a deficit in PPI mRNA after 48 h stimulation with JWH015, islet insulin content actually increased under these conditions. The reason for this is not immediately obvious, but it is likely that there was increased translation of PPI mRNA in the presence of JWH015, resulting in reduced
steady-state PPI mRNA and increased insulin content. Measurements of dynamic insulin secretion indicated that islets still showed significant glucose-induced secretory responses after prolonged exposure to ACEA or JWH015, indicating that changes in mRNA levels of ECS components induced by selective agonists do not have a negative impact on glucose-stimulated insulin output.

Previous studies have reported significant acute effects of cannabinoid agonists on insulin secretion from murine and human islets [8–10,12–14,19,21,38], and on glucose homoeostasis in rats [15,17,18,54]. However, there is no consensus on whether insulin secretion is stimulated [12–14,19,21,37] or inhibited [9,10,19] in response to cannabinoid receptor activation in vitro, partly as a consequence of different concentrations and selectivity of agonists used, and differing methodologies. We determined dynamic insulin secretion in response to acute exposure to 100 nM ACEA and JWH015 from islets that had been maintained for 48 h or 7 days in the absence and presence of these cannabinoid agonists used, and differing methodologies. We determined dynamic insulin secretion in response to acute exposure to 100 nM ACEA and JWH015 from islets that had been maintained for 48 h or 7 days in the absence and presence of these cannabinoid agonists. Both agonists stimulated insulin secretion from islets that had been maintained for up to 7 days in DMEM alone, although the magnitude of response was smaller than we have previously observed following perfusion of islets with ACEA and JWH015 [13,21]. This most likely reflects the lower agonist concentrations used in the present study (100 nM compared with 5–10 μM) to minimize activation of non-cannabinoid receptors. Pre-exposure to ACEA for 48 h did diminish acute stimulation to a subsequent ACEA challenge at 3 mM glucose, which could have been a consequence of reduced endogenous AEA after FAAH up-regulation, but islets showed similar responses to ACEA at 16 mM glucose whether they had been pre-treated with ACEA or not. However, ACEA-stimulated insulin secretion at 16 mM glucose was slow in onset in both the control islets and those that had been pre-exposed to ACEA, and it was sustained even after removal of ACEA. The reason for this is not entirely clear, but we have observed this in our previous experiments [13] and it is possible that endocannabinoid accumulation at elevated glucose levels [8,19] contributes to the maintained secretory output after removing ACEA. JWH015 also stimulated acute insulin secretion to a similar extent irrespective of whether the islets had been pre-exposed to this CB2r agonist, again supporting a model in which ECS regulatory enzyme mRNAs were down-regulated to minimize the impact of sustained activation of CB2r.

There is little information available about the role of the ECS in α-cells. The presence of CB1r [9–11,14,15,19] and/or CB2r [9,11,13,15] in α-cells of murine and human islets is not well defined. It has been reported previously that acute exposure to ACEA and 2-AG stimulated glucagon release from human islets [19] and we have demonstrated that inhibition of MGL in human islets to allow endogenous accumulation of 2-AG is also accompanied by increased glucagon secretion [37]. It has been suggested that activation of cannabinoid receptors impairs glucose homoeostasis by alterations in glucagon secretion [8,11,18], but our results do not support this. We found that chronic exposure to JWH015, for either 48 h or 7 days, decreased islet glucagon mRNA and content, but the islets still showed significant increases in glucagon secretion in response to arginine. This suggests that there was sufficient glucagon available for an appropriate secretory response, consistent with well-established observations that islets maximally secrete only approximately 10% of their hormone content per hour, so reductions in glucagon content would not be expected to reduce short-term glucagon release. Elevation in stimulated glucagon output in response to acute exposure to ACEA was observed in islets that had been pre-exposed to ACEA for 48 h, but this enhanced glucagon output was not observed in 7 day treated islets. These observations suggest that dysfunctional glucose homeostasis in response to excess endocannabinoids is unlikely to be a consequence of increased glucagon release.

In summary these data indicate that chronic exposure of isolated mouse islets to cannabinoid receptor agonists has direct effects on gene expression of ECS components, on glucagon mRNA and protein content, but there are no deleterious effects on islet cell viability and no marked alterations in insulin or glucagon secretion. The impaired glucose tolerance that is observed in response to elevations in circulating endocannabinoids and administration of cannabinoid receptor agonists [1,8,54] may therefore reflect insulin resistance rather than β-cell dysfunction.

**CLINICAL PERSPECTIVES**

- The ECS has physiological signal transduction roles, but it becomes overactive in obesity as a consequence of enhanced fat mass. Chronic elevation of endocannabinoids has been associated with impaired glucose homeostasis, but the direct effects of chronic activation of islet cell cannabinoid receptors have not been previously investigated.

- In the present paper, we report that maintenance of isolated mouse islets for up to 7 days in the presence of agonists of CB1r and CB2r does not exert overt deleterious effects on islet viability or function.

- Our results imply that islet cell death or dysfunction do not contribute to the impaired glucose tolerance that occurs when endocannabinoid levels are chronically elevated, and suggest that research should focus on ameliorating the chronic effects of cannabinoids to induce insulin resistance.

**AUTHOR CONTRIBUTION**

Alonso Vilches-Flores and Shanta Persaud were involved in the study concept and design; Alonso Vilches-Flores was responsible for performing the experiments and data analysis with Astrid Hauge-Evans; Alonso Vilches-Flores and Shanta Persaud drafted the paper; Shanta Persaud and Peter Jones critically revised the paper; Alonso Vilches-Flores and Shanta Persaud obtained funding; all authors read and approved the final paper.

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