OPEN ACCESS

KING'S College LONDON

King's Research Portal

DOI:
10.1016/j.bbacli.2016.03.009

Document Version
Publisher's PDF, also known as Version of record

Link to publication record in King's Research Portal

Citation for published version (APA):
Vilches-Flores, A., Franklin, Z., Hauge-Evans, A. C., Liu, B., Huang, G. C., Choudhary, P., Jones, P. M., & Persaud, S. J. (2016). Prolonged activation of human islet cannabinoid receptors in vitro induces adaptation but not dysfunction. *BBA Clinical*. https://doi.org/10.1016/j.bbacli.2016.03.009

Citing this paper
Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights
Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

•Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
•You may not further distribute the material or use it for any profit-making activity or commercial gain
•You may freely distribute the URL identifying the publication in the Research Portal

Take down policy
If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 25. Nov. 2020

Accepted Manuscript

Prolonged activation of human islet cannabinoid receptors in vitro induces adaptation but not dysfunction

Alonso Vilches-Flores, Zara Franklin, Astrid C. Hauge-Evans, Bo Liu, Guo C. Huang, Pratik Choudhary, Peter M. Jones, Shanta J. Persaud

PII: S2214-6474(16)30013-7
DOI: doi: 10.1016/j.bbacli.2016.03.009
Reference: BBACLI 103

To appear in: *BBA Clinical*

Received date: 22 January 2016
Revised date: 26 March 2016
Accepted date: 29 March 2016

Please cite this article as: Alonso Vilches-Flores, Zara Franklin, Astrid C. Hauge-Evans, Bo Liu, Guo C. Huang, Pratik Choudhary, Peter M. Jones, Shanta J. Persaud, Prolonged activation of human islet cannabinoid receptors in vitro induces adaptation but not dysfunction, *BBA Clinical* (2016), doi: 10.1016/j.bbacli.2016.03.009

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Prolonged activation of human islet cannabinoid receptors in vitro induces adaptation but not dysfunction.

Alonso Vilches-Flores[a,b], Zara Franklin[a], Astrid C. Hauge-Evans[a,c], Bo Liu[a] Guo C Huang[a], Pratik Choudhary[a], Peter M. Jones[a], Shanta J. Persaud[a]

[a]Diabetes Research Group, Division of Diabetes and Nutritional Sciences, King's College London, UK.

[b]Universidad Nacional Autonoma de Mexico, FES Iztacala. Mexico.

[c]Department of Life Sciences, University of Roehampton, London, UK.

Corresponding author: Shanta J. Persaud, Diabetes Research Group, Division of Diabetes & Nutritional Sciences, King's College London, 2.9N Hodgkin Building, Guy's campus, London SE1 1UL, UK. E-mail: shanta.persaud@kcl.ac.uk

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Abstract.

Background: Although in vivo studies have implicated endocannabinoids in metabolic dysfunction, little is known about direct, chronic activation of the endocannabinoid system (ECS) in human islets. Therefore, this study investigated the effects of prolonged exposure to cannabinoid agonists on human islet gene expression and function.

Methods: Human islets were maintained for 2 and 5 days in the absence or presence of CB1r (ACEA) or CB2r (JWH015) agonists. Gene expression was quantified by RT-PCR, hormone levels by radioimmunoassay and apoptosis by caspase activities.

Results: Human islets express an ECS, with mRNAs encoding the biosynthetic and degrading enzymes NAPE-PLD, FAAH and MAGL being considerably more abundant than DAGLα, an enzyme involved in 2-AG synthesis, or CB1 and CB2 receptor mRNAs. Prolonged activation of CB1r and CB2r altered expression of mRNAs encoding ECS components, but did not have major effects on islet hormone secretion. JWH015 enhanced insulin and glucagon content at 2 days, but had no effect after 5 days. Treatment with ACEA or JWH015 for up to 5 days did not have marked effects on islet viability, as assessed by morphology and caspase activities.

Conclusions: Maintenance of human islets for up to 5 days in the presence of CB1 and CB2 receptor agonists causes modifications in ECS element gene expression, but does not have any major impact on islet function or viability.

General Significance: These data suggest that the metabolic dysfunction associated with over-activation of the ECS in obesity and diabetes in humans is unlikely to be secondary to impaired islet function.

Keywords: human islets, endocannabinoid system, gene expression, insulin, glucagon, apoptosis.

ACCEPTED MANUSCRIPT

Abbreviations

ACEA: *N*-(2-Chloroethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eiscosatetraenamide

AEA: anandamide

2-AG: 2-arachidonoyl glycerol

CB1r: cannabinoid receptor type 1

CB2r: cannabinoid receptor type 2

DAGL: diacylglycerol lipase

ECS: endocannabinoid system

FAAH: fatty acid amide hydrolase

JWH015: (2-methyl-1propyl-1*H*-indol-3-yl)-1-napthalenylmethanone

MAGL: monoacylglycerol lipase

NAPE-PLD: N-acyl-phosphatidyl ethanolamide-hydrolysing phospholipase D

PPG: preproglucagon

PPI: preproinsulin

Highlights

- Human islets express an endogenous cannabinoid system (ECS).
- Prolonged activation of human islet CB1 and CB2 receptors modifies expression of ECS elements
- This is not accompanied by marked changes in insulin and glucagon levels or islet viability.
- Metabolic dysfunction following ECS activation is not due to impaired islet function or viability.

ACCEPTED MANUSCRIPT

1. Introduction

Islets of Langerhans have a limited capacity to adapt to different environmental challenges such as hyperglycaemia, hyperlipidaemia, pregnancy and obesity. When this narrow threshold is insufficient to compensate for alterations in metabolic state, inadequate β-cell turnover and islet dysfunction may lead to the development of type 2 diabetes. In this context, accumulating evidence suggests that the endocannabinoid system (ECS) may be involved in the dysregulation of fuel homeostasis through the hypersecretion of the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) from expanding adipocytes in obesity [1-8]. Sustained elevations in endocannabinoid levels in the circulation that occur following ECS over-activation in obese and diabetic patients are reported to have detrimental effects in peripheral organs, leading to impaired insulin sensitivity, glucose intolerance and dyslipidaemia [3, 4, 7, 9, 10]. In addition, central CB1 receptor activation by endocannabinoids has orexigenic effects [11-14], and chronic exposure to cannabinoids is reported to lead to changes in the expression of lipogenic genes and lipid content in hepatocytes [4, 15, 16], and to the induction of apoptosis in sebocytes [5].

Rodent and human islets express a local ECS consisting of the enzymes required for the synthesis (NAPE-PLD and DAGL) and degradation (FAAH and MAGL) of AEA and 2-AG, and the endocannabinoids can activate the CB1 and CB2 cannabinoid receptors that are also expressed by islet cells [10, 17-35]. There is some controversy over whether acute activation of CB1 and CB2 receptors is coupled to stimulation [17, 19, 23-27, 29-31, 33, 34], or inhibition [10, 18, 22, 28, 32] of insulin secretion from rodent islets. However, our earlier studies in isolated human islets indicate that CB1 and CB2 receptor agonists can stimulate insulin secretion [17, 30], suggesting that cannabinoids may have beneficial effects in human β-cells. Most studies investigating the effects of cannabinoid agonists and antagonists on islet function in vitro have focused on acute signalling [10, 18, 19, 22, 23, 25-34], but 2-AG and AEA levels are chronically elevated in the pancreas under conditions of diet-induced obesity [6, 25], and these endocannabinoids are also increased in human islets in response to high glucose concentrations [3, 10]. Excess endocannabinoid production by the local islet ECS in obesity and diabetes could therefore have a chronic impact on islet function [35].

We have previously evaluated the long term effects of mouse islet CB1r or CB2r over-activation on ECS gene expression, on insulin and glucagon secretion and on islet viability, and found that there were no major functional effects [24]. However, similar studies on the effects of chronic exposure of human islets to cannabinoids have not been carried out although cannabinoids have been implicated in mouse β-cell death [22]. Therefore, the aim of the current study was to identify whether isolated human islets maintained chronically with

ACCEPTED MANUSCRIPT

cannabinoid agonists show modifications in expression of genes coding for elements of the ECS, and/or alterations in islet secretory function and viability.

2. Materials and Methods

2.1 Materials.

DMEM (5.5mM glucose), penicillin/streptomycin, L-glutamine, collagenase (Type XI), and PCR primers for human CB1r, CB2r, MAGL, NAPE-PLD, FAAH, DAGL and 18s rRNA were purchased from Sigma Aldrich (Dorset, UK). Primers for human preproinsulin and preproglucagon, RNeasy mini kits, and QuantiFast SYBR Green PCR Kit, were obtained from Qiagen (West Sussex, UK). Real-time PCR master mix and plates were purchased from Roche Diagnostics Ltd. (West Sussex, UK). *N*-(2-chloroethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eiscosatetraenamide (ACEA) and (2-methyl-1propyl-1*H*-indol-3-yl)-1-napthalenylmethanone (JWH015) were from Tocris Biosciences (Bristol, UK). Fetal bovine serum and reverse transcription reagents were purchased from Invitrogen (Paisley, UK), and Caspase-3/7-Glo apoptosis kits were obtained from Promega UK (Southampton, UK).

2.2 Human islet exposure to cannabinoids.

Human islets were isolated and purified from non-diabetic, heart-beating donors (with ethical permission) by the King's College Islet Transplantation Unit, as previously reported [36]. Human islets from eight separate donors with the following characteristics were used in this study: 5 male, 3 female; age 45±4.2; BMI 27±1.0. Hand-picked, size-matched islets were maintained in culture with DMEM containing 5.5mM glucose, 10% FBS, 2mM L-glutamine, and 100 units/ml penicillin/100μg/ml streptomycin, for 2 or 5 days in the presence or absence of either 100nM of the CB1r-selective agonist ACEA, or 100nM of the CB2r-selective agonist JWH015. Control groups of islets were incubated in the presence of 0.001% DMSO, the vehicle used for preparation of ACEA and JWH015. Culture media replacement and microscopic observation of the islets was performed daily.

2.3 Polymerase chain reaction (PCR).

Total RNA was isolated using RNeasy mini kits from groups of 150-200 human islets previously treated for 2 or 5 days in the absence or presence of ACEA or JWH015 except for quantification of mRNAs encoding ECS elements, where four groups of 150-200 human islets were incubated overnight, without treatment, immediately after provision by the King's College Islet Transplantation Unit. RNA samples were adjusted to obtain 20-50ng/μl of cDNA by reverse transcription reactions, as previously reported [24]. Real-time SYBR Green PCR amplifications were performed with a LightCycler 480 96 well-plate system, using the

ACCEPTED MANUSCRIPT

primers and conditions described in Table 1. Relative expression of mRNAs was determined after normalisation against 18s rRNA as an internal reference gene, and calculated by the $2^{-\Delta\Delta}$Ct method [37].

2.4 Insulin and glucagon secretion.

Human islets that had been maintained in culture for 2 or 5 days in the absence or presence of 100nM ACEA or 100nM JWH015 were washed then pre-incubated for one hour at 37°C in 400μl of physiological buffer [38] containing 2mM glucose. For measurements of insulin and glucagon secretion groups of 5 and 10 islets respectively were transferred to Eppendorf tubes and incubated for one hour at 37°C in 500μl of buffer supplemented with 16mM glucose or 10mM arginine. Insulin and glucagon secreted from the islets in these static incubation experiments was quantified by radioimmunoassay [17,39].

2.5 Insulin and glucagon content.

Acidified ethanol (100μl) was added to four groups of 10 human islets that had been exposed for 2 or 5 days to DMEM (5.5mM glucose) alone or with DMEM supplemented with 100nM of either ACEA or JWH015. The islet samples were sonicated and stored at -20°C until radioimmunoassays were performed to measure insulin and glucagon contents.

2.6 Caspase-3/7 activities.

The extent of apoptosis of human islets previously maintained for 2 or 5 days in the absence or presence of cannabinoid agonists was determined by detection of caspase-3/7 activities with a luminometer following cleavage of a proluminescent substrate (Z-DEVD-aminoluciferin), as previously reported [40]. Apoptosis was induced in these experiments by exposing five islets from each group to a cytokine cocktail (0.5U/μl IL-1β, 5U/μl TNFα, and 5U/μl IFNγ) for the final 20 hours of incubation.

2.7 Statistical analysis.

Data are expressed as means ± SEM of 3 or 4 individual experiments. One-way and two-way ANOVA with Bonferroni's test were used for analyses and differences between treatments were considered statistically significant at $p<0.05$.

3. Results

3.1 Relative mRNA expression of the endocannabinoid signalling elements in human islets.

Quantitative RT-PCR using human islet cDNAs indicated that the mRNA encoding FAAH, an enzyme responsible for degradation of the endocannabinoid AEA, was the most

ACCEPTED MANUSCRIPT

abundant component of the endocannabinoid system in human islets. It was expressed at levels similar to the mRNA coding for NAPE-PLD, which catalyses biosynthesis of AEA from the membrane phospholipid *N*-acylphosphatidylethanolamine, and both mRNAs were of similar abundance to preproglucagon mRNA (Figure 1). The mRNAs encoding the enzymes that regulate the synthesis (DAGLα) and degradation (MAGL) of the other major endocannabinoid, 2-AG, were also detected in human islets but these, in particular DAGLα, showed lower levels of expression than NAPE-PLD and FAAH mRNAs (Figure 1). Figure 1 also indicates that CB1 and CB2 receptor mRNAs were detectable in human islets, but their expression levels were only approximately 0.1% of those of preproinsulin mRNA, which encodes the major islet hormone.

3.2 Effects of prolonged exposure to ACEA and JWH015 on ECS element gene expression in human islets.

Quantitative RT-PCR demonstrated that human islets exposed to a CB1r (100nM ACEA) or CB2r (100nM JWH015) agonist for 2 or 5 days affected expression of ECS elements. Thus, as can be seen from Figure 2A, stimulation of CB1r with ACEA (grey bars) induced significant decreases in NAPE-PLD, FAAH and MAGL mRNA levels at 2 days. While exposure of islets to JWH015 for 2 days (black bars) also significantly decreased NAPE-PLD mRNA it did not affect FAAH or MAGL expression, but it did induce significant up-regulation of mRNAs encoding CB1r and CB2r. Exposure of islets to ACEA for 5 days induced a 50% increase in CB1r mRNA levels and a significant reduction in DAGLα mRNA expression (Figure 2B), while 100nM JWH015 caused at least 50% reductions in mRNAs encoding CB1r, FAAH and MAGL after 5days. These gene expression studies indicate that relatively short-term activation of CB1r by ACEA caused down-regulation of mRNAs encoding enzymes involved in endocannabinoid synthesis and degradation in human islets within 2 days, while chronic activation of CB2 receptors over 5 days decreased mRNAs encoding cannabinoid degradation.

3.3 Effects of prolonged cannabinoid receptor activation on insulin and glucagon secretion from human islets

Static incubation secretion experiments were performed to evaluate whether chronic exposure to the cannabinoid agonists affected acute glucose-stimulated insulin secretion (Figure 3A and 3C) or acute arginine-induced glucagon secretion (Figure 3B and 3D) from human islets previously maintained in culture in the absence or presence of 100nM ACEA or JWH015 for 2 (upper panels) or 5 days (lower panels).

Islets that had been maintained in culture for 2 days and 5 days showed significant elevations in insulin secretion in response to 16mM glucose (Figure 3A and 3C, control),

ACCEPTED MANUSCRIPT

although basal and glucose-stimulated insulin secretion were significantly reduced after 5 days in culture, compared to culture for 2 days. Basal insulin secretion at 3mM glucose was significantly increased in human islets after incubation for 2 days with 100nM ACEA, but the magnitude of insulin secretion at 16mM glucose was not affected by 48 hour pre-exposure to ACEA (Figure 3A). Islets that had been treated with ACEA for 5 days showed a reduced secretory response to 16mM glucose, but there was still a significant elevation in insulin release over the low basal insulin output (Figure 3C). Treatment of human islets with 100nM JWH015 for 2 days and 5 days had no significant effect on basal or glucose-stimulated insulin secretion (Figure 3A and 3C).

Islets responded to acute exposure to 10mM arginine after 2 and 5 days in culture with significant elevations in glucagon secretion (Figure 3B and 3D, control), although the extent of glucagon release was significantly reduced after 5 days. Pre-exposure of human islets to 100nM ACEA for 2 days significantly elevated basal glucagon secretion without modifying the amplitude of the glucagon secretory response to 10mM arginine (Figure 3B), while islets incubated with ACEA for 5 days did not show any alterations in basal or arginine-stimulated glucagon output (Figure 3D). Islets that had been exposed to JWH015 did not show a significant glucagon secretory response to 10mM arginine (Figure 3B), but they responded appropriately to arginine after they had been maintained in the presence of JWH015 for 5 days (Figure 3D).

3.4 Effects of chronic exposure to cannabinoid receptors agonists on insulin and glucagon content in human islets.

Since prolonged culture of human islets resulted in changes in insulin and glucagon secretion, as shown in Figure 3, further experiments were carried out to quantify hormone content after 2 days and 5 days culture in the absence and presence of ACEA and JWH015. It can be seen from Figure 4C (white bars) that maintenance of human islets in culture for 5 days resulted in significant reduction in insulin content compared to islets after 2 days in culture (Figure 4A), but there was no significant change in glucagon content of the islets under the same conditions (Figure 4B and 4D, white bars). Exposure of human islets to the CB1r agonist ACEA for 2 and 5 days had no effect on insulin content (Figure 4A and 4C, grey bars). However, ACEA induced a significant increase in glucagon content after 2 days (Figure 4B, grey bars), and this could be responsible for the elevated basal glucagon secretory response seen after maintenance of islets in the presence of 100nM ACEA for 2 days (Figure 3B). The CB2r agonist JWH015 caused a marked increase in insulin content at 2 days (Figure 4A, black bars). This increase was not sustained after 5 days of treatment with JWH015, and islet insulin levels under this condition were not significantly different from

ACCEPTED MANUSCRIPT

the reduced content seen in the control islets. JWH015 also increased islet glucagon content after 2 days, but not at 5 days (Figure 4B, black bars).

3.5 Effects of chronic exposure to cannabinoid receptor agonists on human islet morphology and apoptosis.

The photomicrographs of human islets (Figure 5) indicate that maintenance for 2 days (upper panels) or 5 days (lower panels) in the absence (left) or presence of 100nM ACEA (middle) or JWH015 (right) did not cause any major deleterious effects on integrity, although after 5 days in culture all islets were generally less compact, with the loss of some cells. This was associated with increased basal apoptosis after 5 days compared to that seen at 2 days (Figure 6A control, white bars versus Figure 6B). Evaluation of the effects of chronic exposure to cannabinoid agonists on human islet cell caspase 3/7 activities was carried out in the absence and presence of mixed cytokines (black bars) and these studies indicated that pre-exposure to ACEA and JWH015 for 2 (Figure 6A) or 5 days (Figure 6B) had no effects on human islet cell apoptosis.

4. Discussion

We, and others, have previously identified components of a local ECS in human islets by PCR and immunohistochemistry [10, 17, 20, 21, 30], but the relative expression of these elements has not been fully investigated. Our observations indicate a relatively high mRNA expression of FAAH, NAPE-PLD and MAGL in human islets, while DAGLα and both cannabinoid receptors show lower expression levels. The high levels of FAAH mRNA that we detected in human islets is consistent with our earlier observations that this is the most abundant ECS mRNA in mouse islets [24], as are our observations of low levels of CB1r, CB2r and DAGLα. It has previously been reported that CB1 expression is considerably higher than CB2 in human islets [20], but this differential expression of the cannabinoid receptors was not confirmed in the current study. We did find here that human islets express substantially more MAGL mRNA than mouse islets do [17, 24], suggesting that 2-AG levels are tightly controlled in human islets through MAGL-dependent degradation. Since whole islets were used for mRNA purification in these studies and our earlier work with mouse islets [24], it was not possible to determine the distribution and specific expression of the ECS elements in each islet cell type, but immunofluorescence staining of human pancreas has indicated the presence of MAGL, DAGLα, NAPE-PLD and FAAH in β-cells [10]. It is therefore likely that the endogenous production, accumulation and degradation of endocannabinoids is important in the modulation of islet functions.

ACCEPTED MANUSCRIPT

Previous studies have demonstrated that expression of ECS components is a dynamic process that is modified in response to high fat diet, and high glucose concentrations are reported to increase AEA and 2-AG synthesis in rodent and human islets [10, 20]. In the current study the effects of 100nM of CB1r (ACEA) and CB2r (JWH015) agonists were investigated, to provide a direct comparison with an earlier study in which we had investigated the effects of chronic activation of cannabinoid receptors in mouse islets [24]. The changes that we observed in mRNA levels in isolated human islets of the enzymes involved in the synthesis and degradation of endocannabinoids suggest a negative feedback mechanism in the presence of exogenous agonists, most likely to limit endogenous endocannabinoid levels to minimise over-stimulation of the CB1 and CB2 receptors. However, it is interesting that while chronic activation of human islet cannabinoid receptors led to depletion of mRNAs responsible for regulation of endocannabinoid levels there was a simultaneous increase in the mRNAs encoding CB1r and CB2r, which may have been a mechanism for sustained responses to these ligands. Generation of AEA and 2-AG in the human islets following exposure to ACEA and JWH015 was not quantified in the current study, but it is possible that the up-regulation of CB1r as well as CB2r mRNA following exposure to JWH015 for 2 days was a consequence of altered synthesis of the CB1r ligand AEA by the islets. The role played by endogenous human islet endocannabinoids in the patterns of ECS gene expression observed here will be the subject of future work.

These changes in the ECS mRNAs in human islets in vitro support a model of dynamic regulation of islet endocannabinoid content to maintain the “on-demand” responses of islets to physiological and pathological stimuli [3, 6, 19, 24, 26, 31, 32]. However, the current study does not provide direct information that islets in situ would necessarily respond in the same manner as islets in vitro. Moreover, the use of whole islets, while providing an appropriate physiological model, does not allow identification of the cells in which the alterations in ECS mRNAs occurs.

Although exposure of human islets to ACEA and JWH015 for 2 days increased insulin and glucagon content, there were no marked effects on acute secretion of these hormones, suggesting that islet stores of insulin and glucagon were enhanced, rather than β- or α-cell secretory capacity. Overall, maintenance of human islets with the cannabinoid receptor agonists was without deleterious effects, either on the capacity of islets to secrete insulin and glucagon in response to glucose and arginine respectively, or in terms of islet cell viability. Our observations that apoptosis was not enhanced by exposure of islets to CB1r or CB2r agonists is in contrast to observations that acute and chronic exposure to cannabinoids induces apoptosis in a number of cell types [5, 41-43], including islet β-cells [22]. However, there is also evidence supporting a protective role of 2-AG and AEA in cell survival [44-47],

ACCEPTED MANUSCRIPT

and we have previously reported that pre-exposure of mouse islets to 100nM ACEA or JWH015 for 7 days protects against cytokine-induced apoptosis [24]. A similar protective effect was not observed in the current studies, which may reflect the 5 day time-course of exposure here and 7 days with mouse islets, or it may indicate species-dependent differences in apoptotic signalling downstream of cannabinoid receptor activation in mouse and human islets. Nonetheless, our data clearly demonstrate that chronic treatment of human islets with 100nM ACEA or JWH015 does not induce apoptosis. This apparent discrepancy with an earlier study in islets [22] may be due to the use in that study of 25μM 2-AG and AEA to maximally reduce islet cell viability over 48 hours, a concentration that is several orders of magnitude higher than detected in diabetes [3], and considerably higher than those used in the current study.

5. Conclusion

In conclusion, we report that prolonged maintenance of isolated human islets in the presence of cannabinoid CB1 and CB2 receptor agonists modulates the gene expression of ECS components and promotes short-term increases in islet insulin and glucagon content, but does not exert deleterious effects on islet viability or function. Our results suggest that over-activation of islet endocannabinoid receptors induces an adaptation process through down-regulation of endogenous islet cannabinoid levels, but the maintenance of islet viability and function suggests that islet cell death and dysfunction do not play a role in the metabolic defects arising from over-activation of the ECS in obesity and diabetes. Thus, these data are consistent with the impairments in glucose homeostasis observed following increased production of endocannabinoids being a consequence of insulin resistance rather than β-cell dysfunction or α-cell hypersecretion.

Acknowledgements

We are grateful to the relatives of organ donors for human pancreases for islet isolation. This project was supported by The Novo Nordisk UK Research Foundation; Diabetes UK [BDA: RD07/0003510]; Programa de estancias posdoctorales y sabáticas al extranjero para la consolidación de grupos de investigación 2010-2012, from the National Council of Science and Technology (CONACYT), Mexico [#00166231]; and Programa de apoyos para la superación del personal académico PASPA, from the DGAPA-UNAM, Mexico.

References

ACCEPTED MANUSCRIPT

1. Scheen, A.J. Cannabinoid-1 receptor antagonists in type-2 diabetes. Best. Pract. Res. Clin. Endocrinol. Metab. 2007, **21**: 535-553

2. Li, C., Jones, P.M., Persaud, S.J. Role of the endocannabinoid system in food intake, energy homeostasis and regulation of the endocrine pancreas. Pharmacology and Therapeutics. 2011, **129**: 307-320

3. Matias, I., Gonthier, M.P., Orlando, P., Martiadis, V., De Petrocellis, L., Cervino, C., Petrosino, S., Hoareau, L., Festy, F., Pasquali, R., Roche, R., Maj, M., Pagotto, U., Monteleone, P., Di Marzo, V. Regulation, function, and dysregulation of endocannabinoids in models of adipose and beta-pancreatic cells and in obesity and hyperglycemia. J. Clin. Endocrinol. Metab. 2006, **91**: 3171-3180

4. Osei-Hyiaman, D., DePetrillo, M., Pacher, P., Liu, J., Radaeva, S., Bátkai, S., Harvey-White, J., Mackie, K., Offertáler, L., Wang, L., Kunos, G. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. J. Clin. Invest. 2005, **115**: 1298-1305

5. Dobrosi, N., Tóth, B.I., Nagy, G., Dózsa, A., Géczy, T., Nagy, L., Zouboulis, C.C., Paus, R., Kovács, L., Bíró, T. Endocannabinoids enhance lipid synthesis and apoptosis of human sebocytes via cannabinoid receptor-2-mediated signaling. FASEB J. 2008, **22**(10): 3685-3695

6. Starowicz, K.M., Cristino, L., Matias, I., Capasso, R., Racioppi, A., Izzo, A.A., Di Marzo, V. Endocannabinoid dysregulation in the pancreas and adipose tissue of mice fed with a high-fat diet. Obesity. 2008, **16**(3): 553-565

7. Engeli, S., Böhnke, J., Feldpausch, M., Gorzelniak, K., Janke, J., Bátkai, S., Pacher, P., Harvey-White, J., Luft, F.C., Sharma, A.M., Jordan, J. Activation of the peripheral endocannabinoid system in human obesity. Diabetes. 2005, **54** (10): 2838-2843

8. Cota, D. CB1 receptors: emerging evidence for central and peripheral mechanisms that regulate energy balance, metabolism, and cardiovascular health. Diabet. Metab. Res. Rev. 2007, **23**: 507-517

9. Eckardt, K., Sell, H., Taube, A., Koenen, M., Platzbecker, B., Cramer, A., Horrighs, A., Lehtonen, M., Tennagels, N., Eckel, J. Cannabinoid type 1 receptors in human skeletal muscle cells participate in the negative crosstalk between fat and muscle. Diabetologia. 2009, **52**: 664-674

10. Kim, W., Doyle, M.E., Liu, Z., Lao, Q., Shin, Y.K., Carlson, O.D., Kim, H.S., Thomas, S., Napora, J.K., Lee, E.K., Moaddel, R., Wang, Y., Maudsley, S., Martin, B., Kulkarni, R.N., Egan, J.M. Cannabinoids inhibit insulin receptor signaling in pancreatic β-cells. Diabetes 2011, **60**(4): 1198-1209

11. Scheen, A.J., Paquot, N. Use of cannabinoid CB1 receptor antagonists for the treatment of metabolic disorders. Best. Pract. Res. Clin. Endocrinol. Metab. 2009, **23** (1): 103-116

ACCEPTED MANUSCRIPT

12. Scheen A.J., Finer N., Hollander P., Jensen M.D., Van Gaal L.F.; RIO-Diabetes Study Group. Efficacy and tolerability of rimonabant in overweight or obese patients with type 2 diabetes: a randomised controlled study. Lancet. 2006; **368** (9548): 1660-1672

13. Pi-Sunyer F.X., Aronne L.J., Heshmati H.M., Devin J., Rosenstock J.; RIO-North America Study Group. Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. JAMA. 2006; **295** (7): 761-775

14. Bellocchio, L., Cervino, C., Pasquali, R., Pagotto, U. The endocannabinoid system and energy metabolism. J. Neuroendocrinol. 2008, **20** (6): 850-857

15. Ruby, M.A., Nomura, D.K., Hudak, C.S., Barber, A., Casida, J.E., Krauss, R.M. Acute overactive endocannabinoid signaling induces glucose intolerance, hepatic steatosis, and novel cannabinoid receptor 1 responsive genes. PLoS One. 2011, **6** (11): e26415

16. De Gottardi, A., Spahr, L., Ravier-Dall'Antonia, F., Hadengue, A. Cannabinoid receptor 1 and 2 agonists increase lipid accumulation in hepatocytes. Liver Int. 2010, **30** (10): 1482-1489

17. Li, C., Vilches-Flores, A., Zhao, M., Amiel, S.A., Jones, P.M., Persaud, S.J. (2012) Expression and function of monoacylglycerol lipase in mouse β-cells and human islets of Langerhans. Cell. Physiol. Biochem. **30**(2): 347-358

18. Juan-Picó, P., Fuentes, E., Bermúdez-Silva, F.J., Díaz-Molina, F., Ripoll, C., Rodríguez de Fonseca, F., Nadal, A. Cannabinoid receptors regulate Ca ($^{2+}$) signals and insulin secretion in pancreatic beta-cell. Cell. Calcium. 2006, **39**: 155-162

19. Vilches-Flores, A., Delgado-Buenrostro, N.L., Navarrete-Vazquez, G., Villalobos-Molina, R. CB1 receptor expression is regulated by glucose and feeding in rat pancreatic islets. Reg. Peptides. 2010, **163**: 81-87

20. Bermúdez-Silva, F.J., Suárez, J., Baixeras, E., Cobo, N., Bautista, D., Cuesta-Muñoz, A.L., Fuentes, E., Juan-Pico, P., Castro, M.J., Milman, G., Mechoulam, R., Nadal, A., Rodríguez de Fonseca, F. Presence of functional cannabinoid receptors in human endocrine pancreas. Diabetologia. 2008, **51**(3): 476-487

21. Tharp, W.G., Lee, Y.H., Maple, R.L., Pratley, R.E. The cannabinoid CB1 receptor is expressed in pancreatic delta-cells. Biochem. Biophys. Res. Commun. 2008, **372**: 595-600

22. Kim, W., Lao, Q., Shin, Y.K., Carlson, O.D., Lee, E.K., Gorospe, M., Kulkarni, R.N., Egan, J.M. Cannabinoids induce pancreatic β-cell death by directly inhibiting insulin receptor activation. Sci. Signal. 2012, **5**(216): ra23 1-13

23. Li, C., Bowe, J.E., Jones, P.M., Persaud, S.J. Expression and function of cannabinoid receptors in mouse islets. Islets. 2010, **2**: 1-10

24. Vilches-Flores A., Hauge-Evans A.C., Jones P.M., Persaud S.J. Chronic activation of cannabinoid receptors in vitro does not compromise mouse islet function. Clin Sci (Lond).

ACCEPTED MANUSCRIPT

2013; **124** (7):467-478

25. Flores L.E., Alzugaray M.E., Cubilla M.A., Raschia M.A., Del Zotto H.H., Román C.L., Suburo A.M., Gagliardino J.J. Islet cannabinoid receptors: cellular distribution and biological function. Pancreas. 2013; **42** (7): 1085-1092

26. Anderson R.L., Randall M.D., Chan S.L. The complex effects of cannabinoids on insulin secretion from rat isolated islets of Langerhans. Eur J Pharmacol. 2013; **706** (1-3): 56-62

27. Li, C., Jones, P.M., Persaud, S.J. Cannabinoid receptors are coupled to stimulation of insulin secretion from mouse MIN6 β-cells. Cell. Physiol. Biochem. 2010. **26**: 187-196

28. Nakata, M., Yada, T. Cannabinoids inhibit insulin secretion and cytosolic Ca^{2+} oscillation in islet beta-cells via CB1 receptors. Regul. Pept. 2008, **145**: 49-53

29. Getty-Kaushik, L., Richard, A.M., Deeney, J.T., Krawczyk, S., Shirihai, O., Corkey, B.E. The CB1 antagonist rimonabant decreases insulin hypersecretion in rat pancreatic islets. Obesity. 2009, **17**(10): 1856-1860

30. Li, C., Bowe, J.E., Huang, G.C., Amiel, S.A., Jones, P.M., Persaud, S.J. Cannabinoid receptor agonists and antagonists stimulate insulin secretion from isolated human islets of Langerhans. Diabetes. Obes. Metab. 2011, **13**(10): 903-910

31. González-Mariscal I., Krzysik-Walker S.M., Kim W., Rouse M., Egan J.M. Blockade of Cannabinoid 1 Receptor Improves GLP-1R Mediated Insulin Secretion in Mice. Mol Cell Endocrinol. 2015. pii: S0303-7207 (**15**): 30171-30174.

32. Bermudez-Silva F.J., Romero-Zerbo S.Y., Haissaguerre M., Ruz-Maldonado I., Lhamyani S., El Bekay R., Tabarin A., Marsicano G., Cota D. Cannabinoid CB1 receptors and mTORC1 signalling pathway interact to modulate glucose homeostasis. Dis Model Mech. 2015, **12**. pii: dmm.020750

33. Malenczyk K., Jazurek M., Keimpema E., Silvestri C., Janikiewicz J., Mackie K., Di Marzo V., Redowicz M.J., Harkany T., Dobrzyn A. CB1 cannabinoid receptors couple to focal adhesion kinase to control insulin release. J Biol Chem. 2013; **288** (45): 32685-32699

34. De Petrocellis, L., Marini, P., Matias, I., Moriello, A.S., Starowicz, K., Cristino, L., Nigam, S., Di Marzo, V. Mechanisms for the coupling of cannabinoid receptors to intracellular calcium mobilization in rat insulinoma beta-cells. Exp Cell Res. 2007. **313** (14): 2993-3004

35. Duvivier, V.F., Delafoy-Plasse, L., Delion, V., Lechevalier, P., Le Bail, J.C., Guillot, E., Pruniaux, M.P., Galzin, A.M. Beneficial effect of a chronic treatment with rimonabant on pancreatic function and beta-cell morphology in Zucker Fatty rats. Eur. J. Pharmacol. 2009. **616**: 314-320

36. Huang G.C., Zhao M., Jones P., Persaud S., Ramracheya R., Löbner K., Christie M.R., Banga J.P., Peakman M., Sirinivsan P., Rela M., Heaton N., Amiel S. The development of new density gradient media for purifying human islets and islet-quality assessments. Transplantation. 2004; **77** (1): 143-145

ACCEPTED MANUSCRIPT

37. Livak, K.J., Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001, **25** (4): 402-408

38. Gey, G.O., Gey, M.K. The maintenance of human normal cells and tumor cells in continuous culture, I. Preliminary report: Cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation. Am. J. Cancer. 1936: 27-45

39. Jones, P.M., Persaud, S.J., Howell, S.L. Time-course of Ca2+-induced insulin secretion from perifused, electrically permeabilised islets of Langerhans: effects of cAMP and a phorbol ester. Biochem. Biophys. Res. Commun. 1989, **162** (3): 998-1003

40. Muller, D., Jones, P.M., Persaud, S.J. Autocrine anti-apoptotic and proliferative effects of insulin in pancreatic β-cells. FEBS Lett. 2006, **580**: 6977-6980

41. Sánchez, C., Galve-Roperh, I., Canova, C., Brachet, P., Guzmán, M. (1998) Delta9-tetrahydrocannabinol induces apoptosis in C6 glioma cells. FEBS. Lett. **436** (1): 6-10

42. Lombard, C., Nagarkatti, M., Nagarkatti, P. CB2 cannabinoid receptor agonist, JWH-015, triggers apoptosis in immune cells: potential role for CB2-selective ligands as immunosuppressive agents. Clin. Immunol. 207, **122** (3): 259-270

43. Do, Y., McKallip, R.J., Nagarkatti, M., Nagarkatti, P.S. Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-kappaB-dependent apoptosis: novel role for endogenous and exogenous cannabinoids in immunoregulation. J. Immunol. 2004, **173** (4): 2373-2382

44. Esposito, G., De Filippis, D., Maiuri, M.C., De Stefano, D., Carnuccio, R., Iuvone, T. (2006) Cannabidiol inhibits inducible nitric oxide synthase protein expression and nitric oxide production in β-amyloid stimulated PC12 neurons through p38 MAP kinase and NF-kB involvement. Neurosci. Lett. **399**: 91–95

45. Fernández-Ruiz, J., Romero, J., Velasco, G., Tolón, R.M., Ramos, J.A., Guzmán, M. Cannabinoid CB2 receptor: a new target for controlling neural cell survival. Trends. Pharmacol. Sci. 2007, **28**: 39–45

46. Horváth B., Magid L., Mukhopadhyay P., Bátkai S., Rajesh M., Park O., Tanchian G., Gao R.Y., Goodfellow C.E., Glass M., Mechoulam R., Pacher P. A new cannabinoid CB2 receptor agonist HU-910 attenuates oxidative stress, inflammation and cell death associated with hepatic ischaemia/reperfusion injury. Br J Pharmacol. 2012; **165** (8): 2462-2478

47. Mukhopadhyay P, Rajesh M, Pan H, Patel V, Mukhopadhyay B, Bátkai S, Gao B, Haskó G, Pacher P. Cannabinoid-2 receptor limits inflammation, oxidative/nitrosative stress, and cell death in nephropathy. Free Radic Biol Med. 2010; **48** (3):457-67

ACCEPTED MANUSCRIPT

Table 1. Primer sequences and annealing temperatures used in real-time PCR.

Gene name for *Homo sapiens*	**Primer sequences**	**Annealing temp.**
Cannabinoid receptor 1 (CB1r)	F 5'-CACCTTCCGCACCATCACCAC-3' R 5'-GTCTCCCGCAGTCATCTTCTCTTG-3'	60 °C
Cannabinoid receptor 2 (CB2r)	F 5'-CATGGAGGAATGCTGGGTGAC-3' R 5'-GAGGAAGGCGATGAACAGGAG-3'	62 °C
Diacylglycerol lipase alpha (DAGLα)	F 5'-AGAATGTCACCCTCGGAATGG-3' R 5'-GTGGCTCTCAGCTTGACAAAGG-3'	62 °C
Monoacylglycerol lipase (MAGL)	F 5'-CAAGGCCCTCATCTTTGTGT-3' R 5'-ACGTGGAAGTCAGACACTAC-3'	60 °C
Fatty acid amide hydrolase (FAAH)	F 5'-CCCAGATGGAACATTACAGG-3' R 5'-CAGGATGACTGGTTTTCAGG-3'	60 °C
N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD)	F 5'-CACGGTAATGGTGGAAATGG-3' R 5'-GTCCAGATGGTCATAGTGGTTG-3'	62 °C
18s rRNA	F 5'-GGGAGCCTGAGAAACGG-3' R 5'-GGGTCGGGAGTGGGTAATTT-3'	60 °C
Preproinsulin	Qiagen Quantitect primer	60 °C
Preproglucagon	Qiagen Quantitect primer	60 °C

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure Legends

Figure 1. Groups of 150-200 human islets were incubated overnight after isolation under standard culture conditions and qPCR was used to quantify relative expression of human islet mRNAs encoding preproinsulin (PPI), preproglucagon (PPG), and the enzymes and receptors of the endocannabinoid system. Data are expressed as relative percentage with respect to PPI mRNA levels, obtained from real-time PCR amplification values, normalised to 18s rRNA as an internal reference (means+SEM, n=4).

Figure 2. Quantification of mRNAs encoding elements of the ECS in human islets after maintenance for 2 (A) and 5 days (B) in DMEM (5.5mM glucose) alone (open bars) or supplemented with 100nM ACEA (grey bars) or 100nM JWH015 (black bars). Data are expressed relative to the levels present in control islets in the absence of cannabinoid receptor agonists and normalised to 18s rRNA levels in the same samples; means+SEM, n=4 experiments. Data were analysed by one-way ANOVA; *p<0.05, **p<0.01 compared to the appropriate control.

Figure 3. Acute, one hour insulin (panels A and C) and glucagon (panels B and D) secretion from human islets after maintenance for 2 (upper panels) or 5 (lower panels) days in DMEM containing 5.5mM glucose alone (control), or supplemented with 100nM ACEA or 100nM JWH015. Basal levels of insulin and glucagon secretion at 3mM glucose are shown by the open bars and the black bars show insulin secretion at 16mM glucose (panels A and C) and glucagon secretion at 10mM arginine panels B and D); means+SEM, n=4 experiments. Data were analysed by two-way ANOVA; *p<0.05, **p<0.01 versus the appropriate basal secretion, $^{\#}$p<0.05 versus the appropriate control.

Figure 4. Insulin (panels A and C) and glucagon (panels B and D) hormone contents of human islets maintained for 2 (upper panels) or 5 (lower panels) days in the absence of cannabinoid receptor agonists (open bars) or with 100nM ACEA (grey bars) or 100nM JWH015 (black bars). Data are expressed as ng/islet (means+SEM, n=4). Data were analysed by one-way ANOVA; *p<0.05, **p<0.01 versus the appropriate control.

Figure 5. Micrographs of human islets maintained in culture for 2 (upper panels) or 5 (lower panels) days in DMEM containing 5.5mM glucose (left) or supplemented with 100nM ACEA (middle) or 100nM JWH015 (right). Micrographs are representative of 3 experiments using islets obtained from three different donors.

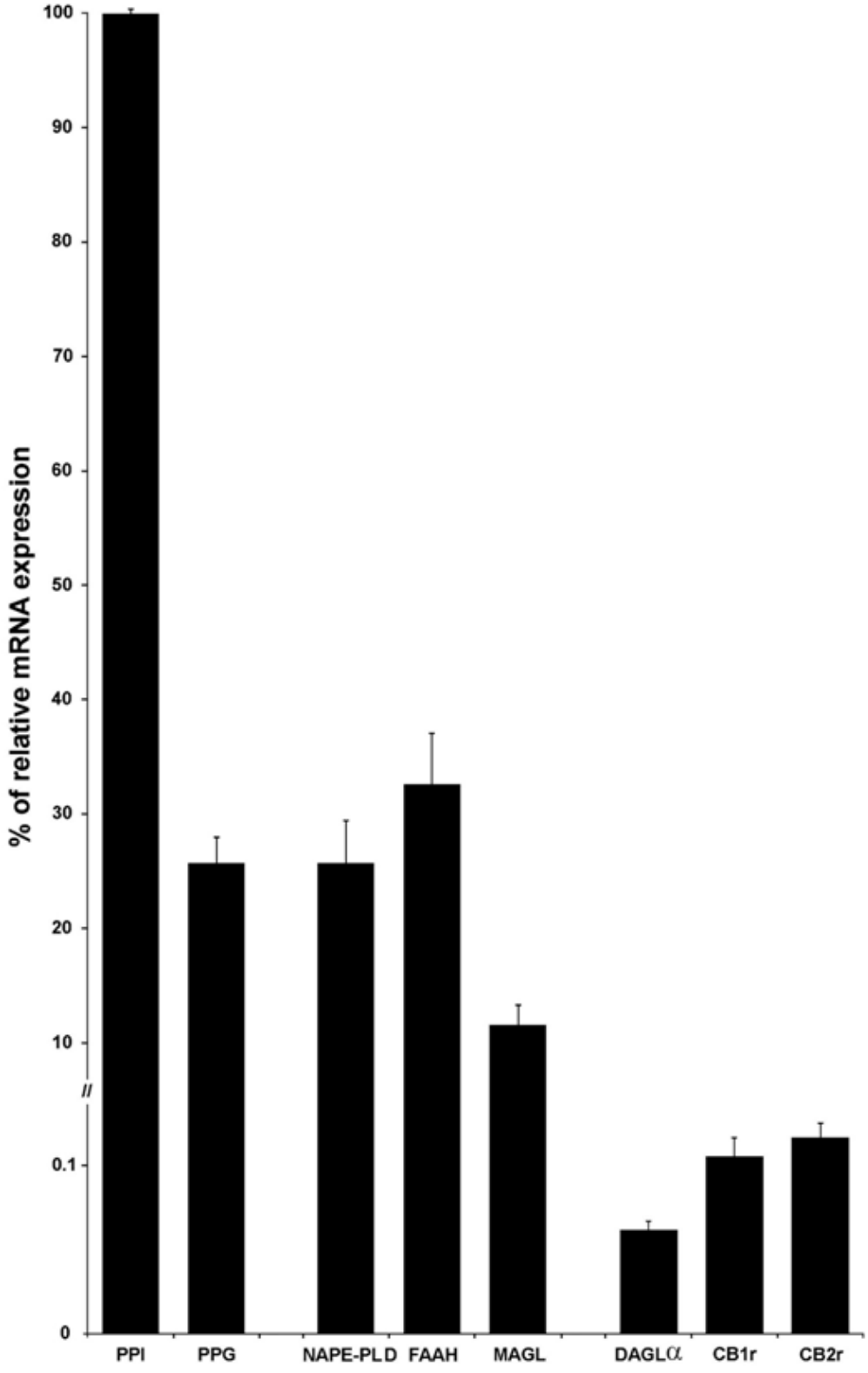
ACCEPTED MANUSCRIPT

Figure 6. Caspase-3/7 activities in human islets after maintenance for 2 (panel A) or 5 (panel B) days in DMEM containing 5.5mM glucose alone (control) or supplemented with 100nM ACEA or 100nM JWH015. Apoptosis was induced by adding a cytokine cocktail (0.5U/ml IL1β, 5U/ml TNFα, and 5U/ml IFNγ) for the final 20 hours of incubation, shown by the black bars. Data are expressed in luminescence units (means+SEM, n=6 for panel A, n=4 for panel B). Data were analysed by two-way ANOVA; *$p<0.05$ with respect to basal apoptosis in the absence of cytokines.

ACCEPTED MANUSCRIPT

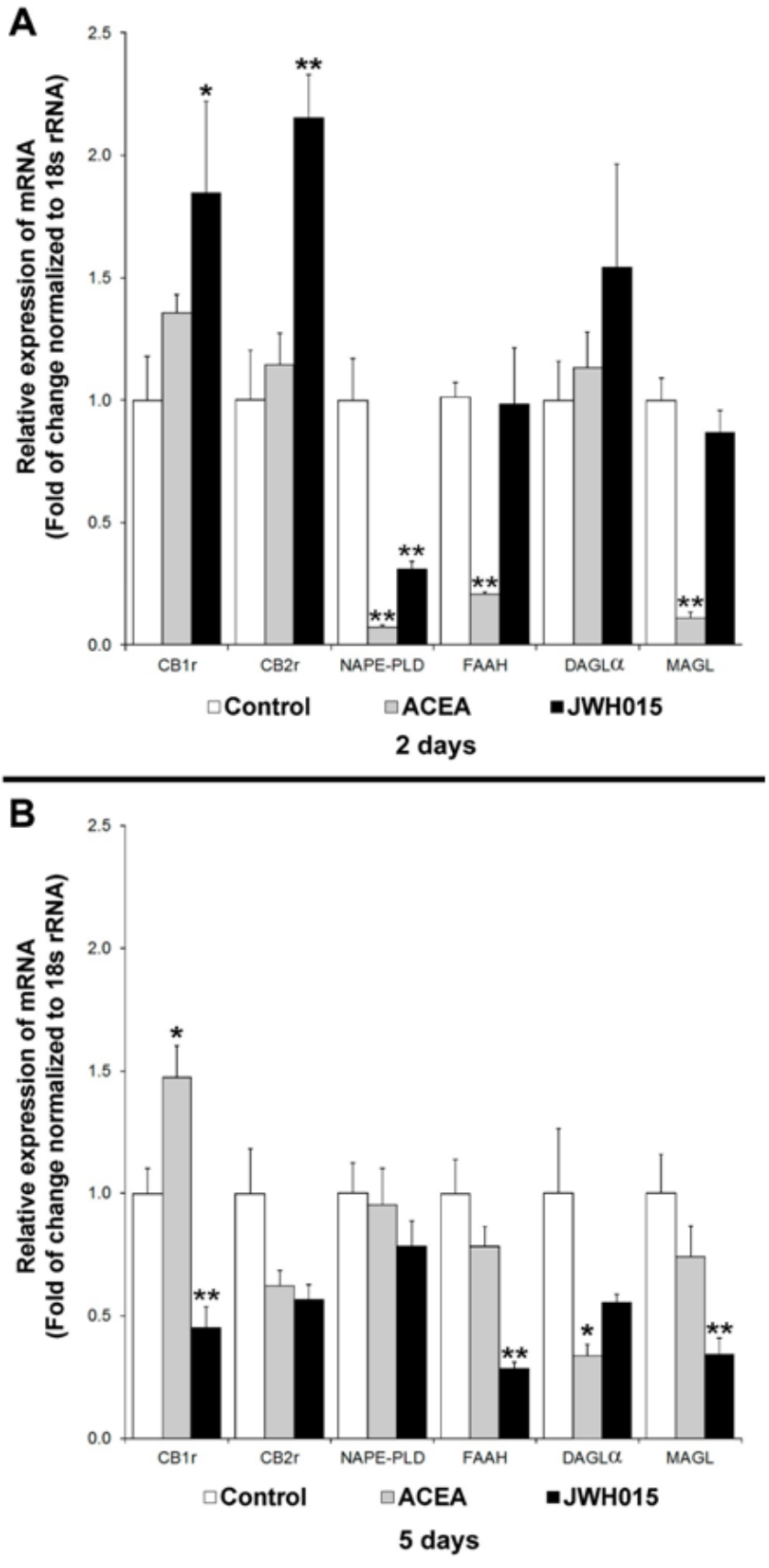
ACCEPTED MANUSCRIPT

Figure 1



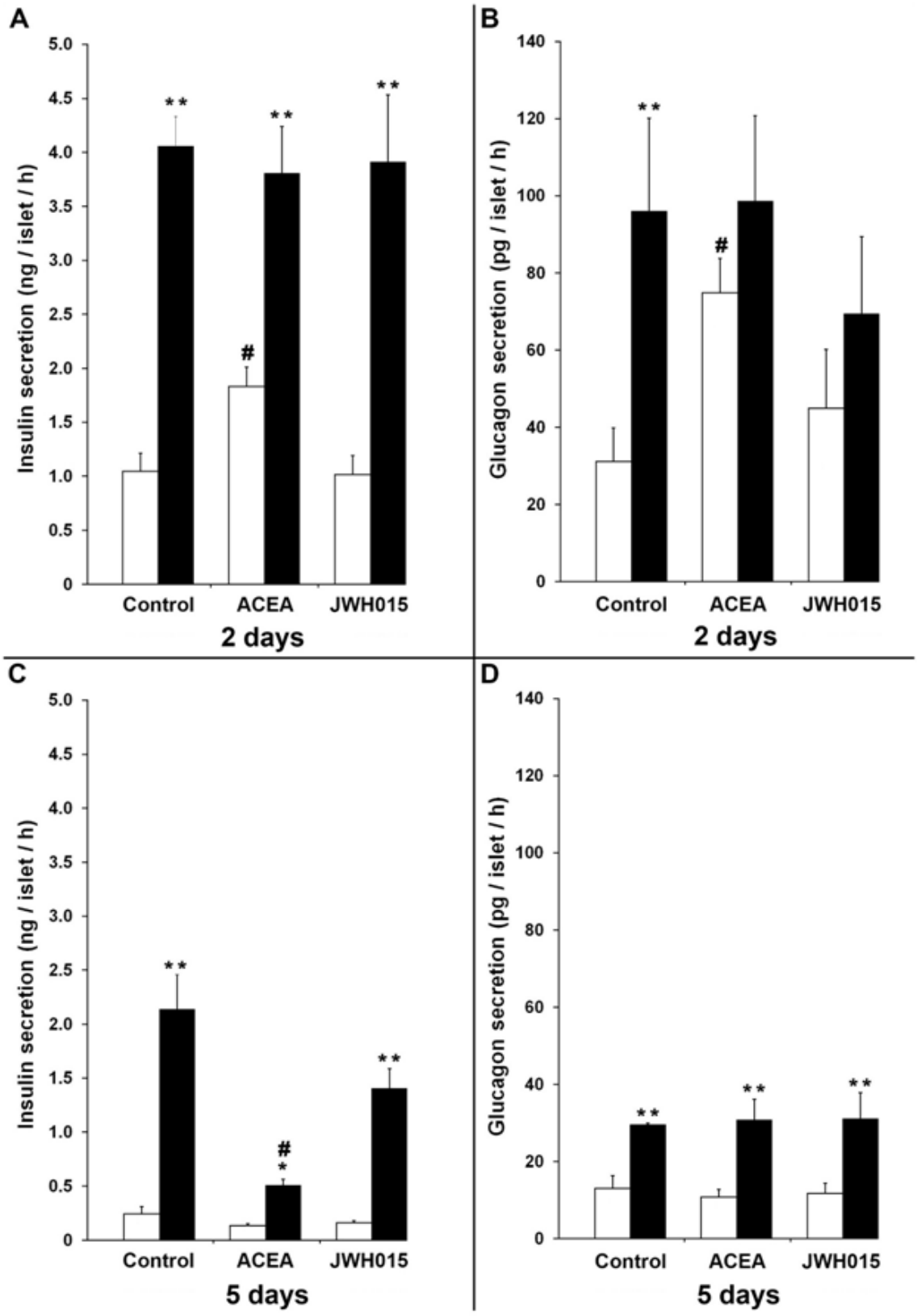
ACCEPTED MANUSCRIPT

Figure 2



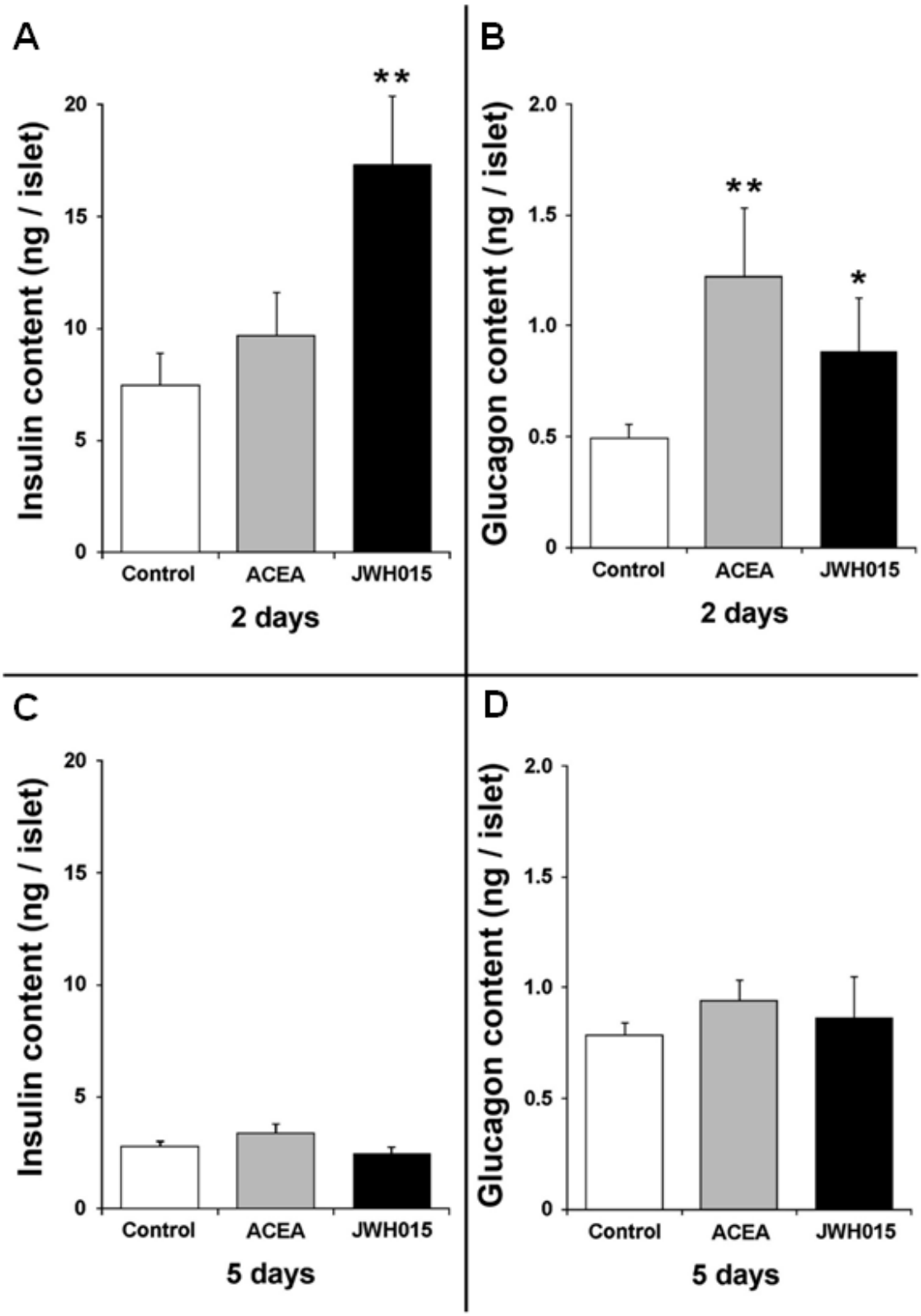
ACCEPTED MANUSCRIPT

Figure 3



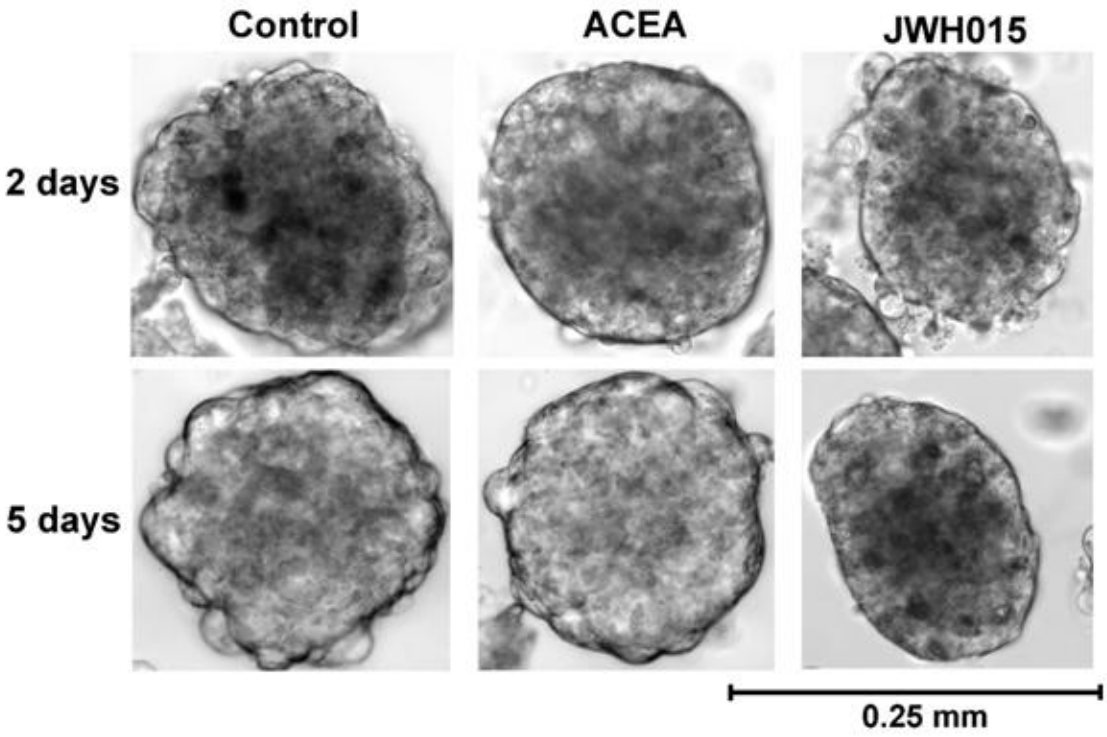
ACCEPTED MANUSCRIPT

Figure 4



ACCEPTED MANUSCRIPT

Figure 5



ACCEPTED

ACCEPTED MANUSCRIPT

Figure 6