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What Does Rimonabant Do in Rat Primary Skeletal Muscle Cells?

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ABSTRACT

Rimonabant decreases body weight in man and improves metabolic parameters in animal models of obesity. The contribution of peripheral targets, particularly skeletal muscle, for CB_1 cannabinoid receptor antagonism in these effects is, however, unclear. The purpose of this study was, therefore, to investigate the impact of CB_1 receptors inhibition upon downstream signalling and to characterize the molecular mechanisms that mediate the direct effects of rimonabant in rat primary skeletal muscle cells. Skeletal muscle cells were cultured from vastus lateralis obtained from 180-200 g male Wistar rats according to Blau and Webster method with slight modification. mRNA expression, and downstream signalling genes were examined by gene expression microarray. QRT-PCR (Taqman) identified CB_1 cannabinoid receptor mRNA expression with Ct values of 26.5-27.5 in myoblasts, myotubes and skeletal muscle tissue. The findings from the present study indicate, for the first time, that rimonabant up-regulates the mRNA content of neuropeptide Y (NPY), apelin (APLN) and lipocalin 2 (LCN2), and down-regulate the mRNA content of G protein alpha subunit (GNAI3) and nuclear receptor subfamily 4, group A (NR4A). These findings provide evidence for potential functional role of rimonabant in skeletal muscle in terms of insulin resistance, glucose and fat metabolism, inflammation and myogenesis. The impact of CB_1 receptor expression in skeletal muscle will be the subject of further investigation

Key words: Rimonabant, skeletal muscle, myotube, signalling.

INTRODUCTION

Obesity has grown in the United States and throughout the world at an unprecedented rate in recent decades (Ogden *et al*., 2006; Singh *et al*., 2011). Obesity, especially fat accumulation in the intra-abdominal region is linked to disease states such as: type 2 diabetes mellitus (Colditz *et al*., 1995), hypertension (Witteman *et al*., 1989), cardiovascular disease (Rimm *et al*., 1995), osteoarthritis, steatohepatitis, and cancer (Calle *et al*., 2004a; Calle *et al*., 2003; Calle *et al*., 2004b). Indeed, obesity has been linked to the development of insulin resistance and other metabolic abnormalities underlying the pathology of Type 2 diabetes mellitus. The pathogenesis of Type 2 diabetes mellitus is the failure of insulin action on metabolic tissues – known as insulin resistance. In other words, insulin resistance is the reduced ability of insulin to effectively stimulate glucose transport due to alteration of insulin receptor expression or insulin release in response to food ingestion (Del Prato *et al*., 2002; Ferrannini, 1998; Mosthaf *et al*., 1991). Moreover, insulin resistance can be associated with altered insulin receptor sensitivity which might be modulated by potential pharmacological agents such as rimonabant (Kahn, 1978).

Skeletal muscle is the largest tissue in the human body and represents ~40% of the human body mass and 35-40% of the total body weight in the rat (Delbono *et al.*, 2007; Pedersen, 2011). Indeed, it plays a crucial role in maintaining body glucose homeostasis (James *et al.*, 1985) and it clears out the majority (70-80%) of ingested glucose since it is the main site for insulin-dependent and non-insulin-dependent or contraction-mediated glucose uptake (Baron *et al.*, 1988; Ferrannini *et al.*, 1983; Toft *et al.*, 1998). Therefore, skeletal muscle is generally considered as the most important site of insulin resistance. Insulin resistance in skeletal muscle participates in glucose intolerance and consequently in compensatory hyperinsulinemia (Nistala *et al.*, 2006).

A novel therapeutic intervention in the treatment of obesity and hyperglycemia might occur through the antagonism of the endocannabinoid system. Indeed, studies from animals and humans have shown that the endocannabinoids are increased in the obese state. In addition, obese animal models showed that levels of endocannabinoids were elevated in the hypothalamus and peripheral tissues (Di Marzo *et al.*, 2001; Matias *et al.*, 2006; Osei-Hyiaman *et al.*, 2005). Moreover, studies showed that circulating levels of anandamide (AEA) and 2-Arachidonoyl glycerol (2-AG) was also found to be elevated in visceral adipose tissue in obese and hyperglycaemic type 2 diabetic patients (Bluher *et al.*, 2006; Engeli *et al.*, 2005; Matias *et al.*, 2006). Furthermore, CB_1 knock-out mice were found to be resistant to diet-induced obesity (Osei-Hyiaman *et al.*, 2005; Ravinet Trillou *et al.*, 2004). Originally, CB_1 receptor antagonism was investigated as a mediator of the hypophagic effect which leads to weight loss (Di Marzo *et al.*, 2001; Vickers *et al.*, 2003). However, independent to hypophagic weight loss attributed to CB_1 receptor antagonism, CB_1 receptor antagonism was also discovered to improve metabolic parameters, such as increased glucose uptake in skeletal muscle (Liu *et al.*, 2005), increased glucose tolerance (Bermudez-Siva *et al.*, 2006; Nogueiras *et al.*, 2008) and decreased hyperinsulinemia (Doyon *et al.*, 2006) as well as effects on lipids (increased HDL/LDL ratio and increased triglyceride) (Despres *et al.*, 2005).

As previously stated, CB_1 receptor mRNA and protein expression has been detected in skeletal muscle myotubes and tissues of rodents and humans (Cavuoto *et al.*, 2007; Pagotto *et al.*, 2006). In addition, in mice fed a high fat diet (HFD), the expression of CB_1 in skeletal muscle was found to be up-regulated (Pagotto *et al.*, 2006).

From agonist and antagonist studies, both *in vitro* and *in vivo*, it seems that the endocannabinoid system plays a role in glucose transport in skeletal muscle. *In vitro*, using cell culture models (L6 mouse myotube cell line and human primary skeletal muscle cells), Esposito et al and Eckardt et al, respectively showed that CB_1 receptor antagonism using rimonabant enhanced basal and insulin-stimulated glucose transport activity (Eckardt *et al.*, 2008; Esposito *et al.*, 2008). *In vivo*, chronic CB_1 receptor antagonism was found to increase insulin-stimulated glucose transport activity in obese mice (Liu *et al.*, 2005). Furthermore, chronic CB_1 receptor antagonism during euglycemic hyperinsulinemic clamp increased glucose uptake in diet-induced obese rats by several skeletal muscle groups (Nogueiras *et al.*, 2008). These data suggest that the endocannabinoid system can play a role regarding glucose transport in skeletal muscle.

The glucose transport into the skeletal muscle is facilitated mainly by the GLUT4 isoform. The mechanism, through which CB_1 antagonists affects glucose levels is unknown. Moreover, the effects of CB_1 cannabinoid receptor antagonism in terms of glucose and fatty acid metabolism on peripheral targets, particularly skeletal muscle are unclear (Eckardt *et al.*, 2009; Lindborg *et al.*, 2011; Lipina *et al.*, 2010). In an attempt to address this issue, the signalling events underlying the inhibition of the CB_1 receptor in rat primary skeletal muscle cells were investigated. In other words, the effect of rimonabant on gene expression was also investigated in rat primary skeletal muscle cells.

AIMS

The main aim of these experiments was to investigate the impact of CB_1 inhibition upon signaling and to characterize the molecular mechanisms that mediate the direct effects of rimonabant on skeletal muscle. In other words, the main aim was to assess

gene expression in rat primary skeletal muscle cells in response to rimonabant.

METHODS

Primary skeletal muscle cells

Muscle culture was performed as Blau and Webster method with slight modification (Blau et al., 1981). Vastus lateralis muscles from Wistar rats were removed and immersed in phosphate buffered saline (PBS), washed to remove the remnants of blood, and minced finely with scissors and scalpel blades on a Petri dish. Then, the minced muscle was transferred to a 50 ml flask containing a "flea and 5-10 ml of 0.25% (W/V) trypsin/EDTA (1X) for incubation at 37ºC for 15 minutes. After that, the supernatant was transferred to a 50 ml flask and neutralised with an equal volume of medium (streptomycin, penicillin, foetal bovine serum and Ham's F10), then centrifuged at 1700 rpm for 5 minutes. The collected cells were filtered through 100 µm nylon mesh ("cell strainers") to purify the cells from the debris, and centrifuged for 10 minutes at 17,000 rpm (g=26) at room temperature. The supernatant layer was removed and the cell pellet (satellite cells) was re-suspended in Ham s F10 growth medium, pre-plated on uncoated flasks for 10 minutes at 37 °C to purify these satellite cells from fibroblasts present in the extract, and then transferred to culture flasks coated with 0.2% (W/V) gelatin. The satellite cells were then grown to confluent myoblasts and differentiated into myotubes in growth medium; 20% (V/V) fetal bovine serum (FBS) and 5 ml of penicillin and streptomycin (10,000 units penicillin and 10 mg streptomycin/ml in 0.9% NaCl) were added to Ham s F10. After one day, the cells were fed with fresh medium, cells require fresh medium every 48 hours. The cells were fed with 20% (V/V) FBS fresh medium for three weeks, then reduced to 10% (V/V) FBS fresh medium for two weeks and then changed to 6% horse serum and 10 mM glucose Ham s F10 for two to three days. Images were taken for the cells using a digital camera connected to microscope (Nikon) at X40 magnification (Figure 1).

RNA Extraction and QRT-PCR (Taqman)

Myoblast and myotube cells were grown and differentiated as described above. The cells were collected in TriReagent, and processed according to the manufacturer's directions. RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen). Then, QRT-PCR (Taqman) was performed as described according to the manufacturer's directions.

Gene expression levels (in arbitrary units) were determined from the mean of triplicate determinants of each sample. Data from Taqman were only used if the slope of the standard curve for each plate was between -3.2 and -3.6 and R^2 values of more than 0.99. In addition, Ct values of triplicate readings for an individual sample, which were more than 0.5 Ct apart, were excluded. The forward primer 5'CCAAAAGTGGAG AGCGAC AAC3', reverse primer 5'CGTCTCGAAGG TCCCAATGT3' and probe 5'ATCCAGATCACC ATGCCGTTCACA3' were used for *Cnr1.*

Microarray

Experimental design

Myotubes were cultured in 25 cm^2 flasks and incubated with rimonabant 100 nM for 24 hours (four flasks/condition). Ethanol 0.01% was used as a vehicle control. Fresh charcoal stripped fetal bovine serum 4% was replaced for four hours before performing the treatment.

Procedure for the microarray

The treated myotubes were lysed using Trizol and stored at -80°C, then total RNA was extracted and cleaned up with the Qiagen Rneasy kit according to the manufacturer's instructions.

All RNA samples were examined using Agilent Bioanalyzer. Samples that had a RNA integrity number (RIN) greater than 8 were included in the analysis. These values indicated that RNA degradation did not occur. Distinct bands of 28S and 18S RNA were visualized in all RNA samples isolated from the vehicle and rimonabant-treated myotubes to confirm that the RNA was suitable for microarray procedure (Table 1).

Briefly, synthesis of labelled cRNA, hybridization, and scanning of microarrays were carried out as described (Schafer *et al.*; Voss *et al.*, 2005) according to established methods in the manufacturer's protocols (Affymetrix, Santa Clara, CA).

Gene expression profiling, data processing and analysis

Global changes in gene expression induced by rimonabant were determined using Affymetrix Rat Genome 230 PM Array.

Pre-analysis data treatment

Before analysis took place, the raw microarray (Cel files) data was pre-processed through RMA (Robust Multichip Averaging) algorithm. RMA has the following components; background correction, normalization and probe summarization.

A) Background correction was based on the distribution of perfect match (PM) values amongst probes on an Affymetrix array. Plate and exon (PM only) arrays contain a set of antigenomic background probes that are not matched to any putative transcript region. By default, the Affymetrix software estimates probe background signal by the median response of all background probes with matching GC content to the probe in question. This background signal is then subtracted from the probe intensity to yield a background-corrected intensity.

B) Quantile normalization was performed within all arrays based on the raw intensities (Raymond *et al.*, 2010). Normalization was performed to remove nonbiological effect among all arrays. This makes all arrays comparable. The RMA used quantile normalization. In this normalization, 1) probe intensities were ranked for each array, 2) the average across all arrays was taken and 3) the corresponding values of probe intensities were all set to the average. Consequently, these steps force the distribution of measurements on all arrays to be equal.

C) Probe summarization was performed through observing probe behavior [i.e., log transformed (PM) after background correction; any values attributed to background were eliminated] on the log scale as the sum of the actual expression value on the log scale (a probe specific term).

The summary of the final steps of data transformation were:

1) Log2 transformation of the intensities.
2) Tukey's median polish was used to summarize the intensity values of individual probes into a single measurement for the corresponding gene.

After raw data was normalized, genespring GX 11 software was used to identify differentially expressed genes.

Initial characterisation of microarray gene expression data

The microarray data were summarized using Principal Components Analysis (PCA) based on gene expression patterns for each of the experimental conditions. All four replicates treated with rimonabant were grouped in the scatter plot.

Correlation was performed between pairs of conditions using Affymetrix IDs common to all microarrays. In order to test the reproducibility of these data, the correlation co-efficient of technical replicates were calculated between conditions on the normalized data. All correlation coefficients were found to be higher than 0.98 across all conditions. The normalized data (not transformed to the median of all samples) was used in all subsequent analyses.

Statistical analysis

After preprocessing the microarray raw data, genespring GX 11 was used to identify differentially expressed genes. Using the normalized microarray data, conditions were compared using one-way ANOVA and Benjamini-Hochberg test. A statistically significant difference was accepted when the treatment effect yielded a $P < 0.05$ to correct for the likelihood of false positives. This *p*-value was used as a cut off for differentially expressed genes. Then, the 2 fold-change approach (increase or decrease) was utilized.

Data were further analysed using Ingenuity Pathways Analysis (IPA) **http://www.ingenuity.com**. The list of differentially regulated genes identified by the microarray analysis using genespring GX 11 was exported into IPA, which predicted biological functions of genes that are associated with particular biological processes.

RESULTS

MRNA expression of CB_1 receptor using QRT-PCR (Taqman)

mRNA for *cnr1* was detected in rat myoblasts, myotubes, skeletal muscle tissue and adipose tissue at Ct ~ 27.5, 27.5, 26.5 and 27.5, respectively.

The effect of the rimonabant on gene expression

The influence of rimonabant on gene expression was investigated using Affymetrix microarray.

Interestingly, treatment with rimonabant altered mRNA expression of a number of genes. These genes represent transcription regulators (ANKRD57, BCL3, CEBPD, EGR2, GBX2, HIVEP1, ID4, IRX3), cytokines (CCL20, CXCL6), transporters (AQP1, LCN2, RBP1, SLC16A7), peptidases (C3, PRSS35), nuclear receptors (NR4A1, NR4A2, NR4A2, NR4A3, NR4A3), GPCR (PTGER4) and growth factor (BMP6). In particular, LCN2 and NPY were up-regulated by rimonabant compared to vehicle while NR4A were down-regulated by rimonabant compared to vehicle (Table 2).

The treatment with rimonabant affected the expression of a number of genes involved in the activation of the following biological functions; adipogenesis of cells, inflammatory response, activation of phagocytes, proliferation of smooth muscle cells and impairment of tumorigenesis (Table 3).

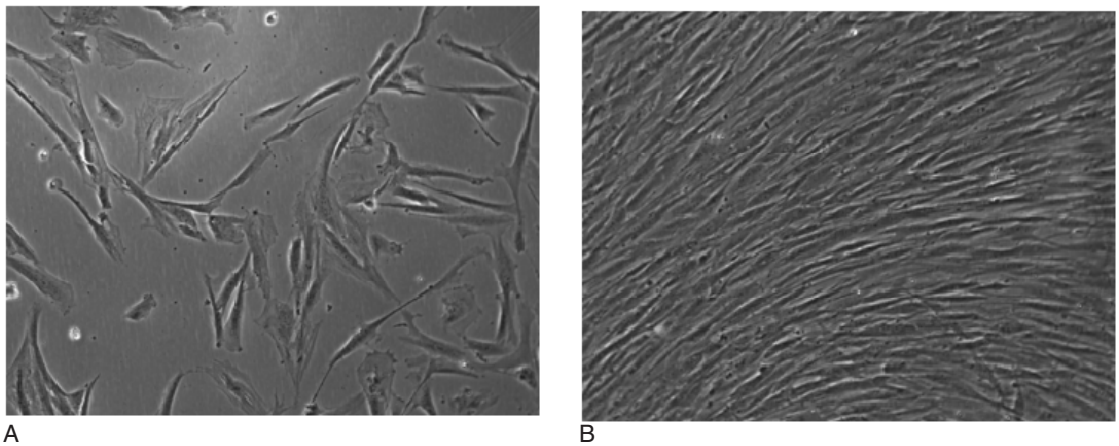
DISCUSSION

Skeletal muscle is a main site of fatty acid and glucose metabolism and involved in energy balance (Zurlo *et al.*, 1990). Skeletal muscle also produces skeletal movement through its contraction and maintains body glucose homeostasis, and so pharmacological tools that target the molecular mechanisms controlling skeletal muscle metabolism, functions and physiological roles may be therapeutically useful for metabolically related disorders.

In the present study, CB_1 receptor mRNA was detected in both skeletal muscle tissue and rat primary cells using QRT-PCR (Taqman). In previous studies, CB_1 receptor was found to be expressed in human and rodent skeletal muscle (Cavuoto *et al.*, 2007). Interestingly, CB_1 receptor protein expression was found to be significantly decreased in soleus muscle from obese compared to lean Zucker rats (Lindborg *et al.*, 2011). However, CB_1 receptor mRNA expression in soleus muscle was found to

Table 1: Quality of RNA isolated from myotubes

Sample	RIN	Sample	RIN
Vehicle 1	9.8	Rimonabant 1	9.5
Vehicle 2	9.5	Rimonabant 2	9.7
Vehicle 3	9.8	Rimonabant 3	9.8
Vehicle 4	10	Rimonabant 4	10



A B

Fig. 1: Representative myoblasts and myotubes derived from Wistar rat skeletal muscle. A) myoblasts taken during the third week of tissue culture and B) myotubes taken during sixth week of tissue culture

Table 2: The fold change in the expression of genes influenced by rimonabant in rat primary skeletal muscle cells (+ means up-regulated and – means down-regulated in response to rimonabant)

Fold Change	Symbol	Entrez Gene Name
+3.79	LCN2	lipocalin 2
+3.71	NPY	neuropeptide Y
+2.77	CCL20	chemokine (C-C motif) ligand 20
+2.75	C3	complement component 3
+2.58	GBX2	gastrulation brain homeobox 2
+2.53	RBP1	retinol binding protein 1, cellular
+2.46	TGM1	transglutaminase 1 (K polypeptide epidermal type I, protein-glutamine-gamma-glutamyltransferase)
+2.42	RASD1	RAS, dexamethasone-induced 1
+2.39	BCL3	B-cell CLL/lymphoma 3
+2.31	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta
+2.30	G0S2	G0/G1switch 2
+2.26	CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
+2.17	MGP	matrix Gla protein
+2.16	C9orf16	chromosome 9 open reading frame 16
+2.14	APLN	Apelin
+2.13	RND1	Rho family GTPase 1
+2.10	BMP6	bone morphogenetic protein 6
+2.08	RASL12	RAS-like, family 12
+2.08	KRT18	keratin 18
-2.01	ANKRD57	ankyrin repeat domain 57
-2.02	PRSS35	protease, serine, 35
-2.03	RGS2	regulator of G-protein signaling 2, 24kDa
-2.06	RAD51	RAD51 homolog (S. cerevisiae)
-2.07	TRIO	triple functional domain (PTPRF interacting)
-2.09	NR4A2	nuclear receptor subfamily 4, group A, member 2
-2.09	BUB1	budding uninhibited by benzimidazoles 1 homolog (yeast)
-2.09	PTGER4	prostaglandin E receptor 4 (subtype EP4)
-2.10	STARD13	StAR-related lipid transfer (START) domain containing 13
-2.11	KIF20B	kinesin family member 20B
-2.12	GNAI3	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3
-2.13	SLC16A7	solute carrier family 16, member 7 (monocarboxylic acid transporter 2)
-2.14	EFEMP1	EGF containing fibulin-like extracellular matrix protein 1
-2.18	LRRN4CL	LRRN4 C-terminal like
-2.21	IRX3	iroquois homeobox 3
-2.21	NR4A2	nuclear receptor subfamily 4, group A, member 2
-2.21	ITGBL1	integrin, beta-like 1 (with EGF-like repeat domains)
-2.24	MKI67	antigen identified by monoclonal antibody Ki-67
-2.33	HIVEP1	human immunodeficiency virus type I enhancer binding protein 1
-2.35	KIF11	kinesin family member 11
-2.42	ARL4C	ADP-ribosylation factor-like 4C
-2.56	TRIB3	tribbles homolog 3 (Drosophila)
-2.56	AQP1	aquaporin 1 (Colton blood group)
-2.58	ECT2	epithelial cell transforming sequence 2 oncogene
-2.72	C1QTNF3	C1q and tumor necrosis factor related protein 3
-2.84	NR4A3	nuclear receptor subfamily 4, group A, member 3
-3.23	NR4A3	nuclear receptor subfamily 4, group A, member 3
-3.56	EGR2	early growth response 2
-3.88	HAS2	hyaluronan synthase 2
-4.23	ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
-5.22	NR4A1	nuclear receptor subfamily 4, group A, member 1

Table 3: The biological functions ascribed to genes that were altered by treatment with rimonabant. For the gene abbreviation, see (Table 2)

Category	Functions Annotation	Predicted Activation State	Molecules
Cellular Development	adipogenesis of cells	Increased	C3,CEBPD,NPY,NR4A1,NR4A2, NR4A3
Connective Tissue Development and Function	adipogenesis of cells	Increased	C3,CEBPD,NPY,NR4A1,NR4A2, NR4A3
Inflammatory Response	inflammatory response	Increased	C3,CCL20,CXCL6,GNAI3,LCN2,NPY,NR4A2,PTGER4
Inflammatory Response	activation of phagocytes	Increased	C3,CXCL6,LCN2,NPY
Cell-To-Cell Signaling and Interaction	activation of phagocytes	Increased	C3,CXCL6,LCN2,NPY
Hematological System Development and Function	activation of phagocytes	Increased	C3,CXCL6,LCN2,NPY
Immune Cell Trafficking	activation of phagocytes	Increased	C3,CXCL6,LCN2,NPY
Antigen Presentation	activation of phagocytes	Increased	C3,CXCL6,LCN2,NPY
Cell-To-Cell Signaling and Interaction	activation of cells	Increased	BCL3,C3,CXCL6,EGR2,KRT18,LCN2,NPY
Cellular Growth and Proliferation	proliferation of smooth muscle cells	Increased	CEBPD,NR4A1,NR4A2,NR4A3
Skeletal and Muscular System Development and Function	proliferation of smooth muscle cells	Increased	CEBPD,NR4A1,NR4A2,NR4A3
Cancer	tumorigenesis	Decreased	AQP1,ARL4C,BCL3,BMP6,BUB1,C3,CEBPD,CXCL6,ECT2,EFEMP1,HAS2,ID4,ITGBL1,KIF11,KIF20B,KRT18,LCN2,MGP,MKI67,NR4A1,NR4A2,NR4A3,PTGER4,RAD51,RASL12,RBP1,RGS2,STARD13,TRIO

be increased after high fat feeding in C56BL/6 mice (Pagotto *et al*., 2006).

In a previous study investigating the effect of rimonabant in overweight or obese patients with type 2 diabetes, rimonabant was found to reduce bodyweight and cause a clinically significant reduction in HbA_{1c} levels (Scheen *et al*., 2006). Gene expression changes were studied in peripheral tissues such as liver and adipose from diet-induced obese mice treated with AM251 (Zhao *et al*., 2010). They found down-regulation of genes within fatty acid and cholesterol synthetic pathways such as sterol regulatory element binding proteins 1 and 2 in both liver and adipose tissues. However, these gene expression changes have not been studied in skeletal muscle. Therefore, in the present study a comprehensive analysis of differential gene expression in response to rimonabant treatments in rat primary skeletal muscle cells was achieved using Affymetrix Rat Genome 230 PM Array. The four technical replicates used for this analysis were found to be reproducible since the Pearson correlations for normalized intensity data for all replicates were above 0.98.

In this study, rimonabant up-regulated the mRNA content of NPY, APLN and LCN2, and down-regulated GNAI3 and NR4A1. These genes were suggested to be related to insulin resistance although the exact mechanisms are not known. There are limited studies on these genes in skeletal muscle; acute administration of apelin in chow-fed mice was associated with enhanced utilization of glucose in skeletal muscle (Dray *et al*., 2008). Similarly, administration of NPY to rats was associated with increased glucose utilization in skeletal muscle (Vettor *et al*., 1998). Although the cross-talk between cannabinoids and GNAI3 has not been studied in skeletal muscle, CB_1 receptor activation was suggested to hinder insulin-stimulated insulin receptor (IR) autophosphorylation dependent on the association between GNAI3 and IR in pancreatic beta-cells (Kim *et al*., 2011). It is worth noting that GNAI2 in skeletal muscle was suggested to have a role in insulin sensitivity through the suppression of protein-tyrosine phosphatase 1B (PTP1B) (Tao *et al*., 2001).

LCN2 knockout mice exhibit significant decrease in fasting glucose levels and insulin sensitivity (Law *et al*., 2010). In addition, LCN2 concentrations correlated with hyperglycemia and insulin resistance in humans (Wang *et al*., 2007), whereas mRNA content of LCN2 was increased in liver and adipose tissue of diabetic/obese mice. Moreover, it was reported that cAMP can affect the mRNA content of NR4A1 in skeletal muscle (Kawasaki *et al*., 2011; Pearen *et al*., 2008; Pearen *et al*., 2006). NR4A1 might modulate fat and glucose metabolism through regulating the expression of genes related to oxidative metabolism in skeletal muscle (Pearen *et al*., 2008). It is worth mentioning that all of the above genes (NR4A1, NPY, APLN, LCN2 and GNAI3) were detected in rat skeletal muscle tissue using Agilent microarray at the following ranking (506, 12597, 1827, 12118 and 15033) out of 41000. However, more research is needed to investigate the role of rimonabant in skeletal muscle metabolism. Moreover, measurement of NPY and apelin in response to rimonabant should be recommended to be investigated in skeletal muscle cells.

There is little information in the literature about the roles of these genes in skeletal muscle. Overall, rimonabant might have a role in skeletal myogenesis through regulating the expression of those genes (CEBPD, NR4A1, NR4A2, NR4A3). Therefore, further research is needed to address this issue.

Moreover, rimonabant might have a role in inflammatory role in skeletal muscle through regulating of those genes (C3, CCL20, CXCL6, GNAI3, LCN2, NPY, NR4A2, PTGER4). In fact, the level of complement C3 was shown to be improved by treatment with antidiabetic agents such as thiazolidinediones (Ebeling et al., 1999). In addition to that, markers of inflammation including the proinflammatory cytokines and acute phase proteins are associated with developing and severity of type 2 diabetes (Donath *et al*., 2011; Spranger *et al*., 2003; Xie *et al*., 2011). Furthermore, type 2 diabetes has been associated recently with subclinical chronic inflammation (Donath *et al*., 2011; Spranger *et al*., 2003; Xie *et al*., 2011). Therefore, rimonabant might play a role with type 2 diabetes through improving the inflammation.

There is also a suggestion that rimonabant might give a response as an agonist through other receptors such as GPR55 (Godlewski *et al*., 2009). It is also worth noting that the serum used in this experiment might also contain low endocannabinoid level that might interact with signalling of rimonabant. Therefore, it is very hard to explain the response of rimonabant. From this data, no conclusion can also be made as to whether the rimonabant effects depend on the inhibition of the CB_1 receptor (a CB_1 receptor dependent manner). Further work should be performed to get a clear comprehensive image in these issues, such as repeating this experiment from different animals using either microarray or QRT-PCR (Taqman) or using delipidated serum instead of charcoal stripped serum. Further work is also required to understand these responses such as using siRNA for CB_1 receptor or using GPR55 antagonist.

CONCLUSION

In summary, the microarray findings revealed that treatment myotubes with rimonabant influenced the mRNA content of genes (NPY, APLN, LCN2, NR4A1 and GNAI3) might be related to insulin resistance, glucose and fat metabolism. Further research is warranted to establish the precise role of endocannabinnoids in the regulation of gene expression in skeletal muscle and the importance of this role in the development of insulin resistance and obesity.

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