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ADRENOCEPTORS IN RAT PRIMARY SKELETAL MUSCLE CELLS

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ABSTRACT

GPCRs are the largest family of proteins in the human genome. One family of GPCRs is adrenoceptors which are divided into two major types: α and β-adrenoceptors. Indeed, GPCRs are targets for huge numbers of therapeutic drugs, although the role of skeletal muscle in the action of these drugs is unclear. The purpose of this research was to identify genes encoding adrenoceptors highly expressed in skeletal muscle and in cultured preparations thereof.

Skeletal muscle cells were cultured from vastus lateralis obtained from male Wistar (180-200 g) rats as Blau and Webster method with slight modification (Blau *et al.*, 1981). mRNA expression, cAMP and ERK phosphorylation were examined by conventional methods (Akaneya *et al.*, 2006; Hudson *et al.*; Millns *et al.*, 2001). Data were reported as means of triplicate or quadruplicate wells generated from two animals. Statistical analysis was conducted using one or two-way ANOVA with Bonferroni's multiple comparisons test.

mRNA encoding G_s- (*adrb2*) and G_i- (*adra2a*) were detected using gene microarray (Agilent, all ranked <24000 out of 41090). QRT-PCR (Taqman) identified α_{2A} receptor mRNA expression with Ct values of 28-31 in myoblasts, myotubes and skeletal muscle tissue. In myotubes, forskolin (1 µM) –evoked elevation of cAMP (17 ± 1) was significantly inhibited in the presence of UK14304 (10^{-7} M) (12 ± 0). However, rauwolscine (10^{-7} M) did not prevent this effect. Treatment with UK14304 increased phosphorylation of extracellular signal-regulated kinase 1/2 (25 ± 1.6 compared to basal ERK phosphorylation of 13 ± 3.5 P-ERK/ERK); these responses were significantly inhibited by rauwolscine (11 ± 1). Interestingly, rauwolscine showed also a significant inhibition of phosphorylation of ERK

(3.6 ± 0.24). Isoprenaline (10^{-5} M) failed to increase cAMP levels in myotube cells, as did the β_2-adrenoceptor-selective antagonist ICI118551 (10^{-7} M).

In summary, we observe expression and functional responses to example members of the adrenoceptor families in rat skeletal muscle preparations.

Key words: GPCRs, cAMP, ERK, calcium and myotubes, beta2 adrenoceptors, alpha2 adrenoceptors

Abbreviations

(GPCRs); G protein coupled receptors, (Adrb2); adrenergic receptor, beta 2, (Adra2a); adrenergic receptor, alpha 2a, (AC); adenylyl cyclase, (cAMP); cyclic adenosine monophosphate, (UK14304); 5-Bromo-6-(2-imidazolin-2-ylamino)quinoxaline, (IBMX); 3-isobutyl-1-methylxanthine, (NR4A); nuclear receptors, subfamily 4, (PDK4); pyruvate dehydrogenase kinase 4, (FOXO1); forkhead box protein O1, (PGC-1α); peroxisome proliferator-activated receptor-γ coactivator, (lipin-1α); phosphatidate phosphatase LPIN1, (Pfkm); muscle phosphofructokinase, (GLUT4); Glucose transporter-4, (MAPK); Mitogen activated protein kinase, (ERK 1/2); extracellular signal-regulated kinase 1/2, (SiRNA); Small interfering RNA, FBS; (fetal bovine serum), FCS; (foetal calf serum), NCS; (newborn calf serum), (DMEM); Dulbecco's Modified Eagle Medium, (PBS); phosphate buffered saline, (SEM); standard error of mean, (AUC); area under the curve, (ANOVA); analysis of variance, (APS); ammonium persulphate, (cDNA); Complementary DNA, (Ct); Cycle threshold, (DMSO); Dimethyl sulfoxide, (mRNA); messenger ribonucleic acid, (PBS); Phosphate buffered saline, (PCR); Polymerase chain reaction; (P-value); Probability, (RT-PCR); Reverse transcription polymerase chain reaction, (SDS-PAGE); Sodium dodecyl sulfate polyacrylamide gel electrophoresis, (SEM); Standard error of the mean, (TBST); Tris-buffered saline with Tween 20.

INTRODUCTION

G protein coupled receptors (GPCRs) are the largest family of proteins in the human genome. One family of GPCRs is adrenoceptors which are divided into two major types: α and β-adrenoceptors. Indeed, GPCRs are the richest targets for pharmaceutical drugs on the market today; it is estimated that they are the targets of 30-50% of all medications due to their vast and varied roles in regulating the body processes, metabolism and signal transduction and their involvement in key biological functions (Kobilka, 2007; Tilakaratne *et al.*, 2005). GPCRs are expressed in every tissue and play a major role in many diseases.

Skeletal muscle is a heterogeneous tissue since it contains a variety of fibres that differ in contractile, functional, metabolic and molecular characteristics (Pette *et al.*, 1997; Staron *et al.*, 1999). Moreover, skeletal muscle is the largest organ 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, skeletal muscle utilizes the majority (70-80%) of ingested glucose since it is the main site for insulin-dependent glucose uptake (Toft *et al.*, 1998). Therefore, it is generally considered a major site of insulin resistance.

Myotubes are primary skeletal muscle cells sharing the morphological, metabolic and biomedical characteristics and properties of adult skeletal muscle (Henry *et al.*, 1995; Raymond *et al.*, 2010).

The purpose of the investigations in this study was to characterize the mRNA expression of beta2 and alpha2 adrenoceptors in rat mixed fibre-type skeletal muscles using DNA microarray and QRT- PCR (Taqman) techniques and to assess the downstream signalling of these GPCRs in rat primary skeletal muscle cells using cAMP assay, calcium imaging and immunoblotting.

MATERIALS AND METHODS

Materials

UK14304, rauwolscine and ICI118551 were purchased from Tocris Company. All other materials were purchased from Sigma unless otherwise mentioned. RNeasy Mini Total RNA Purification kits were purchased from Qiagen (West Sussex, UK). Reagents for reverse transcription of RNA to cDNA were purchased from Invitrogen (Paisley, UK). Taqman reagents were purchased from Applied Biosystems (CA, USA). Primers and probes were purchased from Eurofins MWG GmBH (Ebersberg, Germany). TriReagent was purchased from Invitrogen. 30% Bis-acrylamide was purchased from

Severn Biotech (Kidderminster, UK). Seeblue Protein Ladder was purchased from Fermentas (York, UK). Nitrocellulose membrane was purchased from GE Healthcare (Amersham, UK). Goat 680 anti-rabbit, goat 680 anti-mouse, goat 800 anti-rabbit and goat 800 anti-mouse secondary antibodies were purchased from Li-Cor Biosciences (Cambridge, UK). P-ERK and ERK primary antibodies were purchased from Cell-Signalling company. 3T3-L1 preadipocytes were obtained from American Type Collection (ATCC). FCS (foetal calf serum), NCS (newborn calf serum), FBS (fetal bovine serum) and Ham's F10 were purchased from PAA Laboratories (Somerset, UK). Oil red O stain was purchased from British Drug Houses Ltd (Poole, England). Horse serum was purchased from Invitrogen. cAMP Kit was obtained from Cayman Chemical (Europe).

Tissue Collection

Two male adolescent Wistar rats (180-200 g, 4-6 weeks old) were killed by cervical dislocation without anesthesia. Skeletal muscles were obtained. Skeletal muscle (mixed fibre-type from hindlimb) tissues were then separated bilaterally: tissue was immediately frozen in liquid nitrogen and stored at -80 °C with liver and adipose tissue, muscle tissue was cultured after the isolation of satellite cells in gelatine-coated flasks.

Tissue Culture

3T3-L1 Cell Culture

The mouse 3T3-L1 preadipocytes were seeded at ~1 x 10^5 cells in 35 mm dishes or 6 well plates. The cells were cultured until 100% confluent in Dulbecco's Modified Eagle Medium (DMEM) high glucose with 10% newborn calf serum (NCS) containing 2 mM glutamine and 1% of (10,000 units penicillin and 10 mg streptomycin/ml). After 2 days from reaching confluence, cells were induced to differentiate with medium 2 (DMEM high glucose with 10% foetal calf serum (FCS) containing 2 mM glutamine, 1% of (10,000 units penicillin and 10 mg streptomycin/ml), 0.5 mM 1-methyl-3-isobutylxanthine, 0.25 µM dexamethasone and 166 nM insulin). The cells were maintained in this media for 3 days. Then, the cells were incubated with medium 3 (DMEM high glucose with 10% FCS containing 2 mM glutamine, 1% of (10,000 units penicillin and 10 mg streptomycin/ml) and 166 nM insulin) for 2 days. Differentiated cells were incubated with medium 4 (DMEM high glucose with 10% FCS containing 2 mM glutamine and 1% of (10,000 units penicillin and 10 mg streptomycin/ml)) for 2 days. Oil-Red O staining was used to visualize lipid droplets after differentiation to adipocytes as

described earlier (Ramirez-Zacarias *et al.*, 1992). Images were taken for the cells using a digital camera connected to microscope (Nikon) at X40 magnification (Figure 1).

Primary Muscle Cell Culture

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 37 C° 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 2).

Microarray Procedure

Agilent 4*44K DNA one color whole genome microarrays were used to measure the expression of 41090 genes in mixed fibre-type of skeletal muscle from rat hindlimb. The microarray experiment was carried out according to manufacturing instructions. After assessing the quality control criteria generated from feature extraction software, extracted data were further processed with GeneSpring GX 11.

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' GGCCTCAGCGGACATCCT 3' reverse primer: 5' CATAACCTCGTTGGCCAAA GA 3' and probe 5' TGGCCACGCTGGTC ATTCCCTT 3' were used for adra2a.

cAMP Assay

Accumulated cAMP in the myotube cells was measured by a competitive Enzyme Immunoassay (EIA) kit (Cayman Chemical). Cells were prepared as discussed above. Where indicated, cells were pre-incubated with antagonist 20 minutes prior to agonist addition. At the end of the exposure period for the drugs, 40 µl 5 M HCl was added to each well (2 ml media (Ham's F10), 6% horse serum), before cAMP measurement as described in the manufacturer's instructions.

Western Blot

The samples (20 µl) were loaded onto a 12% SDS polyacrylamide gel. 5 µl of seeblue marker (Invitrogen) was loaded with each gel as a molecular weight marker. The protein was separated by gel electrophoresis in a Bio-Rad gel apparatus (Mini-PROTEAN) filled with SDS-PAGE running buffer. A constant current of 150 volt was supplied for around one hour. The separated proteins from the gel were transferred to pre-soaked nitrocellulose membrane in transfer buffer. A constant current of 105 volt was supplied for around one hour. The protein was stained on the membrane with Ponceau S. Non-specific antibody binding was reduced by blocking the nitrocellulose membrane in blocking buffer (3% gelatin in 0.1% TBS.T) for one hour at room temperature with continuous gentle shaking. The nitrocellulose membrane was subsequently incubated with primary antibody overnight at 4 C° on a roller. After overnight incubation, the membrane was washed with 1XTBST three times each 20 minutes. The membrane was then incubated with secondary antibody diluted 1:10,000 for one hour at room temperature. After

washing three times for 5 minutes with 1XTBST, the membrane was rinsed with distilled water. Finally, the membrane was scanned using The Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE/USA), which equipped with two infrared channels. Using two detection channels, total and phosphorylated forms could be visualized and differentiated between on the same gel (same experiments) using different secondary antibodies. Band intensities were quantified by densitometry, using Odyssey software version 3. Each blot was analyzed for p-ERK to ERK ratios to assess ERK activation.

Statistical Analysis

Data were analyzed using one or two-way ANOVA and Bonferroni post-hoc test. Analysis was performed using GraphPad Prism, version 5.03 (GraphPad Software Inc). Differences were considered significant at $P<0.05$. Data were reported as means ± standard error of mean (SEM) of triplicate or quadruplicate wells generated from two animals (n=2). Statistics was performed from at least two repeats (n=2 rats) for cAMP and western blot experiments.

RESULTS

mRNA Expression of Adrenoceptors Using Microarray

β_2-adrenoceptors and α_2-adrenoceptors GPCR entities were detected in skeletal muscle tissue (ranked higher relative intensity values) than either nicotinic cholinergic alpha subunit (*chrnα1*) or reference gene (TATA box binding protein (Tbp)) (12526 and 10220 ranking out of 41090, respectively)). These GPCR entities include two main families that coupled to different G proteins (G_s and G_i) in this study. Examples of G_s-GPCRs are β_2-adrenoceptors and G_i-GPCRs are α_2-adrenoceptors. These examples of detected GPCR entities was investigated in this paper to examine the possible different signalling in skeletal muscle cells. Indeed, investigation of the different families (G_s and G_i) of GPCRs might help to confirm the expression and understand the expected signalling and functional role of these receptors.

mRNA Expression of α_{2a}-Receptor Using QRT-PCR (Taqman)

mRNA for *adra2a* was detected in rat myoblasts, myotubes, skeletal muscle tissue and adipose tissue at Ct ~ 28, 30, 31 and 30, respectively.

Effect of α_2-adrenoceptor ligands on cAMP levels

Adra2a expression was detected using QRT-PCR (Taqman) and microarray in this paper. Functional expression of the α_{2A}-adrenoceptor as a G_i-coupled receptor was assessed by quantifying cAMP levels. Treatment of myotube cells with UK14304, an α_2-adrenenoceptor agonist (Jasper *et al.*, 1998), inhibited forskolin (1 µM)-evoked elevation of cAMP significantly. However, rauwolscine, the selective α_2-adrenoceptor antagonist (Convents *et al.*, 1989; Uhlen *et al.*, 1994), did not prevent this effect (Figure 3).

Effect of α_2-adrenoceptor ligands on ERK phosphorylation

α_2-adrenoceptor coupling to ERK phosphorylation was investigated as an alternative coupling mechanism. Treatment of myotube cells with UK14304 for 10 minutes showed a significant increase in P-ERK1/ERK1 and P-ERK2/ERK2 ratios compared to vehicle (0.01% ethanol). The molecular weight of the bands for P-ERK1/ERK1 and P-ERK2/ERK2 was detected at 44 kDa and 42 kDa, respectively as expected. Interestingly, treatment of myotube cells with rauwolscine (100 nM), 30 minutes prior to the addition of UK14304 inhibited the effect of UK14304 significantly for both P-ERK1/ERK1 and P-ERK2/ERK2 ratio. However, rauwolscine showed a significant inhibition of basal levels of ERK phosphorylation as well (Figure 4).

Effect of β-adrenoceptor ligands on cAMP levels

The potential coupling of β_2-adrenoceptors to elevation of cAMP levels was assessed, since mRNA encoding β_2-adrenoceptor was detected using microarray in this paper.

Isoprenaline, a non-selective β-adrenoceptor agonist (Bylund *et al.*, 1994), failed to increase cAMP levels in myotube cells, as did the β_2-adrenoceptor-selective antagonist ICI118551 (Bilski *et al.*, 1983) (Figure 5-A). In contrast, isoprenaline evoked a significant elevation of cAMP levels in 3T3-L1 adipocytes (Figure 5-B).

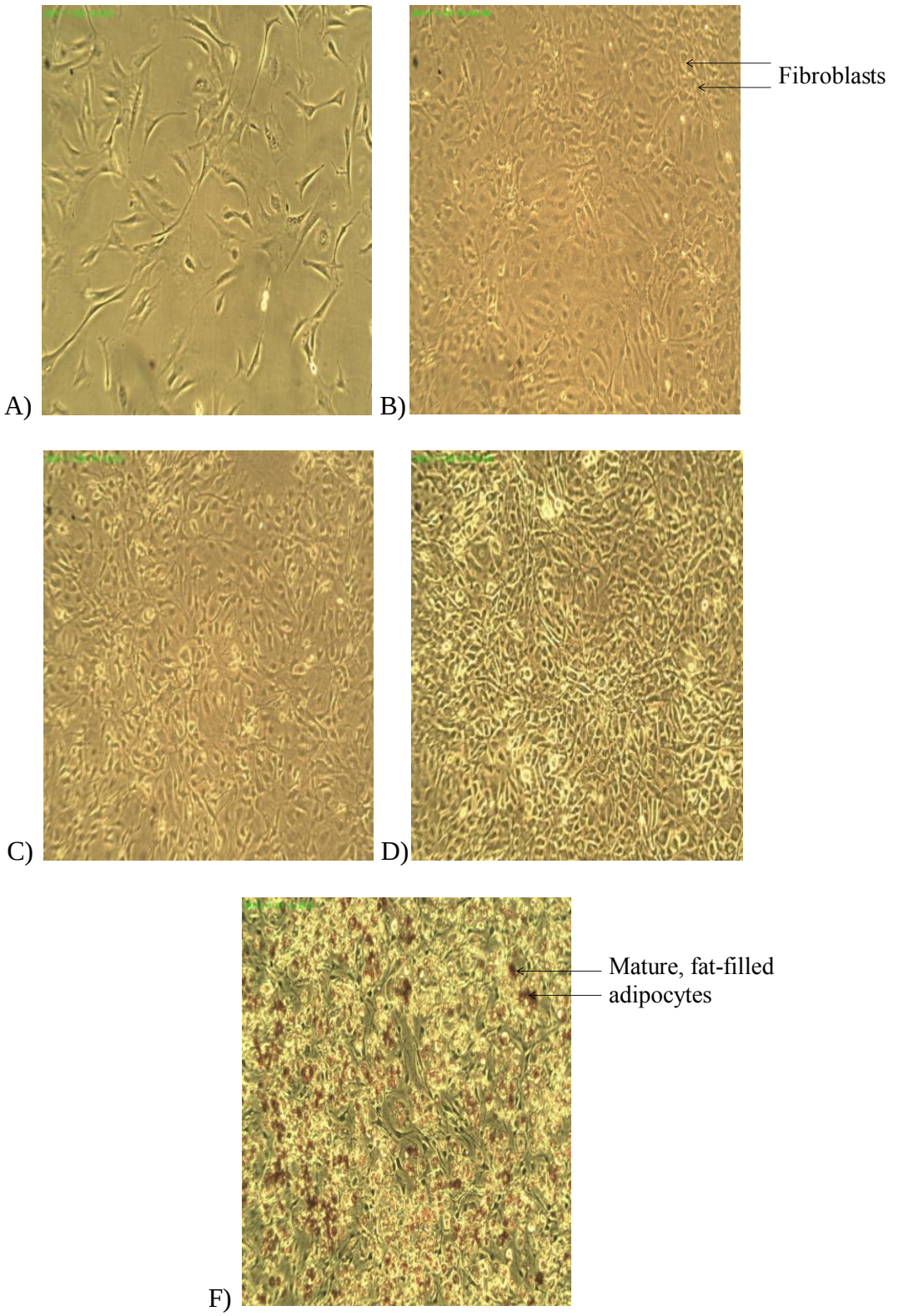


Figure 1: Representative photographs of 3T3-L1-fibroblast cells differentiating into 3T3-L1-adipocytes. A) 3T3-L1-fibroblast cells, B) confluent fibroblast fixed and stained with Oil Red O, C) fibroblast incubated in media 2, D) fibroblast incubated in media 3 and F) 3T3-L1 adipocytes were fixed and stained with Oil Red O

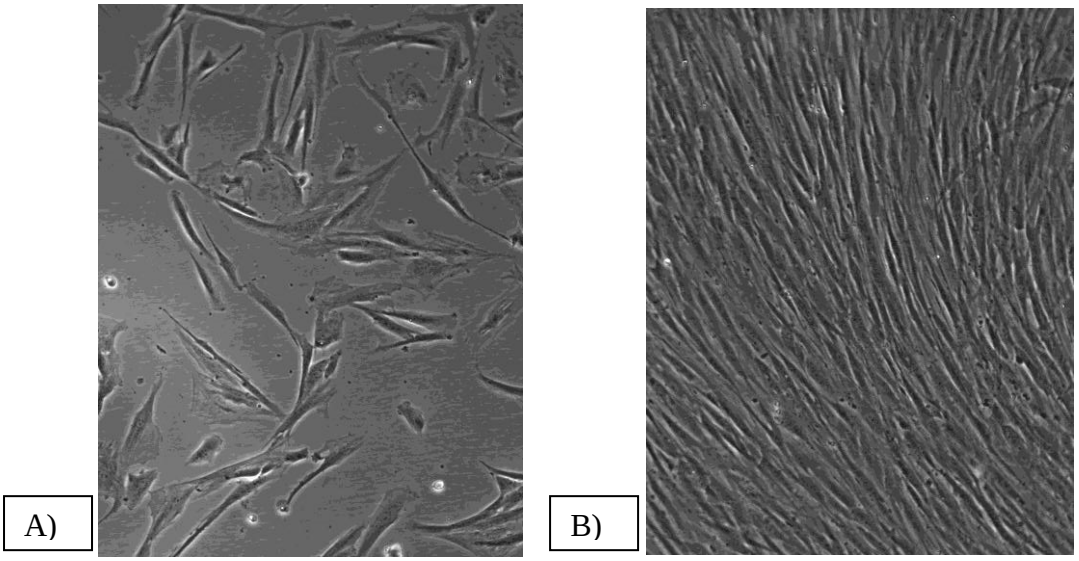


Figure 2: Representative myoblasts and myotubes derived from Wistar rat skeletal muscle. A) myoblasts taken during the third week of tissue culture and B) myotubes taken during sixth week of tissue culture

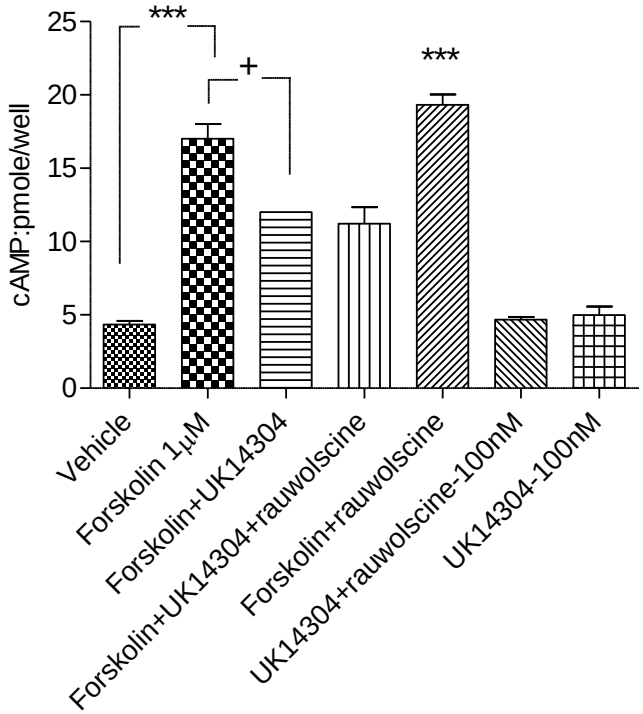
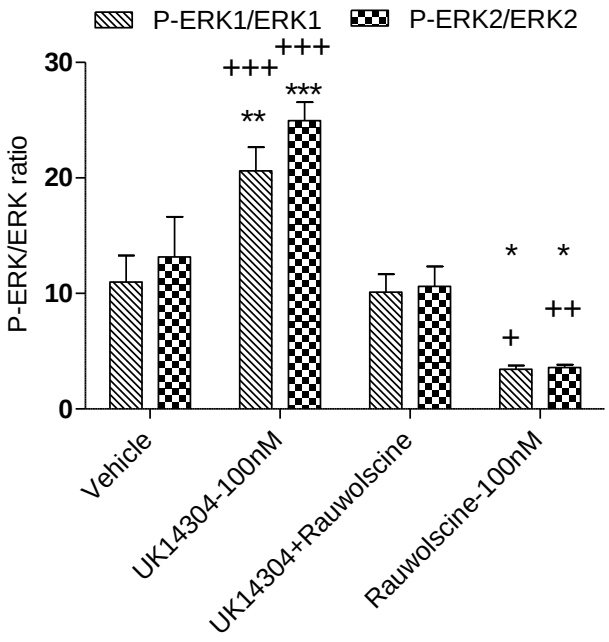
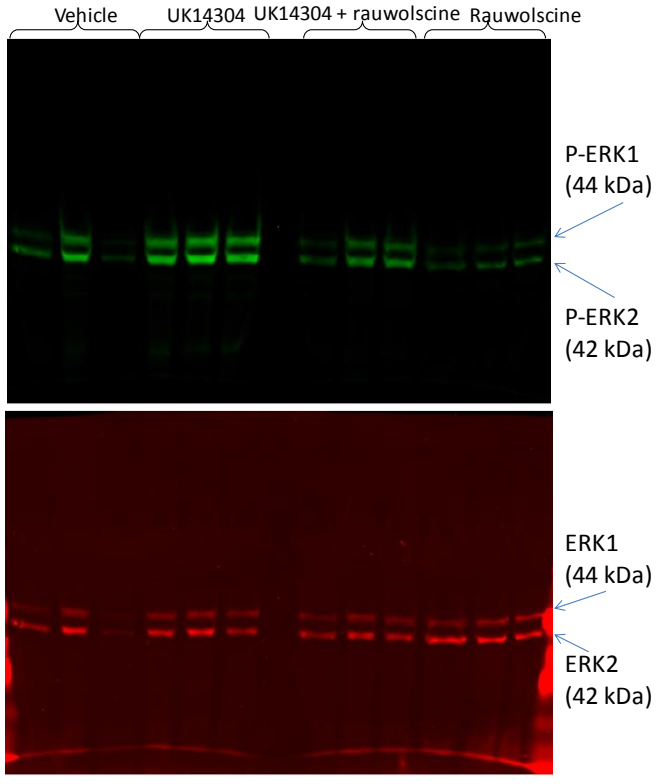


Figure 3: Effect of UK14304 and rauwolscine on cAMP accumulation in myotubes (n=2 rats). cAMP production was measured following incubation of cells for 10 minutes with vehicle (0.01% ethanol), UK14304 (100 nM) and rauwolscine (100 nM). * vs basal. + vs forskolin. Data were analyzed using one way ANOVA test followed by Bonferroni post-hoc.

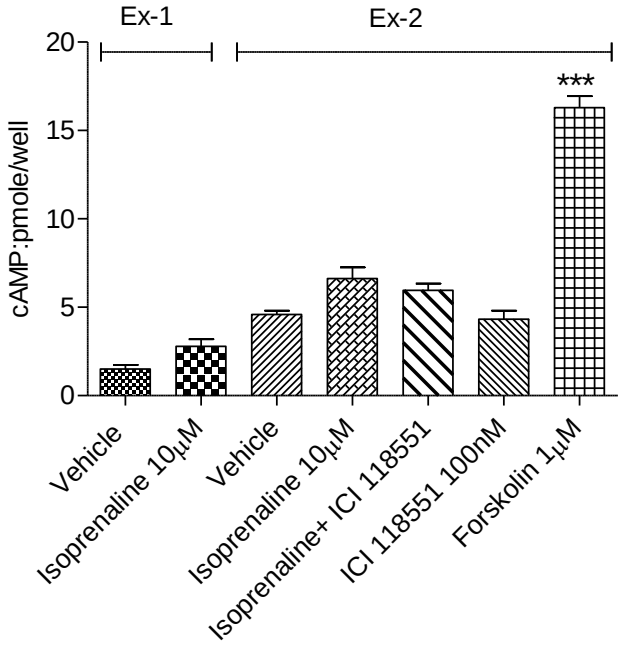


A)

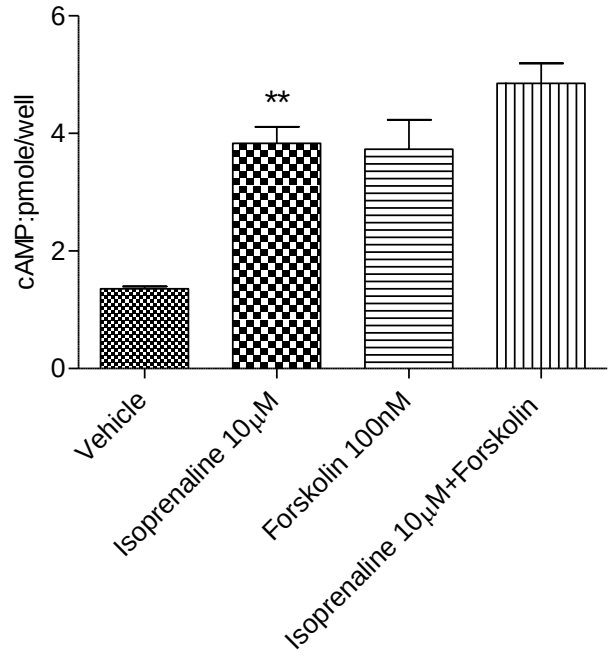


B)

Figure 4: Effect of UK14304 and rauwolscine on phosphorylation of ERK in rat primary skeletal muscle cells (n=2 rats). A) Myotubes were treated with vehicle (0.01% ethanol), UK14304 (100 nM) for 10 minutes, UK14304 for 10 minutes + rauwolscine (100 nM) 30 minutes prior. * ± UK14304. + ± rauwolscine. B) Representative blots showing myotubes treated with vehicle (0.01% ethanol), UK14304 (100 nM) for 10 minutes + rauwolscine (100 nM) 30 minutes prior, phospho-ERK 1/2 (green bands), ERK 1/2 (red bands). Data were analyzed using one way ANOVA test followed by Bonferroni post-hoc



A)



B)

Figure 5: Effect of isoprenaline on cAMP accumulation in myotubes and 3T3-L1 adipocytes (n=2 rats and n=2 experiments, respectively). A) Effect of isoprenaline on cAMP accumulation in myotubes. cAMP production was measured following incubation of cells for 10 minutes with vehicle (0.01% DMSO), isoprenaline (10 µM), ICI118551 (100 nM) and forskolin (1 µM). Ex-1; experiment 1, Ex-2; experiment 2 (***) $p < 0.001$ versus vehicle. B) Effect of isoprenaline on cAMP accumulation in 3T3-L1 adipocytes. cAMP production was measured following incubation of cells for 10 minutes with vehicle (0.01% DMSO), isoprenaline (10 µM) and forskolin (100 nM). (**) $p < 0.01$ versus vehicle. Data were analyzed using one way ANOVA test followed by Bonferroni post-hoc.

DISCUSSION

GPCRs expression was examined in this paper using two different techniques, Agilent microarray and QRT-PCR (Taqman). Using Agilent microarray, β_2-adrenoceptor and α_2-adrenoceptor entities were found to be expressed in skeletal muscle in this paper. These GPCRs are somewhat less well-known in terms of their activity in the skeletal muscle system. Relatively little information in the literature is reported in both normal and disease state about the role of these GPCRs in functional activities and in signal transduction of skeletal muscle tissues. Regarding these CPCRs detected in skeletal muscle in this paper, two main families of GPCRs (G_s and G_i) were detected in skeletal muscle tissue using the microarray. Examples include G_s-GPCR (β-adrenoceptor) and G_i-GPCR (α_2-adrenoceptor). Moreover, *adra2a* mRNA were detected in QRT-PCR (Taqman) and these agreed with the microarray. These GPCRs were investigated in this paper for further signalling and functional roles in skeletal muscle.

The signalling (functionality) associated with these receptors has not previously been investigated in detail in skeletal muscle. Therefore, conventional second messenger studies (investigating cAMP and ERK activation) were conducted to assess their functionality.

cAMP is an important second messenger in skeletal muscle. It was found to increase the expression of members of the orphan family of nuclear receptors, subfamily 4 (NR4A), compared to other nuclear receptors in skeletal muscle (Maxwell *et al.*, 2005). These transcription factors regulate the gene expression of proteins responsible for fat and glucose metabolism through up-regulating the mRNA expression of pyruvate dehydrogenase kinase 4 (PDK4), forkhead box protein O1 (FOXO1), peroxisome proliferator-activated receptor-γ coactivator (PGC-1α), phosphatidate phosphatase LPIN1 (lipin-1α), GLUT4 and muscle phosphofructokinase (Pfkm) (Chao *et al.*, 2007; Kanzleiter *et al.*, 2010; Lessard *et al.*, 2009). The implication of this is that activation of NR4A receptors might improve glucose glycolysis, glucose transport and lipid oxidation in skeletal muscle, and consequently NR4A receptors might be a therapeutic target for diabetes and obesity. Another critical second messenger is calcium, which transduces extracellular signals into numerous intracellular events in many cell types. Indeed, the functions of calcium range from short-term responses, such as contraction and activation of some enzymes (such as adenylyl cyclase), to longer-term responses such as gene expression (Berridge, 1997). In skeletal muscle, calcium has a crucial role

for contraction (Berchtold *et al.*, 2000). When calcium is released from sarcoplasmic reticulum, it binds to troponin and pulls tropomyosin allowing the myosin to bind to the actin, consequently, contraction occurs (Berchtold *et al.*, 2000). Calcium might also improve contraction-stimulated glucose uptake through activating GLUT4 translocation, calmodulin-dependent protein kinases, calmodulin and protein kinase Cs (Ihlemann *et al.*, 1999; Jessen *et al.*, 2005; Wright *et al.*, 2004; Youn *et al.*, 1991). However, the mechanism is still unclear.

One family of GPCRs, called adrenoceptors, was investigated using the cAMP assay. The adrenoceptors are divided into two major types: α and β-adrenoceptors. In terms of α_2–adrenoceptors, *adra2a* expression was found in rat skeletal muscle using northern blot analysis (Lorenz *et al.*, 1990). Consistent with this report, α_{2A}-adrenoceptors were also detected using the microarray and QRT-PCR (Taqman) in this paper. The accepted roles for α_{2A}-adrenoceptors include acting as the major feedback regulator of noradrenaline release at nerve terminals and the regulation of insulin secretion through noradrenaline in pancreatic islets through reducing the cAMP formation (Ahren, 2000; Nakaki *et al.*, 1981). It is noteworthy that a mutation of α_{2A}-adrenoceptors has been shown to be associated with obesity and metabolic alterations (Lima *et al.*, 2007). However, there is a lack of possible roles of the α_{2A}-adrenoceptors in skeletal muscle in the literature. Therefore, a cAMP assay was performed to test the functionality of this receptor.

UK14304 was found to inhibit cAMP elevation evoked by forskolin. However, rauwolscine did not prevent this effect. The concentration which was used in this study for UK14304 is in line with that (100 nM) shown to inhibit forskolin-evoked cAMP level in rat primary superior cervical ganglionic (SCG) cells (Shivachar *et al.*, 1999), and the concentration which was used in this paper for rauwolscine is also in line with that (100 nM) shown to reverse the effect of UK14304 inhibition of secretin-stimulated cAMP level in purified rat bile duct-ligated (BDL) cholangiocytes via α_2-adrenoceptors (Francis *et al.*, 2007). This suggests that the effect of UK14304 regarding cAMP might not function through α_{2A}-adrenoceptors since this effect was not blocked by rauwolscine or it is also possible that the concentration used for rauwolscine did not block the effect of UK14304 in these primary skeletal muscle cells. In other words, the concentration used for rauwolscine might not overcome the effect of UK14304 regarding the cAMP, in particular of the similar relative affinities for

UK14304 and rauwolscine to α_{2A}-adrenoceptors (Kd=10 nM and Ki=3.5 nM, respectively) (Neubig *et al.*, 1988; Wainscott *et al.*, 1998). Different concentration points of rauwolscine and concentration-response curve for UK14304 are required to have a clear comprehensive image of the response in these primary skeletal muscle cells regarding cAMP.

Interestingly, UK14304 evoked a significant elevation of phosphorylated ERK1/2; an effect blocked by rauwolscine. Furthermore, the inhibitory effect of rauwolscine on basal levels of cAMP could be interpreted to mean that α_2-adrenoceptors in this tissue exhibit constitutive activity. Rauwolscine has been reported to be an inverse agonist in stable Chinese hamster ovary cell lines expressing constitutively activated porcine α_{2A}-adrenoceptors in which the suppression of cAMP production in these cells is reversed by rauwolscine (Wade *et al.*, 2001). However, nothing in the literature is reported about constitutively activated rat α_{2A}-adrenoceptors. It is also possible that the influence of rauwolscine on ERK activation, but not for cAMP inhibition in this study, might be ascribed to an antagonist bias (i.e. affecting one pathway and not affecting another) (Kenakin, 2010; Urban *et al.*, 2007). It is also possible that α_2-adrenoceptors mediate the ERK phosphorylation through an adenylyl cyclase-independent cascade in this paper. ERK phosphorylation might be mediated through $G_{\beta\gamma}$ subunit. This is in line with that overexpressed $G_{\beta\gamma}$ subunit in CHO cells was shown to activate MAP kinase (van Biesen *et al.*, 1995). As activation of α_2-adrenoceptors was shown in this paper to stimulate ERK, and ERK signal transduction was traditionally suggested to growth related process (Bennett *et al.*, 1997; Jones *et al.*, 2001; Lopez-Ilasaca, 1998), it is possible that α_2-adrenoceptors have a role in skeletal muscle growth. The implication of this is that long-term treatment of rats with α_2-adrenoceptor agonists should stimulate skeletal muscle growth and lean weight gain.

For β_2-adrenoceptors, in this paper, the microarray experiment showed that the mRNA encoding β_2-adrenoceptors was detected. This agrees with reports of the expression of β–adrenoceptor mRNA in rat skeletal muscle tissues and with expression of β_2–adrenoceptors in rat L6 cells using RT-PCR (Nagase *et al.*, 2001; Sato *et al.*, 2010). However, treatment of myotube cells with isoprenaline, a non selective β–adrenoceptor agonist (Bylund *et al.*, 1994), did not increase cAMP levels. The concentration which was used in this paper for isoprenaline is in line with that (10 µM) shown to increase cAMP production in rat L6 cell membranes (Coppock *et al.*, 1996).

Moreover, the concentration which was used in this paper for ICI118551 is also in line with that (100 nM) shown to inhibit the isoprenaline-evoked cAMP level in rat primary SCG cells (Shivachar *et al.*, 1999). However, it is worth noting that the concentration used for ICI118551 might not block the effect of isoprenaline (10 µM). In other words, the high concentration of isoprenaline might not be antagonized by the concentration used for ICI118551. Therefore, it is suggested to try another concentration for ICI118551 and isoprenaline. Indeed, the concentration ratio between the isoprenaline and ICI118551 is an important issue to consider in this case as the relative affinity of ICI118551 and isoprenaline to the receptor, Ki=1.2 nM and Ki=904 nM, respectively (Kikkawa *et al.*, 1997; Kostka *et al.*, 1989).

The explanation behind the lack of response to isoprenaline in myotubes may be due to the fact that mRNA expression levels might not reflect protein expression levels in rat primary skeletal muscle cells. Moreover, skeletal muscle tissue contains multiple cell types (satellites, myoblasts and myotubes) and receptor expression might be restricted to a specific cell type.

Regarding signalling via G_i-GPCRs (including α_{2A}-adrenoceptors and A_1 adenosine receptor), the activation of these GPCRs did not inhibit forskolin-evoked cAMP. The different explanations behind the lack of response for the activation of G_i-GPCRs might be due to: 1) As skeletal muscle expressed *adcy2 and adcy6*, it is possible that decrease in cAMP level by inhibition of AC2 and AC6 through G_i protein might be neutralized by activation of AC through $G_{\beta\gamma}$ subunit. This is supported by the fact that $G_{\beta\gamma}$ subunit was found to increase AC2 activity in insect ovarian Sf9 cells infected with recombinant baculovirsus (B-rACII) (Tang *et al.*, 1991), and coexpressed G_i protein in Sf9 cells was also found to inhibit AC2 and AC6 activity (Taussig *et al.*, 1994). 2) The receptors might not couple to G_i protein subunit, therefore, no effect was observed for ligands. 3) G_i protein might not be expressed in skeletal muscle; a potential significant influence is that the conditions for culturing myotubes are different from those *in vivo*, including intermittent innervation and variable (time, concentration, etc.) exposure to hormones. 4) The mismatch between mRNA and protein expression of these receptors, as suggested previously. As activation of any of the identified G_i GPCRs failed to decrease cAMP levels, it is strongly supported that these GPCRs did not couple to G_i subunit. However, further investigation is suggested to examine the protein expression of these receptors and G_i subunit using immunoblotting and

immunocytochemistry. Moreover, investigation is recommended to test the signalling of these receptors using cAMP assay in presence of electrode to mimic the *in vivo* conditions for this primary cell culture. Further investigation is also suggested to examine the coupling of these receptors to G_i protein using pertusis toxin, for example, the effect α_{2A}-adrenoceptors of ERK phosphorlyation can be examined if ERK phosphorlyation occurred through coupling to G_i protein. Indeed, inhibition of ERK phosphorlyation should be observed in the presence of pertusis toxin. Furthermore, siRNA for $G_{\beta\gamma}$ subunit is suggested to examine the effect of these GPCRs activation on the cAMP level.

CONCLUSION

These findings provided evidence for functionally-active (potentially) α_2-adrenoceptors in skeletal muscle which might be important for skeletal muscle fat and glucose metabolism and skeletal muscle growth.

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