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The expression of receptors for endocannabinoids in human and rodent skeletal muscle

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Abstract

The endocannabinoid system is a lipid derived signalling system that has been shown to regulate appetite and energy metabolism. The most abundant endogenous endocannabinoid, anandamide, has been shown to activate the cannabinoid receptor type 1 (CB1) and type 2 (CB2) as well as the 'non-cannabinoid' transient receptor potential channel-vanilloid sub-family member 1 (TRPV1), before being rapidly metabolised by fatty acid amide hydrolase (FAAH). We have previously demonstrated the expression of CB1 and studied the effects of CB1 activation and inhibition in human skeletal muscle myotubes, however, not all results could be explained by CB1 mediated effects. This suggests that other receptors which are activated by endocannabinoids may be present in skeletal muscle. In this study we describe the presence of not only CB1, but also CB2, TRPV1 and the degrading enzyme FAAH in human and rodent skeletal muscle using reverse transcription polymerase chain reaction (RT-PCR).

Keywords: CB1; CB2; TRPV1; FAAH; Skeletal muscle; RT-PCR

The endogenous endocannabinoid, anandamide (AEA) is synthesised from arachidonic acid and has an autocrine or paracrine mode of action before being rapidly metabolised by fatty acid amide hydrolase (FAAH) [1,2]. It is a ligand for cannabinoid receptors type 1 (CB1), and type 2 (CB2) which are 7 transmembrane domain G-protein coupled receptors [3,4]. AEA is also a ligand for the transient receptor potential channel-vanilloid sub-family member 1 (TRPV1), activation of which leads to cellular depolarisation and increased intracellular calcium [5–7].

Initially the effects of endocannabinoids were considered to be predominantly central, and certainly the effects to decrease food intake are substantially mediated within the hypothalamus and nucleus accumbens [8], although some effects may also be mediated via peripheral receptors located in the gastrointestinal tract [9]. Initial pair feeding studies suggested that not all of the effects of CB1 antagonists in decreasing adiposity could be explained by a reduction in food intake [10], and effects on metabolism in adipose tissue [11], the liver [12] and skeletal muscle [13] are now known to be an important component of the mechanism of action of CB1 antagonists [14].

CB2 receptors were initially thought to be expressed exclusively in cells of the immune system [4,15], but have now been observed in the pancreas, adipose tissue and cardiac muscle [16–18].

We have recently shown that the CB1 receptor is expressed in human skeletal muscle and that the endocannabinoid AEA modifies the pathways that regulate fatty acid oxidation in human skeletal muscle [19]. However, not all of the effects of AEA can be accounted for by blocking the CB1 receptor with the CB1 receptor specific antagonist AM251, suggesting the presence of other receptors for endocannabinoids in skeletal muscle [19]. We have therefore used RT-PCR to demonstrate the expression of not only CB1, but also CB2, TRPV1 and FAAH in human

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and rodent skeletal muscle using reverse transcription polymerase chain reaction (RT-PCR), suggesting that the endocannabinoid system has regulatory effects in skeletal muscle.

Materials and methods

Human samples. Following informed consent, human tissue was obtained during gastric surgery from the rectus abdominus muscle [20], omental fat (intra-abdominal), and adipose subcutaneous tissue depots. Whole tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C. This study was approved by the Human Ethics Research committee of The Royal Adelaide, Calvary and Burnside Hospitals (SA, Australia).

Primary skeletal muscle cell culture. Human primary skeletal muscle myotubes were cultured according to the methods previously described [19–21]. Prior to RNA extraction, myotubes were grown to confluence at passage 6 then differentiated for 4 days.

Rodent myogenic cell line (L6). L6 cells, kindly donated by Dr. David Cameron-Smith (Deakin University, Melbourne, Australia), were cultured according to methods previously described [22]. Prior to RNA extraction, L6 myotubes were grown to confluence then differentiated for 4 days.

Rodent samples. Following approval from the Animal Ethics committees of the University of Adelaide and Institute of Medical and Veterinary Science, Royal Adelaide Hospital, rodent skeletal muscle and adipose tissue were obtained from male Wister rats aged 20 weeks fed a standard laboratory diet. Immediately after sacrifice, skeletal muscle was obtained from the hind limb and adipose tissue from the mesenteric fat depot, immediately frozen in liquid nitrogen, and stored at −80 °C.

RNA extraction. Human primary skeletal muscle myotubes and L6 myocytes were lysed with TRIzol® (Invitrogen, Melbourne, Australia), then extracted as previously described [23]. Whole tissue portions of skeletal muscle (10–20 mg) or adipose tissue (60–80 mg) were removed from −80 °C storage, placed into 800 μl of TRIzol® containing one 5 mm stainless steel bead (Qiagen, Melbourne, Australia), then homogenised using the Qiagen TissueLyser for 1.5 min at 30 Hz (Qiagen, Melbourne, Australia). RNA was then extracted from the samples as previously described [23]. Total RNA concentration was quantified using nanodrop spectrometry at 260 nm. To ensure purity for mRNA sequences containing a single exon, the RNA was treated with the TURBO DNA-*free*™ kit (Ambion, Austin, Texas).

RT-PCR. RNA (0.5 μg/10 μl) was utilised to generate cDNA by reverse transcription using the Reverse Transcription System (Promega, Madison, WI). RT-PCR was performed using the Eppendorf Mastercycler Gradient (Eppendorf South Pacific, Sydney, Australia) using Qiagen HotStarTaq Plus Master Mix (Qiagen, Melbourne, Australia) with the following cycle profile: activation of Taq polymerase at 95 °C for 5 min, then 94 °C for 30 s followed by 40 cycles at 57 °C for 30 s and 72 °C for 30 s, then 10 min at 72 °C.

Primers were designed using the Primer Express software package Version 3.0 (Applied Biosystems, Foster City, CA). Gene sequences were obtained from GenBank. BLAST searches for all primers confirmed homologous binding to desired mRNA of human or rodent samples for all chosen genes. Primer sequences are shown in Table 1. RT-PCR products were analysed using gel electrophoresis on a 2.5% agarose gel. After electrophoresis the gels were stained with ethidium bromide, placed on top of an ultraviolet transilluminator and visualised with the Kodak EDAS 290 digital camera.

Results

Using RT-PCR, expression of CB1 was observed in all human and rodent samples analysed (Fig. 1). As the open reading frame for CB1 for both human and rodent contains a single exon, the RNA utilised was extracted and DNAse treated as described in the methods. Furthermore, negative controls containing extracted RNA but not the reverse transcriptase enzyme for each sample were applied to ensure no DNA contamination. Product sizes of 66 base pairs (bp) for human and 68 bp for rodent were observed as predicted (Table 1, Fig. 1).

Similarly, the CB2 open reading frame for human was in a single exon. For rodent, while there were three exons, it was not possible to design primers that spanned the exon junctions, possible due to potential sequence variability

Table 1
Primer sequences used for reverse transcription polymerase chain reaction (RT-PCR)

Gene	Species	Primer	Product size (bp)
CB1	Human	F—CGC TTT CCG GAG CAT GTT R—TCC CCC ATG CTG TTA TCC A	66
	Rodent	F—CAA GCA CGC CAA CAA CAC A R—TCT TAA CGG TGC TCT TGA TGC A	68
CB2	Human	F—TAT GGG CAT GTT CTC TGG AA R—GAG GAG CAC AGC CAA CAC TA	141
	Rodent	F—TTC CCC CTG ATC CCC AAC GAC TA R—CTC TCC ACT CCG CAG GGC ATA AAT	369
FAAH	Human	F—AGG CCC AGA TGG AAC ATT ACA G R—GGC AGC CCC ACA CTC TTC T	112
	Rodent	F—CTG CCC AGC TGG TTT AAA AGA R—AGG ACG CAT ACT GTT GAG AAA GG	87
TRPV1	Human	F—TTC ACC ATG GCT GCC TAC TAC A R—TCC TCC TAA CAC AGA CAG GAT CTC T	111
	Rodent	F—GGA CGT TGC CCG GAA GAC AGA CA R—GAC AGG GGC AGC TCA CCA AAG TAG	228

CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; TRPV1, transient receptor potential channel-vanilloid sub-family member 1. Numbers (NM_) of the genes are NCBI accession numbers obtained from the NIH Database for human (H) and rodent (R): CB1,(H) NM_016083, (R) NM_012784; CB2, (H) NM_001841, (R) NM_020543; FAAH, (H) NM_001441, (R) NM_024132; TRPV1, (H) NM_018727, (R) NM_031982.

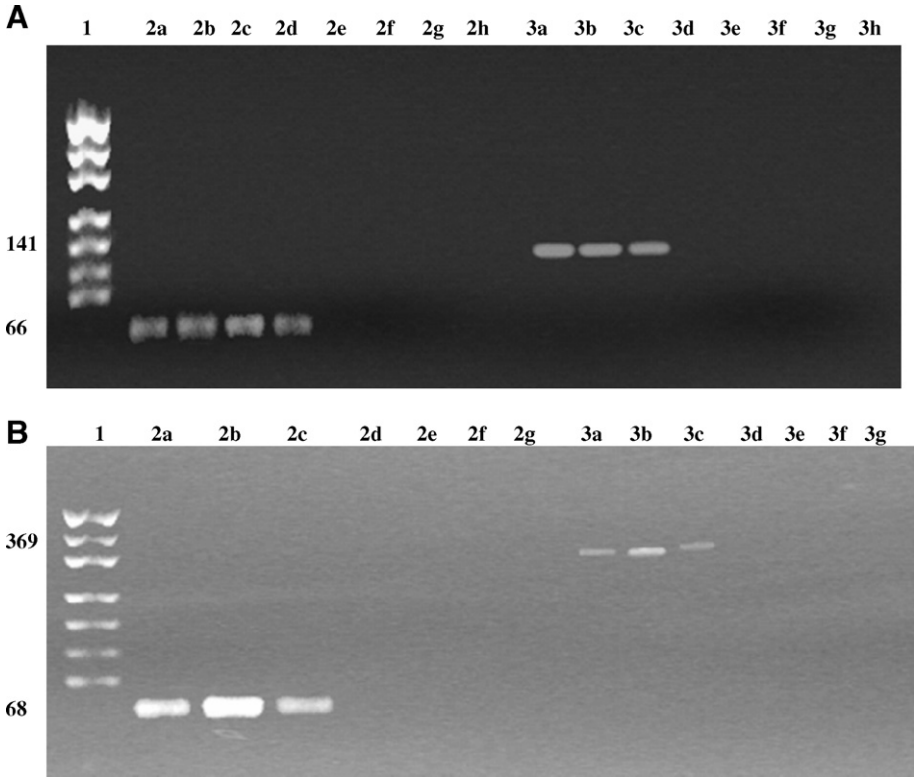

Fig. 1. Gel electrophoresis of RT-PCR products. Products were analysed on a 2.5% agarose gel stained with ethidium bromide. (A) Human tissue samples; lane: (1) pUC 19 DNA size marker, (2a–h) CB1, (3a–h) CB2. Samples were loaded alphanumerically: (a) primary skeletal muscle myotubes, (b) rectus abdominus whole muscle, (c) intra-abdominal adipose tissue, (d) subcutaneous adipose tissue, (e) reverse transcriptase negative (rt−) myotubes, (f) rt− whole muscle, (g) rt− combined adipose tissue, (h) RT-PCR template negative. (B) Rodent tissue samples; lane: (1) pUC 19 DNA size marker, (2a–g) CB1, (3a–g) CB2. Samples were loaded alphanumerically: (a) L6 cells, (b) hind limb whole muscle, (c) adipose tissue, (d) rt− L6 cells, (e) rt− whole muscle, (f) rt− whole adipose tissue, (g) RT-PCR template negative.

and the small size exons 2 and 3. As such, all samples for CB2 underwent the same DNAse treatment and control set as mentioned above. The RT-PCR yield for CB2 produced was very low; to counter this, the amount of cDNA loaded for RT-PCR was increased 10 times over the standard loading amount. Using this technique, CB2 was detected in human cell culture, whole muscle, intra-abdominal adipose tissue, but not in subcutaneous adipose tissue (Fig. 1). In rodent tissue CB2 expression was detected in all samples (Fig. 1). Product sizes for human (141 bp) and rodent (369 bp) matched predicted values (Fig. 1, Table 1).

The human and rodent primers for FAAH and TRPV1 were designed across multiple exons. In human samples, FAAH was detected in all tissues at the predicted product size (111 bp); similarly FAAH was detected in all rodent tissues at the expected product size (87 bp) (Fig. 2, Table 1). TRPV1 was also detected in all human and rodent tissues at the expected product sizes (112 bp and 228 bp, respectively) (Fig. 2, Table 1).

Discussion

When CB1 was first cloned in 1990 [3], it was thought to be expressed only in the brain [24]. This has since changed to include the peripheral organs such as the thyroid gland, adrenal gland, liver, adipose tissue, the gastrointestinal tract and skeletal muscle [9–11,25–27]. Until recently, the expression of CB1 had only been reported in skeletal muscle of rodent [26], but we have demonstrated CB1 receptor expression in human primary skeletal muscle myotubes [19]. In this study we confirm the presence of CB1 in both human and rodent adipose tissue and skeletal muscle. The observation that CB1, as well as CB2, TRPV1 and FAAH are expressed in primary culture of human primary skeletal muscle myotubes and L6 cells indicates that their expression is myocyte specific and not the result of contaminating adipose tissue or another infiltrating cell type.

Initially the expression of CB2 was thought to be limited to cells of the immune system [4]. Recently, CB2 has been observed in brain microglia cells under inflammatory conditions [28], on sensory nerve fibres and adnexal structures in human skin [29], in neonatal cardiomyocytes [18] and in zebrafish muscle [30]. Here, we demonstrate for the first time the expression of CB2 in both human and rodent, skeletal muscle, as well as both human primary skeletal muscle myotubes and rodent myocyte cell culture, albeit at low levels. The expression of CB2 in human fat was detected solely in intra-abdominal and not subcutaneous adipose tissue; however, a recent study has detected low levels of CB2 expression in subcutaneous adipose tissue

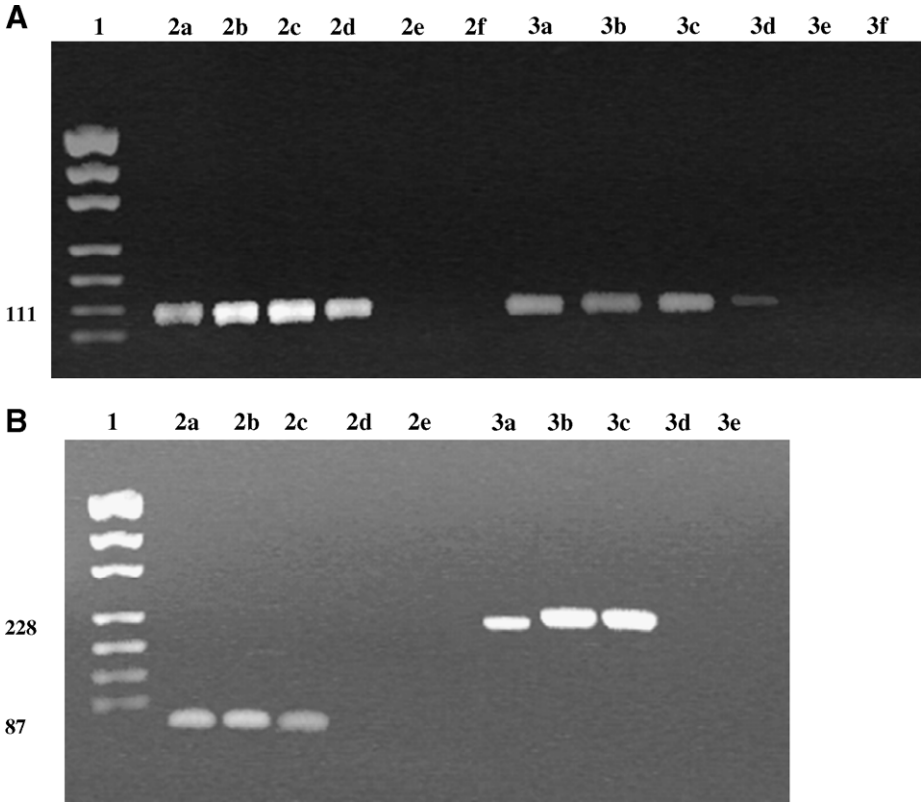

Fig. 2. Gel electrophoresis of RT-PCR. Products were analysed on a 2.5% agarose gel stained with ethidium bromide. (A) Human tissue samples; lane: (1) pUC 19 DNA size marker, (2a–f) FAAH, (3a–f) TRPV1. Samples were loaded alphanumerically: (a) primary skeletal muscle myotubes, (b) rectus abdominus whole muscle, (c) intra-abdominal adipose tissue, (d) subcutaneous adipose tissue, (e) reverse transcription template negative, (f) RT-PCR template negative. (B) Rodent tissue samples; lane: (1) pUC 19 DNA size marker, (2a–e) FAAH, (3a–e) TRPV1. Samples were loaded alphanumerically: (a) L6 cells, (b) hind limb whole muscle, (c) whole adipose tissue, (d) reverse transcription template negative, (e) RT-PCR template negative.

[31]. It may be that the constitutive expression of the receptor is very low, and the expression of the receptor is regulated and increased only in response to specific physiological or pathological situations, for example during exercise or inflammation; this remains to be determined.

FAAH, the enzyme responsible for the breakdown of AEA, has been shown to be highly conserved between human, rodent and mouse [32]. Recent studies have observed that FAAH mRNA expression is down regulated in obesity which is associated with higher levels of circulating AEA [33]. Also, polymorphisms in the gene encoding FAAH have been linked with obesity [34,35]. Therefore, the confirmation of the presence of FAAH in human and rodent skeletal muscle substantiates this enzyme as a possible important regulator of whole body energy balance as skeletal muscle is the primary site for fatty acid oxidation and glucose metabolism [36,37].

The initial cloning of TRPV1 occurred in 1997, when it was shown to be a receptor for capsaicin [38]. Shortly thereafter, it was discovered that AEA could also activate TRPV1 [5]. Since this has raised speculation regarding a pathway that links TRPV1 to FAAH through regulation of AEA [39]. Interestingly, it has been shown in goldfish retinal amacrine cells that TRPV1 and FAAH co-localise when TRPV1 is activated, suggesting an auto regulatory function [40]. It has previously been shown that TRPV1 is expressed in the sarcoplasmic reticulum of rodent skeletal muscle, and is likely responsible for release of Ca^{2+} into the cell [41]. Here we show that TRPV1 is also expressed in human skeletal muscle and adipose tissue. As TRPV1 and FAAH are both expressed in human and rodent skeletal muscle, the pathway linking them may be involved in regulating the metabolism of endocannabinoids in muscle.

There is increasing evidence that the endocannabinoid system has a role in the regulation of whole body energy balance [26]. In skeletal muscle we have previously shown that endocannabinoid activity regulates mRNA expression of key genes involved in nutrient oxidation that could not be explained solely by CB1 mediated effects, making it likely that the effects of AEA in the primary cultures of human myotubes was being mediated by more than one receptor [19]. In this study, have demonstrated the expression of CB2 and TRPV1 in human and rodent skeletal muscle as potential receptor sites for the previously observed effects of AEA [19]. Drugs targeting CB1, CB2, FAAH and TRPV1 for the treatment of obesity, pain and inflammation are currently under development [26,42–44]. The expression of CB2, FAAH and TRPV1 along with CB1 in human and rodent skeletal muscle is

therefore an important consideration in the development of these therapeutic targets. Endocannabinoids may have a complex role in skeletal muscle biology.

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