The effect of cannabinoids on APP, BACE1, and ApoE mRNA expression

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Abstract
Alzheimer’s disease (AD) is an age-related neurodegenerative condition associated with cognitive decline. The pathological hallmark of this disease is the deposition of β-amyloid protein plaques (Aβ) in the brain, which evoke neuronal cell death and impair inter-neuronal communication. Past studies have suggested that cannabinoids reduce the levels of Aβ in the brain; however, little is known about the mechanisms involved in this process. In this study, the SH-SY5Y cell line was first examined for expression of amyloid precursor protein (APP), beta-site APP cleaving enzyme 1 (BACE1), and apolipoprotein E (ApoE), genes involved in Aβ production and clearance. All three genes were expressed and detected in the cell line. We then observed the effects of the endocannabinoid anandamide, a CB1 receptor agonist, on the mRNA expression of APP, BACE1, and ApoE in SH-SY5Y cells. After 48h exposure to anandamide, mRNA levels of APP and BACE1 significantly decreased, which could contribute to reduced Aβ levels. The mechanism of action by which anandamide reduces mRNA levels of APP and BACE1 should be further investigated. ApoE mRNA levels were not found to be significantly changed, suggesting that anandamide does not affect mRNA expression of this gene. The effects of cannabinoids on ApoE levels should be further studied as the effects may occur at a level different from mRNA expression and may even occur via a pathway unrelated to CB1 receptor activation.

Keywords: Alzheimer’s disease; β-amyloid; anandamide; amyloid precursor protein; beta-site APP cleaving enzyme 1; apolipoprotein E

Introduction
Alzheimer’s disease (AD) is known to be a major cause of dementia (Tarawneh and Holtzman 2012). It is characterized by the accumulation of amyloid plaques, comprised of β-amyloid (Aβ) protein, which play a central role in the pathophysiology of AD (Harvey et al. 2012). Aβ comes from a larger protein called amyloid precursor protein (APP) found in the cell membrane of nerve cells. APP can be cleaved by two pathways: the non-amyloidogenic pathway, in which Aβ is not produced, and the amyloidogenic pathway, in which the end product is Aβ. In patients with AD, there is increased cleavage of APP via the amyloidogenic pathway involving beta-secretases such as beta-site APP cleaving enzyme 1 (BACE1). Cleavage of APP by BACE1 produces a sAPPβ fragment and a C-terminal fragment (Cole and Vassar 2008), which is then cleaved by gamma secretase to produce Aβ (Cole and Vassar 2008). In AD, it is thought that higher concentrations of Aβ are due to an impaired clearance of Aβ from the brain, a process normally facilitated by apolipoprotein E (ApoE) (Cramer et al. 2012).

As incidence rates of AD are projected to triple in the next fifty years, the development of therapeutics to slow or cure the disease have become crucial to improve the quality of life of patients as well as reduce the health care expenses associated with AD (Hebert et al. 2013). Recently, there has been increasing interest in the use of cannabinoids as a potential treatment for AD as a result of their neuroprotective and anti-inflammatory effects.

Cannabinoids are a class of chemical compounds that activate cannabinoid receptors in the body of which there are two main subtypes – CB1 and CB2. High concentrations of CB1 receptors exist in brain structures such as the hippocampus and basal ganglia. These receptors, which are important in memory, are also impaired in individuals with AD (Mechoulam and Parker 2013). CB2 receptors are found mainly in peripheral tissue and in microglial cells of the brain, but not in neurons (Mechoulam and Parker 2013). Hence, the CB1 receptor is the focus of this study.
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Table 1. Primers used for reverse transcriptase and real-time polymerase chain reactions

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>APP-Forward</td>
<td>5'-TCCGAGGGGTAGAGTTTGGAAG-3'</td>
</tr>
<tr>
<td>APP-Reverse</td>
<td>5'-TCCACCTGACCCCTTCTCCTC-3'</td>
</tr>
<tr>
<td>BACE1-Forward</td>
<td>5'-TGGAGGGCTTCTAGTTGCT-3'</td>
</tr>
<tr>
<td>BACE1-Reverse</td>
<td>5'-CAGATGGCGACGTGAAAGAG-3'</td>
</tr>
<tr>
<td>ApoE-Forward</td>
<td>5'-CTGCTCTCCCCAGGAGCGGA-3'</td>
</tr>
<tr>
<td>ApoE-Reverse</td>
<td>5'-GGCACCAGTGCGAGTTCCC-3'</td>
</tr>
<tr>
<td>G6PD-Forward</td>
<td>5'-CAACACCCCTGAGCCTGAC-3'</td>
</tr>
<tr>
<td>G6PD-Reverse</td>
<td>5'-TGGAACCGGGACACATC-3'</td>
</tr>
<tr>
<td>B2M-Forward</td>
<td>5'-TGACCCTGATCTTTCTGGTG-3'</td>
</tr>
<tr>
<td>B2M-Reverse</td>
<td>5'-ATCTGAGTTGGTGGAACCTG-3'</td>
</tr>
</tbody>
</table>

Cannabinoids, particularly CB1 receptor agonists, have previously been shown to prevent experimental Aβ-evoked brain pathology in rats in vivo (Ramirez et al. 2005) and reduce Aβ levels in the brains of APP transgenic mice (Martín-Moreno et al. 2012). However, there is currently very little information about the mechanism of action in this process. Cannabinoids have been known to affect the expression of various genes in the brain (Bosier et al. 2007). This study examined whether cannabinoids have a direct cellular effect on the mRNA expression of APP, BACE1, and ApoE in SH-SY5Y cells, which are known to carry the CB1 receptor. This would shed light on the mechanism by which cannabinoids reduce Aβ levels in the brain and exert their neuroprotective effects.

The first objective of this study was to test whether the SH-SY5Y cells, a human neuroblastoma cell line, expressed the genes of interest: APP, BACE1, and ApoE, as well as to test for a good housekeeping gene in this cell line. The second objective was to treat the cells with anandamide, a CB1 receptor agonist, and see whether there were changes in the mRNA expression of APP, BACE1, and ApoE. By examining the direct effects of anandamide on these genes, it may be possible to identify a mechanism through which cannabinoids decrease Aβ levels in the brain: by decreasing production of Aβ, increasing clearance of Aβ or both. It was hypothesized that anandamide lowers Aβ levels through decreased expression of APP and BACE1 and increased expression of ApoE. The present study focused on mRNA expression. Studies examining the effects of anandamide on APP, BACE1 and ApoE protein levels as well as Aβ production will be needed to test this hypothesis further.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, Trizol, trypsin, trypan blue, DNA purification kits, reverse transcription reagents, and PCR reagents were purchased from Invitrogen (Carlsbad, CA). Anandamide was supplied by Dr. Linda Parker at the University of Guelph. PerfeCTa SYBR Green Fastmix with ROX (Quanta BioSciences) was purchased from the Genomics facility at the University of Guelph.

SH-SY5Y cell culture

SH-SY5Y human neuroblastoma cell lines were maintained in 75 cm² culture flasks with DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL of streptomycin. Cells were plated or divided twice weekly using trypsin to detach adherent cells. For plating, cell counts were performed using a haemocytometer and trypsin blue was used to stain non-viable cells, which were excluded from the count. Cell counts typically fell between 155-200 cells for every four fields on the haemocytometer. All experiments and cell maintenance was performed in a laminar flow hood under sterile conditions. Cells were grown in an incubator with 5% CO₂ at 37°C. Cells were plated onto Corning CellBind coated dishes at a density of 2.0 million cells per 100 mm dish for RNA analysis, 48 hours prior to experiments.

Treatment of SH-SY5Y cells

Anandamide was stored at -20°C in 100% ethanol medium. Six sets of cell plates were treated with 0.1 µM, 1 µM, or 10 µM anandamide. Plates treated with 10 µL of 100% ethanol medium were used as a control.

RNA isolation and reverse transcription

Total RNA was isolated from each SH-SY5Y cell plate using Trizol 48h after treatment. RNA was purified with RNase-free chloroform and precipitated using RNase-free isopropanol. The RNA levels were subsequently quantified using a spectrophotometer. Extracted RNA (5 µg) was reverse transcribed with Superscript II for 75 min at 43°C, using oligo-dt as the primer. The cDNA generated was then used for reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR).
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Figure 1. Representative expression of APP, BACE1, ApoE, G6PD, and B2M in SH-SY5Y cells. (A) APP expression in water blank (wb), control sample (cntl), sample treated with 10 μM anandamide (10 μM), and sample treated with 1 μM anandamide (1 μM). (B) BACE1 expression in wb, cntl, 10 μM, and 1 μM anandamide. (C) ApoE expression in wb, cntl, 10 μM, and 1 μM anandamide. (D) G6PD expression in wb, cntl, 10 μM, and 1 μM anandamide. (E) B2M expression in wb, cntl, 10 μM, and 1 μM anandamide.

Figure 2. (A) APP sequencing results. (B) Comparison of APP sequence (query) with Homo sapiens APP transcript variant 7 sequence (subject) published under National Centre for Biotechnology Information. Nucleotides 18-148 from the APP sequencing results were compared against the published sequence.
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Figure 3. (A) BACE1 sequencing results. (B) Comparison of BACE1 sequence (query) with BACE1 transcript variant f sequence (subject) published under National Centre for Biotechnology Information. Nucleotides 17-198 from the BACE1 sequencing results were compared against published sequence.

Figure 4. RT-PCR analysis using housekeeping gene G6PD. (A) RT-PCR visualized using gel electrophoresis for independent sample sets 1-3 (reverse transcribed twice) treated with 100% ethanol for control, 0.1 μM anandamide, 1 μM anandamide, or 10 μM anandamide. (B). RT-PCR visualized using agarose gel electrophoresis for independent samples sets 4-6 treated with 100% ethanol for control, 0.1 μM anandamide, 1 μM anandamide, or 10 μM anandamide.
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Figure 5. Analysis of APP, BACE1, and ApoE mRNA expression (A) Real-time PCR analysis of total APP mRNA relative to G6PD levels following treatment with 0.1, 1, or 10 μM anandamide. APP mRNA expression was significantly decreased with 10 μM anandamide treatment compared to control (*p<0.05). (B) Real-time PCR analysis of total BACE1 mRNA relative to G6PD levels following treatment with 0.1, 1, or 10 μM anandamide. mRNA expression of BACE1 was significantly decreased with 10 μM anandamide treatment when compared to control (***p<0.01). (C) Real-time PCR analysis of total ApoE mRNA relative to G6PD levels following treatment with 0.1, 1, or 10 μM anandamide. Changes in mRNA expression of ApoE were not statistically significant (p>0.05). Statistical significance was determined by performing a one-way ANOVA followed by the Dunnett Multiple Comparisons Test. Data are presented as the mean ± standard error of mean (n=6).

RT-PCR and gel electrophoresis

The RT-PCR reactions were performed to determine whether the SH-SY5Y cell line expressed APP, BACE1 and ApoE gene products at detectable levels, as well as to test whether either glucose-6-phosphate dehydrogenase (G6PD) or beta-2-microglobulin (B2M) would make a better housekeeping gene. For each sample, 5 μL of cDNA was mixed with a master mix containing 15 mM MgCl2, 10 mM dNTPs, 5 μM of forward and reverse primers, 10x PCR buffer, Platinum Taq and water to create a final volume of 50 μL. For all genes, RT-PCR reactions were performed in a MyCycler (Bio-Rad Laboratories) repeating 35 cycles of 94°C for 30s, 56°C for 30s, and 72°C for 1 min. The primers used for each gene are listed Table 1.

The PCR products of each gene were electrophoresed on a 1.5% agarose gel against a 100 bp DNA ladder. The products of APP and BACE1 were purified using a DNA purification kit and sent for sequencing to verify that the gene products amplified from these primers were, in fact, those of APP and BACE1. Gene products of ApoE and G6PD primers had already been sequenced and verified to be those of their corresponding genes. The sequences were compared with published sequences from the National Centre for Biotechnology Information database.

qPCR

The qPCR reactions were carried out to quantify changes in mRNA expression of APP, BACE1, and ApoE. The qPCR reactions were performed with Steponeplus (Applied Biosystems) and Via7 Software (Applied Biosystems) using 96-well plates. The cDNA (5 μL) of each sample was mixed with SYBRGreen Fastmix, primers, and water for a final volume of 15 μL. The cycling conditions were 1 cycle of 30s at 95°C followed by 40 cycles of 3s at 95°C and 30s at 60°C. The primers used to amplify APP, BACE1, ApoE, and G6PD were the same as those used for RT-PCR (Table 1).
Data analysis and statistics

Data from qPCR were analysed using StepOne Software (Applied Biosystems) and Viia7 Software (Applied Biosystems). APP, BACE1, and ApoE levels were quantified relative to G6PD levels in the same sample. Data are representative of six experiments and are presented as the mean ± standard error of the mean (SEM). All data were tested for normality, and statistical analysis was carried out on GraphPad InStat (GraphPad Software Inc.) using a one-way analysis of variance (ANOVA). ANOVA was followed by the Dunnett Multiple Comparisons Test to determine significant differences between treatment groups. Mean values were considered different if p<0.05.

Results

Expression of target genes in SH-SY5Y cell line

To qualitatively test for expression of APP, BACE1, and ApoE as well as the housekeeping genes G6PD and B2M in the SH-SY5Y cell line, a few of the RNA samples collected were subjected to RT-PCR. Products from the RT-PCR reactions were visualized by agarose gel electrophoresis. One control sample, one sample treated with 10 μM anandamide, and one sample treated with 1 μM anandamide, were used for each gene. As seen in Figure 1, amplification of DNA products was detected for each primer pair, indicating that all of the genes were expressed in the cell line. From the intensity of the bands, it is also evident that G6PD (Figure 1D) was amplified at higher concentrations compared to B2M (Figure 1E). Thus, the G6PD gene was used as a housekeeping gene in further experiments. Although this qualitative analysis revealed there was some contamination of the water blank of G6PD (Figure 1D-wb), subsequent PCR and qPCR analysis did not result in the detection of any PCR products in the water blank controls.

Gene sequencing of APP and BACE1

The gene products of APP and BACE1 were purified and sent for sequencing to verify that the gene products amplified from these primers were, in fact, those of APP and BACE1 (Figures 2A and 3A). Gene products of ApoE and G6PD primers had been sequenced previously and verified to be those of their respective primers. The sequences were compared with published sequences from the National Centre for Biotechnology Information database. As seen in Figures 2B and 3B, sequences of gene products of APP as well as BACE1 matched published sequences of these genes with 99% identity.

Effect of anandamide on APP, BACE1 and ApoE mRNA levels

Six sets of SH-SY5Y cell plates were treated with 0.1 μM, 1 μM, or 10 μM anandamide (or 100% ethanol for the control samples), and RNA was isolated 48h after treatment. The samples were subjected to RT-PCR to qualitatively test for sufficient quantity and integrity of cDNA of each sample prior to running qPCR with the samples. The housekeeping gene, G6PD, was used for relative quantification. As seen in Figures 4A and 4B, bands were visible for all samples, indicating that there was a sufficient quantity of gene product in each sample; in addition, it also verified that the integrity of the gene products of all the samples had been conserved.

Next, qPCR was carried out on all the samples to measure changes in mRNA expression of APP, BACE1, and ApoE. The APP mRNA levels were significantly decreased (*p<0.05) with 10 μM anandamide treatment in comparison to control (Figure 5A). The BACE1 mRNA levels also showed a very significant decrease (**p<0.01) with 10 μM anandamide treatment (Figure 5B). The ApoE mRNA levels appeared to increase with anandamide treatment; however, this increase was not statistically significant (Figure 5C).

Discussion

In the present study, APP, BACE1, and ApoE mRNA were successfully amplified by RT-PCR from the SH-SY5Y cell line. These genes have not been extensively analyzed in this cell line, so determination of their expression was crucial in the steps following in this study. As all genes were expressed in the cell line, the human neuroblastoma cell line can be used as a model to test the regulation of expression of APP, BACE1, and ApoE. This study also demonstrated that anandamide significantly decreased the mRNA levels of APP and BACE1 but not ApoE. This suggests that anandamide-mediated decreases in Aβ result from decreased synthesis, rather than increased clearance of this peptide. The physiological concentration of anandamide in various parts of the brain ranges from 0.35-0.45 μM (Muguruza et al. 2013). Studies that previously found decreased Aβ levels in the brain following cannabinoid treatment administered doses both above and below this range (Ramirez et al. 2005; Harvey et al. 2012). In the present study, the doses of anandamide administered were not only those that had decreased Aβ levels in previous studies, but were also doses that fell near the physiological range.

Initial RT-PCR reactions indicated that APP, BACE1, ApoE, B2M and G6PD mRNA were all present in control and anandamide-treated cells. There was some contamination in the water blank of the G6PD as seen in Figure 1D-wb. This was most likely due to contamination of the primers that were used, or possibly transfer of gene product while loading the wells. The use of newly prepared G6PD primers in subsequent experiments did not indicate contamination. Furthermore, as the bands for G6PD were of much greater intensity compared to B2M, it was decided that G6PD should be used as the housekeeping gene in future experiments conducted on SH-SY5Y cells.

The APP and BACE1 gene products were sequenced to verify their identities and to test the efficiencies of the primers used. If the APP and BACE1 sequencing results were not a definite match with published sequences for their corresponding genes, then this would have indicated an issue.
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with primer specificity. The sequencing of APP and BACE1 resulted in a 99% match with the genes published in the National Centre for Biotechnology Information database (Figures 2B and 3B), signifying that the primers were amplifying the correct genes.

In the sequencing results, N represented nucleotides that were undetermined. The nucleotide was marked as undetermined if there was more than one signal detected during the sequencing. However, the graphs of the sequencing results (Figures 2A and 3A) could be retrospectively analyzed to determine the nucleotide that was most likely in that position. In the APP sequencing graph (Figure 2A), the 25th nucleotide was found to be undetermined. At this position in the graph, a red peak, representative of the nucleotide thymine (T), can be observed. Thymine was also the nucleotide found in the same position in the published sequence (Figure 2B). From the BACE1 sequencing graph (Figure 3A), the 29th and 189th nucleotide were undetermined. The BACE1 sequencing graph clearly depicts a green peak at the position of the 29th nucleotide, representing adenine (A), and a red peak at the position of the 189th nucleotide, representing thymine (T). These were also the nucleotides found in the same positions in the BACE1 published sequence. This indicates that the match between the sequencing results and the published sequences is in actuality greater than 99% due to the identity of the previously undetermined nucleotides during sequencing.

Treatment with 0.1 μM and 1 μM anandamide did not show any statistically significant changes in the mRNA expression of APP when compared to control. However, as shown in Figure 5A, treatment with 10 μM anandamide corresponded to a significant decrease in APP mRNA levels (p<0.05). This is in accordance with our hypothesis. If APP mRNA levels decrease, then perhaps there is less APP protein being produced, and consequently less APP being cleaved by the amyloidogenic pathway. As a result, one would expect decreased Aβ formation, which is consistent with what has previously been reported to occur during cannabinoid treatment (Bisogno et al. 2008). The effects of cannabinoids on APP mRNA expression have not been studied at length, so there is very little information as to how APP mRNA levels might decrease due to anandamide administration. However, it has been found that the cytokine interleukin-1 stimulates the promoter region of the APP gene (Donnelly et al. 1990). Since anandamide has been shown to significantly decrease pro-inflammatory cytokines, such as interleukin-1 expression (Ortega-Gutiérrez et al. 2005), it is possible that anandamide down-regulates the transcription of the APP gene by decreasing interleukin-1. If future studies find that this mechanism of action holds true, it might provide an explanation for the anti-inflammatory and neuroprotective effects of cannabinoids.

Changes in mRNA levels of BACE1 were not statistically significant with 0.1 and 1 μM of anandamide treatment, but there was a significant decrease corresponding to treatment with 10 μM anandamide (p<0.01) (Figure 5B). This was also supportive of our original hypothesis. If lower levels of BACE1 mRNA correspond to lower levels of BACE1 synthesis, there should be less APP being cleaved through the amyloidogenic pathway, thus resulting in lowered Aβ levels. Ly et al. (2013) reported that specific inhibition of glycogen synthase kinase 3 (GSK3) reduced Aβ production by decreasing BACE1 gene transcription and expression. However, no research has yet been published on the effect of cannabinoids on GSK3 or BACE1 mRNA expression. Researchers conducting studies on this subject may find that it is through inhibition of GSK3 signalling that cannabinoids exert their effects on BACE1 mRNA, which could in turn lower Aβ levels. Thus, the effect of cannabinoids on GSK3 signalling merits further study.

This study determined slightly higher ApoE mRNA levels with anandamide treatment (Figure 5C), which once again supported our hypothesis. However, these results were not statistically significant. Synthetic cannabinoids, such as WIN 55,212-2 and JWH-133 have previously been shown to enhance Aβ transport across choroid plexus cells in vitro. This suggests that administration of cannabinoids can increase Aβ clearance (Martín-Moreno et al. 2012). Because ApoE plays a key role in the clearance of Aβ from the brain, the increased clearance of Aβ seen by Martín-Moreno and colleagues may have been due to the ability of cannabinoids to increase ApoE levels. However, an increase in ApoE may also be a result of cannabinoids exerting their effects at the level of translation or protein stability, as opposed to at the level of transcription. If this were the case, no changes in ApoE would be detected at the mRNA level, as occurred in this study. Changes in ApoE that occur at levels of regulation other than transcription should be further studied.

There is strong supporting evidence that cannabinoids provide neuroprotection through the CB1 receptors (Ramirez et al. 2005). Most of the effects of cannabinoids that take place in the central nervous system rely on CB1 receptor activation, as it is particularly abundant in discrete areas of the brain (Howlett et al. 2007). However, new receptors have been found in the brain, such as the novel GPR55 receptor, through which cannabinoids could potentially exert their effects (Pertwee et al. 2010). Thus, using a cannabinoid other than anandamide that would target receptors selectively may demonstrate significant changes in mRNA and protein levels of ApoE and consequently reduce Aβ levels.

It is important to note from the trends in data that mean values of APP, BACE1, and ApoE mRNA levels were most different from control when cells were treated with 10 μM anandamide. This observation may indicate that there is an optimal concentration at which cannabinoids exert their effects. Future studies should include finding an optimal concentration of cannabinoids that is both physiologically relevant and appropriate for use in patients with AD.

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Conclusion

As treatment of SH-SY5Y cells with anandamide significantly reduced the mRNA levels of both APP and BACE1, anandamide has the potential to considerably reduce levels of Aβ in vitro and also in the brain, as hypothesized. However, anandamide does not appear to significantly alter mRNA levels of ApoE. The mechanism of action by which anandamide reduces the mRNA levels of APP and BACE1 should be further investigated. Results from these studies would contribute to a better understanding of how cannabinoids reduce Aβ levels. This may lead to the identification of novel therapeutic targets that would be useful in treating AD.

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References


