ELSEVIER

European Journal of Pharmacology 396 (2002) 189-198

ejp

www.elsevier.nl/locate/ejphar

Stoners eat your broccoli: Folic acid enhances the effects of cannabinoids at behavioral, cellular, and transcriptional levels

Nora Rossi[a], Alana Pighin[b], Jamie Clegg[a], James Hui[a], Elizabeth Houlahan[a], David Ng[a*]

[a]*Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI, USA*
[b]*Botanisches Institut, Universitat Essen Universitatsstrasse 5 D-45117 Essen, Germany.*

Received 18 July 2002; received in revised form 23 September 2002; accepted 13 October 2002

Abstract

Recent interest in cannabinoid receptors as therapeutic targets has spurred the investigation into the physiochemical responses to their activation. The cannabinoid pathways in mammalian systems of adult male CD1 mice were investigated using a combinatorial approach based on folic acid mediated response. Various tests of cannabimimetic activity of folic acid showed that the combination of folic acid with either AEA (N-arachidonoylethanolamine, ananamide) or THC (Δ^9-tetrahydrocannabinol) amplified the effects of the cannabinoid. Levels of cyclic AMP (cAMP) in brain tissue also show a marked reduction after the addition of folic acid to either AEA or THC at both 1 and 2.5 µM dose levels in comparison to the cannabinoid alone. Genetic studies using CB1 -/- knockout mice suggest the existence of a CB3 receptor localized in the brain tissue of mice that has yet to be cloned or characterized. In the CB1 knockout mice, addition of folic acid and THC resulted in a 129% increase of [^{3}H]THC binding in the cortex and significant increases in all other areas of the brain when compared to THC alone. This indicates that folic acid is indeed affecting the neurotransmitter pathways of the cannabinoids by potentially acting with a third cannabinoid receptor. Using serial analysis of gene expression, it was found that transcription levels of genes shown to be up-regulated by cannabinoids were further increased upon addition of folic acid.
©2002 Elsevier Science B.V. All rights reserved.

Keywords: Cannabinoid receptors, Folic acid, Tetrahydrocannabinol, Anandamide, Glucokinase, Cannabimimetic activity

1. Introduction

The endocannabinoid system includes cannabinoid receptors, endogenous cannabinoid receptor ligands and enzymes for the biosynthesis and degradation of these ligands. Compounds which effect this system are defined as having cannabimimetic activity (Maccarrone *et al.*, 2002). Two cannabinoid receptors have been cloned from many vertebrates (Gerard *et al*, 1991; Matsuda *et al*, 1990; Abood *et al,* 1997). Cannabinoid receptors, CB1 and CB2, are differentially expressed in the nervous system and the periphery (mostly localized to the immune system) respectively (Goutopoulos and Makriyannis, 2002). The unique characteristics of these receptors and their conserved nature among vertebrates, has created a renewed interest in the actions of classical cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC, hereafter referred to as THC; found in hashish and marijuana). In addition, extensive studies have been conducted to identify endogenous compounds exhibiting cannabin-oid activity. Such studies have identified the endo-cannabinoids anandamide (AEA) (Devane *et al,* 1992), 2-arachidonoyl-glycerol (2-AG) (Mechoulam *et al,* 1995), as well as implicating several endogenous brain lipids as CB1 ligands (Wilson and Nicoll, 2002).

Both classical and endogenous cannabinoids produce biological activity through G-coupled cannabinoid receptor activation, downregulating cAMP and MAP kinase production (Felder *et al,* 1995). Genetic studies using CB1-/- mice have suggested the existence of a CB3 receptor localized to the brain tissue of mice that has yet to be cloned or characterized (Wilson and Nicoll, 2002).

While studying the murine endocannabinoid system, Pighin *et al.* (2001) discovered enhanced cannabinoid activity after mice were fed a diet

*Corresponding author. Tel.: +1-959-824-6264; fax: +1-949-824-6263
E-mail address: ehoulahan@mcw.edu (E. Houlahan).

0014-2999/00/$ - see front matter *© 2002 Elsevier Science B.V. All rights reserved*

supplemented with folic acid. Following these observations, the two diets were analyzed by HPLC and mass spectrometry. The only significant difference in the profiles of the two samples was a peak corresponding to folic acid (Pighin *et al.,* 2001). This confirmed that folic acid was the active agent in the diet.

Folic acid is a water-soluble B vitamin that is necessary in forming coenzymes for purine and pyrimidine synthesis, erythropoiesis, and methionine regeneration (Tanaka *et al,* 1980). Folic acid deficiencies have been linked to breast and pancreatic cancer, heart disease, atherosclerosis, and neural tube defects such as spina bifida and anencephaly (Souza *et al,* 1999). To ensure adequate consumption of this vitamin, staple foods such as bread, rice, noodles, and other grain products in the United States are enriched with folic acid. Furthermore, it has been found that leafy dark green vegetables such as broccoli, contain large amounts of folate, the natural form of folic acid (Souza *et al,* 1999). While much research has been done to characterize the cellular mechanisms of folic acid uptake, to our knowledge there has been no previous link between folic acid and cannabimimetic activity.

In this study, we used *in vivo* behavioral studies, and *in vitro* binding assays and molecular approaches to further characterize the functional link of cannabinoid signaling pathways and folic acid consumption in mammalian systems. Serial analysis of gene expression (SAGE) generates a better understanding of regulatory changes by generating detailed mRNA expression profiles (Velculescu, 1995). This technique allows simultaneous, quantitative analysis of a large number of transcripts. We were able to identify genes that are differentially regulated during cannabinoid binding and examine the effects of folic acid on the regulation of these genes.

2. Materials and Methods

2.1. Chemicals

Folic acid was obtained from ICN Biomedicals Inc. (Irvine, CA, USA) in crystalline form at 98% purity. THC was also obtained from ICN Biomedicals Inc. at 99% purity. AEA was obtained from Fisher Scientific (Nepean, ON, Canada) at 97.5% purity.

2.2. Animal Treatments

Adult male, *Mus muscula* CD1 mice (BSVV, Berlin Germany) and CB1 -/- knockout mice (supplied by Dr. M. Madowski, Dept. Pathology, Washington State University, Pullman, WA), weighing 18-24g, were housed two per cage in standardized conditions (12/12h light/dark cycles at 23˚C). Mice were acclimatized to the animal facility with access to food and water *ad libitum* for a minimum of 1 week prior to tissue harvesting. Mice were maintained on Nutribalance (San Diego, CA. USA) diet for the duration of the experiment. Euthanasia was performed via sedation with isoflo (5%) and decapitation (Tyler *et al.*, 1999).

Mice were injected with 20 mg/kg of either THC, AEA, folic acid, or isotonic saline solution (control). Combinations of 10 mg/kg of THC + 10 mg/kg folic acid, 10 mg/kg AEA + 10 mg/kg folic acid were also administered. Tissue homogenates were prepared from neural cortical (cortex, hippocampal) and peripheral brain (cerebellar, straital) slices using 0.1% phenylisothiocyanate as an amendment to reperfusion.

2.3. Cannabimimetic activity of folic acid

Upon administration of folic acid, THC, AEA and isotonic saline solution to CD1 adult male mice, the following tests were conducted and each was replicated six times.

Analgesic: The analgesic effect was assessed using the hot plate test with highly accurate thermostatic control. The mice were placed on the heated (55°C) surface and paw reaction (rapid lifting and licking or jumping) was used as the endpoint. There was a 30s cutoff time to avoid blistering. Control values were determined before drug administration. Responses were monitored at intervals of 5 min for a total of 60 minutes.

Locomotility: Spontaneous motor activity was measured in an activity cage (41x41x32 cm), made entirely of Perspex (U. Basile, Varese, Italy) and placed in a sound-attenuated room. The cage was fitted with two parallel horizontal and vertical infrared beams, at 2 and 6 cm from the floor, respectively. Interruption of the beams resulted in an increment count registered by online input microprocessor and a multifunction printer (U. Basile, Varese, Italy). Cumulative counts were recorded at intervals of 5 min during a 40 minute test session.

Hypothermia: Baseline rectal temperatures were determined before drug or vehicle injection with a telethermometer and a thermistor probe (Ellab a/s

type PRR, Ellab Instruments, Roedovre, Denmark) inserted 3 cm. Body temperatures were monitored at intervals of 5 min for a total of 60 minutes.

Catalepsy: Mice were placed on a metal ring (5.5 cm in diameter) that was attached to a stand at a height of 16 cm (modified from Pertwee, 1972). The amount of time (sec) spent by the mouse motionless over a period of 60 minutes was recorded. The criterion for the immobility was the absence of all voluntary movements (excluding respiration, but including whisker movements). The results were expressed as total area under the time-response curve.

2.4. *[^{3}H] THC binding and cAMP concentration*

Brain tissue of both CD1 and CB1 -/- knockout mice was dissected and resuspended in 2mM Tris-EDTA, 320 mM sucrose, 5mM $MgCl_2$, 100μM PMSF buffer (pH 7.4), and homogenized in a Potter homogenizer and centrifuged at 1000g for 10 minutes (Maccarrone *et al.,* 2000). The supernatant, containing the protein fraction, was recovered. The procedure was repeated twice and the three supernatants were combined. The combined supernatant fractions were then centrifuged at 40000g for 30 minutes and the resulting pellet was resuspended in assay buffer (50mM Tris-HCl, 2mM Tris-EDTA, 3mM $MgCl_2$, 100μM PMSF, pH 7.4), to a concentration of 1mg/mL (Maccarrone *et al.*, 2000). The tissue preparation was divided in aliquots, quickly frozen in liquid nitrogen, and stored at -80°C. These tissue fractions were used in rapid filtration assays with radiolabelled THC as described previously (Maccarrone *et al.,* 2000). Binding of [^{3}H]THC to the different brain areas was assessed and nonspecific binding was determined in the presence of 10-5M 'cold' THC using autoradiography.

Mouse brain cortex slices (200-300nm thick) were prepared from tissue blocks using a Vibratome (Fischer Co.) and were maintained at 35°C in Krebs' solution gassed with 95% O_2 and 5% $C0_2$ to maintain tissue integrity (Calabresi *et al.,* 2000). Cortical slices were incubated for 15 minutes at 35°C with 1μM forskolin and each treatment (saline solution control, folic acid, AEA, THC, folic acid + AEA, folic acid + THC), washed and homogenized as described above (Maccarrone *et al.,* 2000). Cyclic AMP levels in acetylated tissue extracts were determined by the Cayman Chemical cAMP enzyme immunoassay kit (Alexis Corporation, Texas, USA).

2.5. *Serial analysis of gene expression* (SAGE)

SAGE was used to determine gene expression of cannabinoid receptors in *M. muscula*. Mice were sacrificed following 7 consecutive daily injections of folic acid and THC treatments. Total RNA from the four ipsilateral brain hemispheres of adult male mice was pooled after 14 h of reperfusion that followed 2 h of middle cerebral artery occlusion (MCAO). RNA extractions were performed using the RNAeasy kit (Qiagen Inc. Santa Clarita CA., USA). As a control, total RNA was pooled from four whole brains of the same mouse strain with a mean weight of 20 ± 2 g. For each SAGE procedure 50 μg of mRNA was used, as recommended (Detailed Protocol, version 1.0c; kindly provided by Dr. Klaus Heckelsmiller and colleagues at Harvard University Medical School, Center for Oncology Boston MS), with the tagging enzyme, *Nla*II. PCR was performed for 26 cycles with 600x50 μl reactions in parallel to minimize the percentage of redundant ditags. Ligation to form polytags was performed with 1U of the T4 DNA ligase (Invitrogen, Eggenstein Germany) for 15 min at 25°C. Cloned concatemers were sequenced with dye primer chemistry V.3. and the Primer Cycle Sequencing Kit (Amersham Biosciences, Braunschweig, Germany) using automated ALFexpress DNA sequencer (Pharmacia Biotech, Freiburg, Germany). Dye terminator chemistry with Ampli-Tag FS enzyme (PerkinElmer Life Sciences Vatterstetten, Germany) in combination with an automated ABI 373A DNA sequencer (ABI PerkinElmer Weiterstadt, Germany) was also used.

2.5. *Statistical analysis*

Hot plate analgesia, body temperature and locomotory abilities were analyzed in response to folic acid and cannabinoids using analysis of variance, ANOVA (Zar, 1999). Pair-wise analysis between individual treatments and combinations were performed using the Scaeffe's F-test (Zar, 1999). Tags were analyzed by using SAGE software version 3.01 (available through NCBI http://www.ncbi.nlm.nih.gov/SAGE/). The average *P* value was determined by Monte Carlo analysis using the SAGE software (Zhang *et al.*, 1997) in

combination with automated ALFexpress DNA sequencer and the Primer Cycle Sequencing Kit (Amersham). Transcript abundance was calculated after analysis of 40,611 tags (control mice), 39,324 tags (saline treated), 39,723 tags (THC treated mice), and 41,369 tags (folic acid + THC treated mice).

3. Results

3.1. Cannabimimetic activity of compound

To evaluate the cannabimimetic nature of folic acid, the effects induced by folic acid alone or in combination with other cannabimimetic compounds were compared in the tetrad of behavioral tests considered to be highly predictive of cannabimimetic compounds (Pertwee, 1972; Smith *et al.*, 1994). The effects of folic acid alone were compared with those induced by AEA or THC and in combination with AEA or THC. As shown in Fig. 1, the time courses were similar for AEA and THC alone, and for AEA + folic acid and THC + folic acid, but the effects of folic acid alone were similar to the controls. This was true of these four parameters examined. Interestingly, addition of folic acid to AEA or THC enhanced and prolonged the response when compared with that of folic acid given alone ($p<0.01$ and $p<0.05$, respectively). These results indicate that the folic acid alone is not cannabimimetic, but rather it acts to amplify the activity of other cannabimimetic compounds.

In the hot plate test the analgesic effect of all trials peaked at 5 minutes after the administration, but disappeared within 20 minutes for AEA and within 35 minutes for THC (Fig. 1A). Addition of folic acid to either AEA or THC resulted in prolonged analgesic effects, with response time for each combination remaining significantly above the initial levels for the entire 60 minutes of the time trial ($p<0.001$ and $p<0.01$, respectively). In the motor activity test, all treatments produced a significant inhibition (average 63%, $p<0.001$ in all comparisons) compared with control and folic acid alone, which was evident 5 minutes after injection and lasted for 30 minutes for AEA and 40 minutes for THC (Fig. 1B). Addition of folic acid to either AEA or THC decreased motility noticeably but not

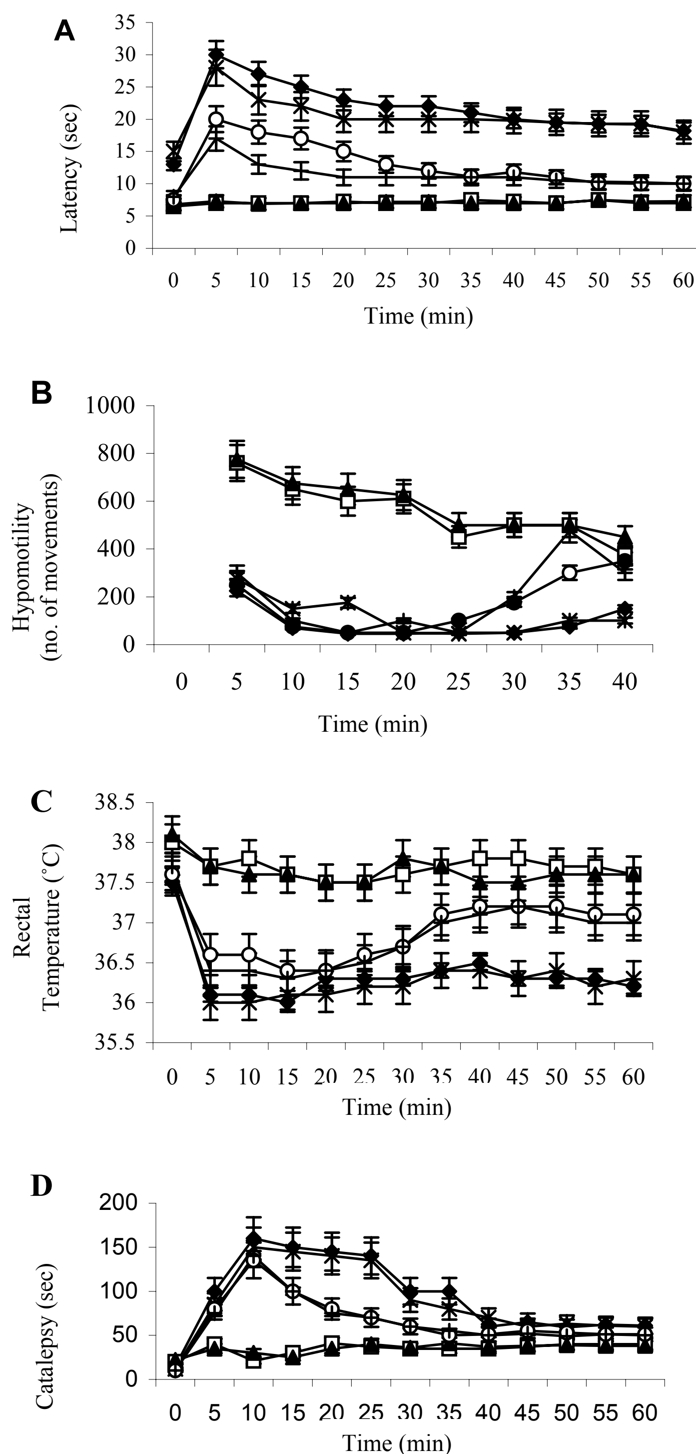

Fig. 1. Cannabimimetic activity of folic acid. Time courses of administration of control (white squares), folic acid (black triangles), folic acid plus THC (black diamonds), folic acid plus AEA (black stars), THC (white circles), and AEA (black "+").

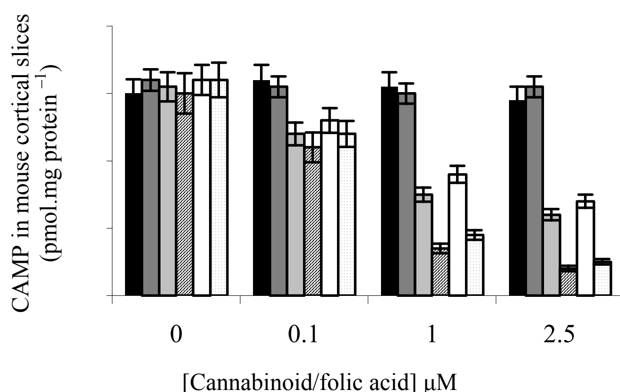

Figure 2: Decrease in cAMP concentration enhanced by folic acid. Effect of folic acid (dark grey), THC (light grey), AEA (white), folic acid + THC (cross-lines), folic acid + AEA (spotted) on forskolin induced cAMP concentration in mouse cortical slices P<0.05 when compared with vehicle treated controls (solid black).

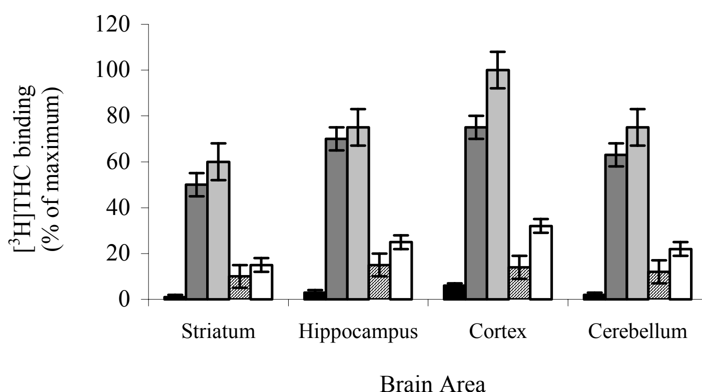

Figure 3: Binding of 500 pM [^{3}H]THC to different areas of mouse brain in wildtype and CB1 knockout mice expressed as a percentage of the maximum (100%=310 fmol mg protein^{-1}). Solid black bars represent binding to wildtype brain areas in the presence of 1μM 'cold' THC. Treatments with [^{3}H]THC include wildtype mice (dark grey), wildtype mice with folic acid (light grey), CB1 knockout mice (cross-lines), CB1 knockout mice with folic acid (white). P<0.01.

significantly. It did however prolong the effects of both AEA and THC given alone, resulting in significant inhibition (average 79%) compared with control and folic acid alone, $p<0.01$ and $p<0.05$ (Fig. 1B). The hypothermic effect of the combination of folic acid and either AEA or THC had a slightly faster onset and much longer duration than that of either cannabinoid alone (Fig. 1C). After 5 min, a significant decrease in rectal temperature was observed in mice treated with folic acid in combination with either AEA or THC, when compared with mice treated with either AEA or THC alone (0.5, $p<0.01$ and 0.4°C, $p<0.001$, respectively). This effect lasted for 60 min in the mice treated with combined compounds, whereas after 20 min the effects of AEA and THC alone were no longer significant. Ring immobility after injection of AEA alone was significantly increased after 5 min and peaked at 10 min ($p<0.001$) but was no longer significant at 20 min (Fig 1D). In contrast, ring immobility induced by addition of folic acid with AEA reached a maximum within 10 min, but declined significantly after 35 min ($p<0.01$) and was no longer significant at 45 min (Fig 1D).

3.2. Properties of folic acid-cannabinoid binding sites

Both AEA and THC both induced the CB1 receptor-mediated decrease in forskolin-induced cAMP concentration in cortical slices, with strong effects seen at 1 and 2.5 μM [cannabinoid] (Fig 2). Addition of 0.1 μM folic acid produced no significant effect on either AEA or THC treated cortical slices.

Strong effects were seen when 1 and 2.5 μM folic acid concentrations were combined with the high doses of AEA and THC. Levels of cAMP was markedly reduced after addition of folic acid to AEA at both 1 and 2.5 μM (50% and 64%, respectively; Fig. 2). There was also a strong decrease in cAMP levels in combination of folic acid and THC compared with THC alone, at both 1 and 2.5 μM dose levels (63% and 67% respectively; Fig 2).

Different areas of wildtype mouse brain were able to bind [^{3}H]THC in the order cortex > hippocampus > cerebellum > striatum, whereas only small amounts of binding were observed in the CB1 knockout mice (Fig 3). The addition of folic acid to THC in these trials caused an observable difference in binding of [^{3}H]THC in all areas of the brain, with a significant difference observed only in the cortex. Interestingly, in the CB1 knockout mice, addition of folic acid and THC resulted in a significant increase in binding of [^{3}H]THC in all areas of the brain when compared to THC alone (50% striatum, 67% hippocampus, 129% cortex, 83% cerebellum) (Fig 3). These results indicate that folic acid binding is perhaps due to a third CB receptor that is present throughout the brain, and densely concentrated in the cortex.

3.3. SAGE Tag library analysis

The results of SAGE analysis on wildtype, saline control treated, THC treated, and folic acid+THC treated mice are summarized in Table 1. Tags representing linker DNA as well as tags representing poly-A tails from PCR products were excluded from analysis. The total number of reliable tags were then used to determine percentage expression levels of each transcript. Evaluation of each treatment on gene expression levels was based on a comparison of saline treated and THC treated mice to wildtype mice. THC treated mice were used as the baseline for evaluation of tags obtained from mice treated with THC+folic acid. Expression of down-regulated genes decreased by the percentage shown whereas expression of up-regulated tags increased by the percentage calculated.

Table 2 lists the tags representing known genes that were differentially expressed with $p<0.01$ (values not shown) between wildtype mice and those treated with the saline control. Among these genes, those encoding various signal transduction pathways were preferentially affected. Genes encoding products such as the LDL receptor-related protein I, calcium/calmodulin-dependent protein kinase I and tyrosine phosphatase were down-regulated whereas those encoding TNF receptor-associated factor 5 and cyclin-dependent protein kinase I were up-regulated. These results were taken as background transcriptional variation due to either the stress of injection or to natural transcriptional fluctuation and were consequently disregarded in analysis of the cannabinoid analyses.

Table 3 illustrates the tags representing known genes that were differentially expressed with $p<0.01$ (values not shown) between wildtype, THC treated and THC+folic acid treated mice. THC alone caused a down-regulation in genes encoding cytoskeletal components, such as microtubule-associated protein 2 and kinesin heavy chain member 1A. There was an increase of tags representing genes coding for glucokinase and melanocyte-specific gene–related gene 1, both of which are known to be correlated with the cannabinoid receptors. Furthermore, mice treated with both THC and folic acid, showed an increase in the transcription of both glucokinase and melanocyte-specific gene-related gene 1.

4. Discussion

In this investigation, we report an apparent interaction between folic acid, cannabinoids, and the cannabinoid receptors that have resulted in increased and prolonged effects of both ananamide and THC as compared to the saline control. Folic acid alone exhibits no cannabimimetic activity in cannabinoid receptor pathways of mice, and the responses in mice in these experiments were not significantly different from those of control mice. However, folic acid combined with cannabinoids showed a significant increase in cannabinoid activity. Levels of cAMP activity in brain tissue also showed a marked reduction in the presence of folic acid and cannabinoid combinations, indicating that folic acid has an effect the neurotransmitter pathway of the cannabinoids.

Analyses of gene expression support the notion

Table 1. Summary of SAGE results.

SAGE library	***Tags total***	***Tags after clean-up***	***Data pre-processing***			***Unique tags***
			Linker-based tags extracted	Poly A tags extracted	Reliable tags	
Wildtype	41,532	41,029	366	52	40,611	12,804
Saline control	40,229	39,782	425	33	39,324	11,682
THC	40,653	40,326	458	45	39,823	13,327
THC + folic acid	41,789	41,284	376	39	40,869	12,591

Table 2. SAGE tags representing known genes differentially expressed in saline treated mice.

Tag sequence	Identified gene	Accession No.	Percentage[1]
	Down-regulated genes		
CGCAAGCTGG	Ubiquitin specific protease 9, X-chromosome	GN545549	3.9259
TCCTGTAAAG	RNA binding protein	R25910	1.9555
GAGACTCCTG	FK506 binding protein 8	GK74062	1.1518
CTCTTCGAGA	Alternative splicing factor	GN511678	0.8037
CTTGACACAC	Calcium/calmodulin-dependent protein kinase I	GN950451	0.5814
ATGTAGAGTG	Protein tyrosine phosphatase	GN390155	0.3333
CAAGGGCTTG	Small GTP-binding protein Rabphillin 3C	GN893444	0.2592
ATCCCTCAGT	ADP ribosylation factor-like 2	R75229	0.2592
AGCTTGCGCT	Small inducible cytokine subfamily D1	SF57882	0.2592
AAAGTCATTG	Protein phosphatase 2A, α isoform	GN510122	0.2222
GTGGACCCTG	N-myc downstream-regulated gene 4	GN233104	0.2222
GGATGAGTAC	Mouse myelin proteolipid protein gene, exon 7	GN388581	0.1851
ACATCCTCAC	Arsenite-translocating ATPase	GC50074	0.1851
CTAATGCAAA	LDL receptor-related protein 1	GN516401	0.1481
	Up-regulated genes		
TGGAGAGCAA	Aspartyl-tRNA synthetase	SC5755	2.2962
AACTGCTTCA	Protein folding kinase	GC90955	1.6212
AATCCTCCTT	Heat shock 27 kDa protein 3	GN790412	0.8518
CAAGCAGGAC	Cyclin-dependent protein kinase 1	GN764575	0.5555
GATTGATGTC	Endothelial differentiation G-protein receptor 3	GN241328	0.2592
GGTGGCTTTG	TNF receptor-associated factor 5	H85778	0.2592
TCCCGACATC	Netrin 1	X-13451	0.1481
TTGGGGTGCC	Microtubule-associated protein tau	GN02308	0.1481
AACCAGGTGT	Myelocytomatosis oncogene	SF16750	0.1481
ATTCCAATCT	Replication factor c, 140 kDa	R71110	0.1481
ACCATCCTGC	Mouse mCAF1 protein (homolog yeast CCR4)	GN659285	0.1481

[1] Tag sequence abundance shown as % total tag sequences isolated

that cannabinoids in combination with folic acid induce distinct but overlapping transcriptional responses in mouse brain, measured 7 days after treatment. The overlapping responses are likely to reflect effects of cannabinoid receptor activation. CB1 is the predominant established cannabinoid receptor in the brain and can bind easily to THC, so transcriptional responses shared by response-induced treatments are likely to be triggered by CB1 activation. Non-CB1/non-CB2 ('CB3') cannabinoid receptors are also thought to exist in brain (Breivogel *et al*., 2001) but have low sensitivity to THC. Therefore, some or all of the genes induced by this cannabinoid, in combination with folic acid might be induced through CB3 receptors.

Cannabinoids affect a variety of tissues, which helps to account for the diversity of their actions. Genes regulated by cannabinoids encode products involved in neuronal signaling, neuronal growth and structure, myelination, or glial differentiation and metabolism (Alverez *et al,* 2002). Certain genes known to be up-regulated during cannabinoid binding, were of particular interest. These included the enzyme glucokinase (Roncero *et al*., 2000) and the transcription factor melanocyte-specific gene-related gene 1 (MRG1) (Sun *et al.,* 1998).

Glucokinase is a member of an enzyme family that catalyzes the first step of glycolysis. It is found at high levels in the brain, especially in the hypothalamus (Roncero *et al*., 2000). Cannabinoid receptors are also abundant in this region, where they seem to mediate the appetite-stimulating effect of cannabinoids (Di Marzo *et al*., 2001; Jamshidi and Taylor, 2001).

MRG1 is an isoform of the cyclic AMP response element binding protein 35srj, which is induced by hypoxia-inducible factor-1 (HIF-1) as part of the transcriptional response to hypoxia (Mazzari *et al.*, 1996). Previous studies demonstrated that endo-cannabinoids accumulate in the brain following hypoxia, and may be involved in protecting the neurons against excitotoxins (Skaper *et al.*, 1996)

Table 3. SAGE tags differentially expressed in the presence of (a) THC and (b) folic acid + THC.

Tag sequence	Identified gene	Accession No.	Percentage[1] (a)	(b)
	Down-regulated genes			
GCAACAGCAA	Neural precursor developmentally down-regulated 4b	GN51891	3.1814	1.1212
GTGAAACCTT	Embryonal Fyn-associated substrate	R8028	1.9296	
GTGAAACCTT	Mortality factor-regulated gene 15	GN76902	1.0000	0.8518
GTGGGAGACC	Reticulon 3	GK2056	0.5555	
ATCGTGCCAC	Microtubule-associated protein 2	GN948602	0.5185	
ATGAAACCCC	Protease nexin-1	GN495259	0.5185	0.0114
GGAGTGTGCG	Neuronal protein 25	GN610462	0.4444	0.0114
CCAACCGTGC	Acidic ribosomal phosphoprotein	GC12131	0.2349	
CCACTGCGCT	Silent mating type information regulation 2	G22910	0.2349	0.5185
CTGCCAACTT	Leucine-rich, glioma inactivated 1	H4057	0.2349	0.3636
TACCCTAAAA	Kinesin heavy chain member 1A	GC91827	0.2222	1.5555
CCGTGCTCAT	Transforming growth factor β1-induced transcript 4	R9803	0.2222	
CTGGTCCTCC	Apoptosis-associated tyrosine kinase	GC27449	0.2222	
TCCTCAGCAT	Stearoyl-CoA A desaturase 2	R8258	0.2139	
GGGGACTGAA	APP-binding protein	GN125662	0.1114	0.0333
	Up-regulated genes			
AAAGACCAAA	Neuromedin	GK0745	1.8444	0.0114
AAGCCCCTGG	Glucokinase	GC10001	1.1139	1.5454
GCGAAAACCC	Gioblastoma-expressed ring finger protein	GN333319	0.8139	
TATCCCAGAA	Melanocyte-specific gene-related gene 1	R0272	0.8139	0.9636
TATGAATGCT	P53 apoptosis effector related to Pmp22	H0757	0.3333	
TTGACCCTGG	Interferon-induced transmembrane protein3	GN054185	0.1251	
TGGACACAAG	low molecular mass ubiquinone-binding protein	GN585817	0.1251	0.3333

[1] Tag sequence abundance shown as % total tag sequences isolated

The observed up-regulation of both glucokinase and MRG1 transcription indicates that SAGE is an accurate method of confirming cannabimimetic activity of a given compound. Furthermore, the increase up regulation of these genes upon addition of both THC and folic acid confirms the results of behavioral and binding assays. It must then be concluded that folic acid enhances the cannabimimetic effect mediated through cannabinoid pathways.

Cannabinoids are neuroprotective in conditions such as cerebral ischemia (Nagayama *et al*., 1999) and trauma (Panikashvili *et al*., 2001), in which vascular factors play an important role and a vascular component to cannabinoid-induced neuroprotection has been proposed (Panikashvili *et al*., 2001). Our finding that cannabinoids induce different genes in neurons (e.g. glucokinase) and in blood vessels (e.g. MRG1) suggests that the molecular basis for vascularly and neuronally mediated neuroprotection may be different. More generally, if cannabinoids activate different signaling pathways in different types of cells, therapeutic approaches that target such downstream events may be capable of dissociating desirable from undesirable effects of cannabinoids.

Cortical binding of cannabinoids in CB1 knockout mice treated with folic acid indicate that the effects of this vitamin on cannabinoid activity may be due to an interaction with a putative CB3 receptor. This interaction is hypothesized to occur either through interaction with the cannabinoid itself or through interaction with the receptor.

The stereochemical structure of cannabinoid compounds is in constant fluctuation (Iwamura *et al.,* 2000; Shim *et al.,* 2002). Certain conformations of the molecules expose the acyl chain, which has been shown to be essential for cannabinoid receptor binding, thereby increasing the binding affinity of the cannabinoids to their receptors (Shim *et al.,* 2002). Folic acid may interact directly with the cannabinoid compound to stabilize a conformation that is most favorable for binding with the CB3 receptor. This might account for the late discovery of this third class of cannabinoid receptors if, in the absence of folic acid, cannabinoids preferentially bind to the CB1 receptors.

Alternatively, folic acid may interact with the 'CB3' receptor, altering its binding site to be specific for the cannabinoids. In this case, CB3 may in fact be a receptor for another compound (perhaps folic acid itself) that does not bind cannabinoids in its native conformation.

Much research has focused on the mechanism of folic acid uptake and use by cells, but there has been no previous evidence that folic acid affects neuro-transmitter pathways. Clearly much more research is needed to elucidate the precise mechanism of this interaction.

Acknowledgements

We thank Drs. Andrew Griffiths, Felice Nueava and Fernando Fonescrea for their critical reading of the manuscript. Mr. Samuel Lacey for editorial assistance and Dr. Jacob Brently for his help with the SAGE analysis. Support from the National Institute of Medical Research (under grant number 11439) is gratefully acknowledged.

References

Abood, M., Dido, K., Noel, M., Showalter, V., Tao, Q. 1997. Isolation and expression of a mouse CB1 cannabinoid receptor gene. Biochem. Pharmacol. 53, 207-219.

Alverez, E., Roncero, I., Chowen J.A., Vazquez, P. and Bazquez, E. 2002. Evidence that glucokinase regulatory protein in expressed and interacts with glucokinase in rat brain. J. Neurochem. 80: 45-53.

Breivogel, C.S., Griffin, G., Di Marzo, V. and Martin, B.R. 2001. Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. Mol. Pharmacol. 60: 155-163.

Calabresi, P., Gubellini, P., Centozone, D., Picconi, B., Bernardi, G., Chergui, K., Svenningsson, P., Fienberg, A., Greengard, P. 2000. Dopamine and cAMP-regulated phosphoprotein 32kD controls both striatal long-term depression and long-term potentiation, opposing forums of synaptic plasticity. J. Neurosc. 20, 8443-8451.

Devane, A., Fernandez-Ruiz, J.J., Munoz, R.M., Romero, J., Villanua, M.A., Makriyannis, A., Ramos, J.A. 1992. Time-course of the effects of different cannabinmimetics on prolactin and gonadotrophin secretion: evidence for the presence of CB1 receptors in hypothalamic structures and their involvement in the effects of cannabimimetics. Biochem. Pharmacol. 53, 1919-1927.

Di Marzo, V., Goparaju, S.K., Wang, L., Liu, J., Batkai, S., Jarai, Z., Fezza, F., Miura, G.I., Palmiter, R.D., and Sugiura, T. 2001. Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature (Lond.) 410: 822-825.

Felder, C., Joyce, K., Briley, E., Manaouri, J., Mackie, K., Blond, O., Lai, Y., Ma, A., Mitchell, R. 1995. Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. Mol. Pharmacol. 48, 443-450.

Gerard, C., Mollereau, C., Vassart, G., Parmentier, M. 1991. Molecular cloning of a human cannaninoid receptor which is also expressed in testis. Biochem. J. 279, 129-134.

Goutopoulos, A., Makriyannis, A. 2002. From cannabis to cannabinergics: new therapeutic opportunities. Pharmacology & Therapeutics. 95, 103-117.

Iwamura, H., Suzuki H., Ueda, Y., Kaya, T., Inaba, T. 2000. In vitro and in vivo pharmacological characterization of JTE-907, a novel selective ligand for cannabinoid CB_2 receptor. J. Pharm. Exp. Therap. 295(2), 420-425.

Jamshidi, N. and Taylor, D.A. 2001. Anandaminde administration into the ventromedial hypothalamus stimulates appetite in rats. Br. J. Pharmacol. 134: 1151-1154.

Kendall, D.A., Cadogan, A.K. and Alexander, S.P.H. 1996. The neurochemistry of the cannabinoids. J. Psycho-pharmacol. 10, A66-A74.

Maccarrone, M., Cartoni, A., Parolaro, D., Margonelli, A., Massi, P., Bara, M., Battista, N., Finazzi-Agro, A. 2002. Cannabimimetic activity, binding, and degradation of stearoulethanolamide within the mouse central nervous system. Mol. Cell NeuroSc. 21, 126-140.

Matsuda, L., Lolait, S., Brownstein, M., Young, A., Bonner, T. 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature. 346, 561-564.

Mazzari, S., Canella, R., Petrelli, L., Marcolongo, G., Leon, A. 1996. N-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cells. *European Journal of Pharmacology*, 300(3), 227-36.

Mechoulam, R., Devane, W., Glasser, R. 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem. Pharmacol. 50, 83-90.

Nagayama, T., Sinor, A.D., Simon, R.P., Chen, J., Graham, S., Jin, K. and Greenberg, D.A. 1999. Cannabinoids and neuroprotection from global and focal cerebral ischemia and in vitro. J. Neurosci. 19: 2987-2995.

Panikashvili, D., Simeonidou, C., Ben-Shabat, S., Hanus, L., Breuer, A., Mechoulam, R. and Shohami, E. 2001. An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. Nature (Lond.) 413: 527-531.

Pertwee, R. 1972. The ring test: a quantitative method for assessing the "cataleptic" effect of cannabis in mice. Br. J. Pharmacol. 46, 753-763.

Pighin, J., Houlahan, N., Clegg, A., Ng, D. 2001. Folic acid displays cannabinoid activity. Science. 253, 132-135.

Roncero, I., Alverez, E., Vazquez, P., and Blazquez, E. 2000. Functional glucokinase isoforms are expressed in rat brain. J. Neurochem. 74: 1848-1857.

Shim, J-Y., Welsh, W.J., Carter, E., Edwards, J., Howlett, A. 2002. Molecular interaction of the antagonist N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide with the CB1 cannabinoid receptor. J. Med. Chem. 45, 1447-1459.

Skaper, SD., Buriani, A., Dal Toso R., Petrelli, L., Romanello, S., Facci L., Leon A. 1996. The ALIAmide palmitoethanolamide and cannabinoids, but not anandamide, are protective in a delayed postglutamate paradigm of excitotoxic death in cerebellar granule cells, PNAS USA. 93(9), 399984-9.

Smith, P., Compton, D., Welch, S., Razdan, R., Michoulam, R., Martin, B. 1994. The pharmacological activity of anandamide, a putative indogenous cannabinoid, in mice. J. Pharmacol. Exp. Ther. 270, 219-227.

Souza, C., Kumar, R., Hooker, T., Rensing, K. 1999. Folic acid and its link to disease: the need for a supplemented diet. FDA Ann. Rev. 129, 324-332.

Sun, H.B., Zhu, Y.X., Yin, T., Sledge, G. and Yang, Y.C. 1998. MRG1, the product of a melanocyte-specific gene related gene, is a cytokine-inducible transcription factor with transformation activity. Proc. Natl. Acad. Sci. USA 95: 13555-13560

Tanaka, H., Hidemi, M., Okuzaka K. 1980. Folic acid: an analytical review. PNAS. 74(1), 118-132.

Tyler, K., Hillard, C.J., Greenwood-Van Meerveld, B. 2000. Inhibition of small intestinal secretion by cannabinoids is CB1 receptor-mediated in rats. Eur. J. Pharmacol. 409, 207-211.

Velculescu, V., Zhang, L., Vogelstein, B., Kinzler, K. 1995. Serial analysis of gene expression. Science 270: 484-487.

Williams, G. 1985. Quantitation of RNA transcripts in actively dividing cells. Cell. 29, 311-321.

Wilson, R., Nicoll, R. 2002. Endocannabinoid signaling in the brain. Science. 296, 678-682.

Zar, J. 1999. Biostatistical Analyses. Prentiss-Hall, Inc. California, USA.

Zhang, P., Thompson, J., Rossi, J., Hui, E. 1997. Statistical analysis of RNA transcripts derived from SAGE tagging. Bioinfo. 50, 110-119.