

Endocannabinoid metabolism in human glioblastomas and meningiomas compared to human non-tumour brain tissue

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Abstract

The endogenous levels of the two cannabinoid receptor ligands 2-arachidonoyl glycerol and anandamide, and their respective congeners, monoacyl glycerols and *N*-acylethanolamines, as well as the phospholipid precursors of *N*-acylethanolamines, were measured by gas chromatography-mass spectrometry in glioblastoma (WHO grade IV) tissue and meningioma (WHO grade I) tissue and compared with human non-tumour brain tissue. Furthermore, the metabolic turnover of *N*-acylethanolamines was compared by measurements of the enzymatic activity of *N*-acyltransferase, *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D and fatty acid amide hydrolase in the same three types of tissue. Glioblastomas were characterized by enhanced levels of *N*-acylethanolamines (eightfold, 128 ± 59 pmol/ μ mol lipid phosphorus) including anandamide (17-fold, 4.6 ± 3.1 pmol/ μ mol lipid phosphorus) and several species of *N*-acylphosphatidylethanolamines (three to eightfold). This was

accompanied by a more than 60% reduction in the enzyme activities of *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D and fatty acid amide hydrolase. By contrast, meningiomas were characterized by a massively enhanced level of 2-monoacyl glycerols (20-fold, 2293 ± 361 pmol/ μ mol lipid phosphorus) including 2-arachidonoyl glycerol (20-fold, 1524 ± 361 pmol/ μ mol lipid phosphorus). This was accompanied by an enhanced *in vitro* conversion of phosphatidylcholine to monoacyl glycerol (fivefold). The enhanced level of the 2-arachidonoyl glycerol, anandamide and other *N*-acylethanolamines detected in the two types of tumour tissue may possibly act as endogenous anti-tumour mediators by stimulation of both cannabinoid and non-cannabinoid receptor-mediated mechanisms.

Keywords: anandamide, 2-arachidonoyl glycerol, fatty acid amide hydrolase, *N*-acylethanolamine, *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D.

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In the year 2000, the estimated number of cases worldwide of tumours in the central nervous system was 176 056. In the same year, 127 614 patients died from this type of cancer with an almost even distribution between the more developed countries and the less developed countries (data from International Agency for Research on Cancer at <http://www-depdb.iarc.fr/globocan/GLOBOframe.htm>). Treatment results vary depending on the specific tumour type. However, due to the general poor survival rate after diagnosis of a brain tumour, a better knowledge of tumour biology is essential for more specific targeting and the development of specific pharmacological methods of treatment.

The biological effects of the major active principle of *Cannabis sativa* (marijuana), Δ^9 -tetrahydrocannabinol (THC) (Gaoni and Mechoulam 1964) on the G-protein-coupled cannabinoid receptors CB1 (Matsuda *et al.* 1990) and CB2

(Munro *et al.* 1993) have formed a basis for one of the possible therapeutic targets for tumour intervention (Jones and Howl 2003). In addition to the characteristic effects of THC, which include a variety of psychotropic effects, the compound

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Abbreviations used: 2-AG, 2-arachidonoyl glycerol; CB, cannabinoid receptor; FAAH, fatty acid amide hydrolase; GC-MS, gas chromatography-mass spectrometry; GFAP, glial fibrillary acidic protein; MAG, monoacyl glycerol; NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; NAPE-PLD, *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D; P, Phosphorus; THC, Δ^9 -tetrahydrocannabinol.

has been demonstrated to have wide therapeutic application for a number of medical conditions including pain, anxiety, glaucoma, nausea, emesis, muscle spasms and wasting diseases (Porter and Felder 2001). Furthermore, publications have increasingly focused on THC and the endocannabinoid system in relation to cancer therapy (Guzmán *et al.* 2001; Bifulco and Di Marzo 2002; Jones and Howl 2003).

Several plant-derived, synthetic and endogenous cannabinoids are known to exert anti-proliferative actions on a wide spectrum of tumour cells in culture (Guzmán *et al.* 2002). Activation of CB1 receptors is involved in this inhibition of proliferation in different types of tumour cells, such as *ras* oncogene-dependent growth of thyroid tumours (Bifulco *et al.* 2001; Portella *et al.* 2003) and growth factor-responsive human breast and prostate cancer cells (De Petrocellis *et al.* 1998; Melck *et al.* 2000; Mimeault *et al.* 2003). In these cancer cells, the CB1-mediated anti-proliferative effect was shown to involve down-regulation of receptors responsive to prolactin, nerve growth factor and epidermal growth factor, leading to cell cycle arrest in the G₁ phase (Melck *et al.* 2000; Mimeault *et al.* 2003).

The endogenous cannabinoid, anandamide (Devane *et al.* 1992), has been found to exert not only anti-proliferative but also pro-apoptotic effects in cancer cells, either via activation of the vanilloid receptor (Maccarrone *et al.* 2000; Contassot *et al.* 2004) or by *de novo* ceramide synthesis mediated through combined CB1 and CB2 receptor activation (Mimeault *et al.* 2003), while THC was shown to induce regression of malignant gliomas in rodents via apoptotic mechanisms after intratumoral administration (Galve-Roperh *et al.* 2000). In transformed neural cell cultures, THC induced apoptosis by stimulation of sphingomyelin breakdown (Sánchez *et al.* 1998a). Further *in vivo* studies showed inhibition of glioma growth by selective activation of the CB2 receptor, mediated by enhanced ceramide synthesis *de novo* (Sánchez *et al.* 2001; Gómez del Pulgar *et al.* 2002). In addition, the level of CB2 receptors was increased in relation to malignancy of human astrocytomas (Sánchez *et al.* 2001), and human leukaemia and lymphoma cells were found susceptible to apoptosis at least partly via activation of the CB2 receptor (McKallip *et al.* 2002).

Cannabinoids have also been shown to target vascular endothelial cells and thereby inhibit growth of gliomas *in vivo*, resulting in a dual effect of cannabinoid administration by promoting apoptosis of the tumour cells and by inhibition of tumour angiogenesis (Blázquez *et al.* 2003). This cannabinoid-induced restriction of tumour blood vessel supply was shown to be mediated by inhibition of the vascular endothelial growth factor pathway in thyroid cancer cells (Portella *et al.* 2003), skin tumours (Casanova *et al.* 2003) and gliomas (Blázquez *et al.* 2004). Recently, the Spanish government allowed an unprecedented clinical study aimed at investigating the effect of intratumoral THC administration on glioma in humans (Bifulco and Di Marzo 2002).

In order to understand the role of the endocannabinoid signalling system in tumour development and its potential use for targeted intervention, it is important to know the levels of the common endocannabinoids, anandamide (Devane *et al.* 1992) and 2-arachidonoyl glycerol (2-AG), (Mechoulam *et al.* 1995; Sugiura *et al.* 1995) in human cancer. In the present study we have determined the levels of these endocannabinoids and the anandamide congeners *N*-acylethanolamines (NAEs) and precursors *N*-acylphosphatidylethanolamines (NAPEs), as well as the 2-AG congeners monoacyl glycerols (MAGs), in human glioblastomas and meningiomas, and compared them with those of non-tumour brain tissue. Furthermore, we compared the metabolic turnover of NAPE and NAE by measuring the enzymatic activity of *N*-acyltransferase, NAPE-hydrolysing phospholipase D (NAPE-PLD) and fatty acid amide hydrolase (FAAH) in these tissues. These enzymes comprise the metabolic pathway illustrated in Fig. 1.

Materials and methods

Patients and tissue sampling

Scientific study of resected human brain tissue was performed after approval through the ethics committee in Copenhagen (KF 01-206/01). Informed consent was obtained before surgery from each of 71 patients with an assumed brain tumour characterized as glioblastoma (WHO grade IV) or meningioma (WHO grade I). Only 44 patients subsequently diagnosed with one of these tumour types were included in the study. Non-tumour brain tissue was also resected from patients who had undergone surgical removal of a glioblastoma. Samples of non-tumour tissue were taken only if (i) it was necessary to penetrate normal brain in order to remove the tumour or (ii) the tumour was located peripherally in a non-eloquent lobe and it was therefore possible to remove a small sample without compromising normal function. Upon resection, the tissue samples to be used for gas chromatography-mass spectrometry (GC-MS) analysis and enzyme assays were immediately placed in liquid nitrogen in the operating room. The frozen samples were weighed (wet weight; typically 200–2000 mg of tumour tissue and 100–800 mg of non-tumour brain tissue) and stored at -80°C until analysed. Tissue samples for histological diagnosis were handled according to the usual procedure at the laboratory of neuropathology. The material was formalin fixed

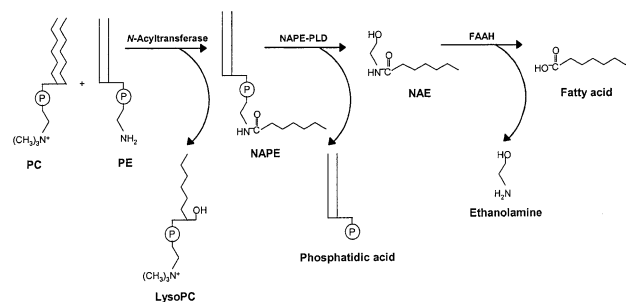


Fig. 1 Schematic view of NAPE and NAE metabolism. PC, phosphatidylcholine; PE, phosphatidylethanolamine.

and paraffin embedded and cut in 4 µm sections. Haematoxylin and eosin, Van Gieson and Van Gieson alcian staining was performed routinely for diagnosis. Furthermore, the glial tumours were stained with Klüver Barrera for myelin, and immunohistochemical with antibodies towards glial fibrillary acidic protein (GFAP) for identifying astrocytes, p53 and epidermal growth factor receptor. In case of doubt, the meningiomas were stained with Vimentin and epithelial membrane antigen to confirm the mesenchymal character of the tumour.

Histology

The tumours were classified according to the WHO 2000 classification (Kleihues and Cavenee 2000). The glioblastomas fulfilled the WHO criteria with pleomorphic, varying differentiated tumour cells and hyperchromatic atypical nuclei. Numerous mitotic cells, also atypical, were found together with malignant vessel proliferation and necrosis. The tumour cells were GFAP positive. The meningiomas had different appearances but were mostly of the meningothelial, fibrous and transitional type, all WHO grade I tumours. The mesenchymal cells were largely uniform containing nuclei without atypia and only occasionally with mitosis. In some meningiomas, characteristic cellular whorls and psammoma bodies were found. There was no necrosis.

GC-MS procedures

Lipids were extracted and analysed essentially as described earlier (Schmid *et al.* 2000; Hansen *et al.* 2001). Frozen tissue was homogenized in chloroform with a Tissuemizer (Tekmar, Cincinnati, OH, USA) and the homogenate was centrifuged to remove debris. Then, the solution was mixed with methanol and 2.5% NaCl (Folch *et al.* 1957) to separate the phases. After centrifugation, the lower chloroform phase was transferred to a screw-capped glass tube, blown to dryness under nitrogen, and the residue re-dissolved in chloroform. Aliquots were taken for the assay of lipid phosphorus (Bartlett 1959). Internal standards of *N*-heptadecanoyl-PE, deuterated NAE and deuterated MAG (16 : 0, 18 : 0, 18 : 1, 18 : 2 and 20 : 4 d_4 NAE, and 18 : 1, 18 : 2 and 20 : 4 d_5 MAG) were then added and the lipid extract applied to a 100 mg silica cartridge for elution of MAG, NAE and NAPE (in a cold room in order to minimize acyl migration of MAG). After elution of the neutral lipids with 4 mL chloroform, NAE plus MAG was eluted with chloroform/methanol (8 : 2, v/v). NAE, NAPE and MAG were derivatized and analysed by GC-MS as previously described in detail (Schmid *et al.* 2000). Briefly, NAE and MAG, including the deuterated internal standards, were converted to *t*-butyldimethylsilyl ethers and analysed with a Hewlett-Packard 5890 gas chromatograph equipped with a 5972 mass selective detector and 7673 autosampler. The HP5MS column, 30 m by 0.25 mm (Hewlett-Packard, Palo Alto, CA, USA); was programmed from 150°C to 280°C at 50°C per min. The M-57 ions were monitored in selected ion monitoring mode. NAE derived from NAPE by phospholipase D hydrolysis was isolated by solid phase extraction and processed as described above.

Enzyme assays

For the enzyme measurements, a membrane preparation (consisting of microsomes and mitochondrial membranes) of each tissue

sample was isolated as described previously (Moesgaard *et al.* 2002). Protein determination was performed by the method Bradford (1976) using γ -globulin as protein standard modified for membrane samples (boiling with NaOH) (Kirazov *et al.* 1993).

The *N*-acyltransferase assay was carried out as previously described (Moesgaard *et al.* 2002). Briefly, 150 µg membrane protein were incubated (in triplicate) with 5.4 µM 1,2-di[1'- 14 C]decanoyl-*sn*-glycero-3-phosphocholine (custom synthesized by Amersham Biosciences, Amersham, UK) for 0, 10, 20 and 30 min at 37°C in a total volume of 250 µL buffer solution. Lipids were separated by TLC and the radioactive-labelled products NAPE and NAE were quantified as percentage of total radioactivity using a PhosphorImager scanner (STORM, Molecular Dynamics, Sunnyvale, CA, USA).

Assay conditions and detection of phosphatidylcholine-hydrolysing PLD activity was as described above for the *N*-acyltransferase assay (in duplicate), with 0, 5, 10 and 20 min of incubation and addition of 0.5% 1-butanol. The radioactive-labelled product phosphatidylbutanol, arising from the well characterized transphosphatidyl reaction of PLD (Vinggaard *et al.* 1996), was quantified in these samples.

NAPE-PLD activity was determined by quantification of product formation (NAE), using zirconium precipitation of the substrate following the incubation, according to a previously published procedure (Petersen *et al.* 2000) with a modification (Moesgaard *et al.* 2003). Briefly, 50 µg membrane protein were incubated (in triplicate) with 10.5 µM 1,2-dilauroyl-*sn*-glycero-3-phospho (*N*-[1'- 14 C]palmitoyl)ethanolamine [prepared according to Moesgaard *et al.* (2000)] for 0, 30, 60 and 90 min at 37°C in a total volume of 200 µL buffer solution.

For the FAAH assay, a method based upon that of Omeir *et al.* (1995) was used. Briefly, 50 µg membrane protein were incubated (in triplicate) with 28 µM 3 H-anandamide {10 dpm/pmol obtained from 60 Ci/mmol [1- 3 H-ethanolamine] anandamide (American Radiolabelled Chemicals, Inc., St Louis, MO, USA) diluted with non-labelled anandamide (Biomol Research laboratories, Inc., Plymouth Meeting, PA, USA)} for 0, 10, 20 and 30 min at 37°C in a total volume of 200 µL 60 mM bis-tris propane buffer (pH 8.25) containing 0.9 mM EDTA and 1.5 mg/mL fatty acid-free bovine serum albumin. The reaction was terminated by addition of 400 µL chloroform : methanol (1 : 1 v/v) and placing the samples on ice. Following 10 min of low speed centrifugation, 100 µL of the upper phase were extracted and the radiolabelled product ethanolamine formed was quantified by subsequent liquid scintillation counting.

Statistics

All data are presented as mean \pm SEM. Statistical analysis for comparison of data from non-tumour brain tissue and glioblastoma tissue was performed using the paired *t*-test followed by the Wilcoxon signed rank test when the normality test failed. Statistical computation of data from meningioma tissue compared with any of the two other tissue types was performed using the *t*-test followed by Mann-Whitney rank sum test when the normality test or equal variance test failed; *p*-values less than 0.05 were considered significant. SigmaStat version 2.03 (SPSS Inc., Chicago, IL, USA) was used for all statistical computations.

Results

Non-tumour human brain tissue was collected from 22 patients when resection of the brain was necessary in order to surgically remove a glioblastoma. Another 22 patients donated a part of the resected tissue from meningiomas. Ten samples of each type were analysed for their content of NAEs and their phospholipid precursor molecules, as well as MAGs. The lipid phosphorus content was determined in all of these samples. Non-tumour brain tissue contained 21 ± 11 μmol phosphorus (P)/g wet weight while tumour tissue, regardless of type, contained significantly less lipid phosphorus ($p < 0.05$; 5.2 ± 5.0 μmol P/g wet weight of glioblastoma tissue and 5.3 ± 2.7 μmol P/g wet weight of meningioma tissue), thereby suggesting that tumour tissue has a higher content of water per gram wet weight.

Total NAPE in resected human non-tumour brain tissue amounted to 1321 ± 237 pmol/g wet weight, corresponding to 88 ± 27 pmol/ μmol P (Table 1 and Fig. 2a). In glioblastoma tissue, total NAPE accounted for 237 ± 105 pmol/ μmol P, while total NAPE in meningioma tissue was significantly reduced, compared with non-tumour brain tissue, to 38 ± 17 pmol/ μmol P (Fig. 2a). Although the increase in total NAPE in glioblastoma tissue was not significant, the individual species *N*-stearoyl-PE (18 : 0), *N*-linoleoyl-PE (18 : 2) and *N*-arachidonoyl-PE (20 : 4) were all significantly increased eight, three and sevenfold, respectively (Table 1 and Fig. 2b). Additional information regarding the significant increases in these NAPE species was obtained when the *N*-acyl composition of NAPE for each sample was calculated in mol% of total *N*-acyl groups. (Table 1). A substantial increase in mol% of these three metabolites was evident in glioblastoma tissue as well as in meningioma tissue. Furthermore, this increase was counteracted by a significant decrease in *N*-palmitoyl-PE (16 : 0) from 64 ± 1.6 mol% to 54 ± 1.9 mol% in glioblastoma

tissue. In meningioma tissue, the decrease in *N*-palmitoyl-PE was even more pronounced (42 ± 2.2 mol%) and was counteracted by a more substantial increase in *N*-linoleoyl-PE (from 6.6 ± 1.5 mol% to 20 ± 1.8 mol%) compared with the content of *N*-linoleoyl-PE in glioblastoma tissue (11 ± 2.4 mol%).

Total NAE in non-tumour brain tissue expressed as pmol/ μmol P amounted to 16 ± 2.9 , while an eightfold higher level was detected in glioblastoma tissue (128 ± 59 pmol/ μmol P; Fig. 3a). Most of the fatty acid species of NAE detected increased significantly in glioblastoma tissue, while the same increase was not detected in meningioma tissue (Table 2). The mol% of *N*-palmitoylethanolamine was decreased significantly in both types of tumour tissue (Table 2), as was the case with mol% of the precursor molecule, *N*-palmitoyl-PE (Table 1). In glioblastoma tissue, this was not counteracted by a significant increase in any of the other NAE species. Instead, a more general increase in the remaining five NAE species was seen, including anandamide (Fig. 3b). In meningioma tissue, the two most noticeable of the significant increases counteracting the *N*-palmitoylethanolamine decrease were those of *N*-linoleoylethanolamine and anandamide (20 : 4).

The amount of total *sn*-2 MAG was substantially increased in meningioma tissue (20-fold), while in glioblastoma tissue, the increase was more moderate (threefold) (Fig. 4a). This increase in total *sn*-2 MAG was found to be evenly distributed among the four species of ester-linked fatty acids, with a two to fourfold increase in the individual *sn*-2 acyl group of MAG measured in glioblastoma tissue (Table 3). A greater variation, amounting to a nine to 38-fold increase, was detected in meningioma tissue compared with non-tumour brain tissue with a 20-fold increase in 2-AG (Fig. 4b). These differences in fold increase of individual *sn*-2 MAG in tumour tissue did not result in significant changes in distribution when calculated as mol% of the four

Table 1 *N*-Acyl composition of NAPE in resected human brain tissue

Acyl group	Non-tumour brain tissue	Non-tumour brain tissue	Glioblastoma	Meningioma	Non-tumour brain tissue	Glioblastoma	Meningioma
	pmol/g wet wt		pmol/ μmol P			mol%	
16:0	850 ± 163	57 ± 18	123 ± 53	$17 \pm 7.9^{\text{a,b}}$	64 ± 1.6	$54 \pm 1.9^{\text{a}}$	$42 \pm 2.2^{\text{a,b}}$
18:0	132 ± 21	4.3 ± 1.2	$36 \pm 21^{\text{a}}$	2.8 ± 0.8	5.6 ± 1.4	$9.0 \pm 2.2^{\text{a}}$	9.4 ± 1.4
18:1 n-9	177 ± 28	12 ± 3.7	37 ± 16	$6.4 \pm 3.2^{\text{b}}$	14 ± 0.6	15 ± 1.0	15 ± 0.8
18:1 n-7	111 ± 21	6.7 ± 1.6	12 ± 4.4	$2.6 \pm 0.5^{\text{a,b}}$	8.2 ± 0.7	7.2 ± 1.0	9.6 ± 2.2
18:2	22 ± 5.3	6.4 ± 3.4	$16 \pm 4.8^{\text{a}}$	8.3 ± 4.1	6.6 ± 1.5	$11 \pm 2.4^{\text{a}}$	$20 \pm 1.8^{\text{a,b}}$
20:4	29 ± 6.7	1.9 ± 0.6	$14 \pm 7.0^{\text{a}}$	$1.5 \pm 0.6^{\text{b}}$	2.1 ± 0.2	$4.1 \pm 0.5^{\text{a}}$	$4.1 \pm 0.6^{\text{a}}$

^a $p < 0.05$ relative to non-tumour brain tissue; ^b $p < 0.05$ relative to glioblastomas. Measurements of individual *N*-acyl species of NAPE in lipid extracts of tissue samples were carried out by GC-MS in the presence of deuterated internal standards, after determination of lipid phosphorus (P) in each sample. Values are means \pm SEM of non-tumour brain tissue ($n = 10$) and glioblastomas from the same patients ($n = 10$) and meningiomas from separate patients ($n = 8$).

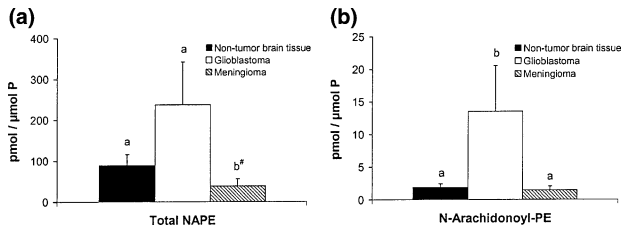


Fig. 2 Total NAPE (a) and *N*-arachidonoyl-PE (b) in lipid extracts of tissue samples, measured by GC-MS in the presence of deuterated internal standards, after determination of lipid phosphorus (P) in each sample. Values are means \pm SEM of non-tumour brain tissue and glioblastomas from the same patients ($n = 10$) and meningiomas from separate patients ($n = 8$). Columns with a letter in common are not significantly different ($p = 0.05$); b#, only significantly different from glioblastoma tissue.

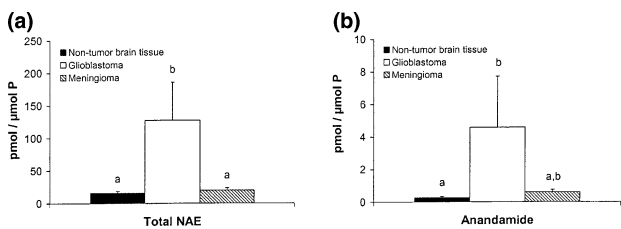


Fig. 3 Total NAE (a) and anandamide (b) in lipid extracts of tissue samples, measured by GC-MS in the presence of deuterated internal standards, after determination of lipid phosphorus (P) in each sample. Values are means \pm SEM of non-tumour brain tissue and glioblastomas from the same patients ($n = 10$) and meningiomas from separate patients ($n = 10$). Columns with a letter in common are not significantly different ($p = 0.05$).

measured species of *sn*-2 MAG. Measurements of the same four species of acyl groups of *sn*-1 MAG in the same tissue samples showed a pattern similar to those of *sn*-2 MAG, although levels were approximately 7–19% of the *sn*-2 MAGs (data not shown). As described earlier (Schmid *et al.* 2000), about 20% of *sn*-2 MAG were isomerized to *sn*-1

MAG during sample preparation, suggesting that essentially, all of the measured *sn*-1 MAGs may have arisen through acyl migration.

A membrane fraction of 12 samples of each of the three tissue types was prepared and the activity of NAPE and NAE metabolizing enzymes was measured (Figs 5a–c). *N*-Acyltransferase activity was determined based on the amount of NAPE plus NAE formed. NAE constituted the main part of total product formation, with 85–93% on average for the three tissue types, indicating much higher activity of NAPE-PLD relative to *N*-acyltransferase. LysoNAPE, another possible product of *N*-acyltransferase activity, was not detectable. The level of *N*-acyltransferase activity was very low in non-tumour brain tissue, as well as in glioblastoma and meningioma tissue. Nonetheless, a significant 2.1-fold increase in *N*-acyltransferase activity was seen in meningioma tissue (Fig. 5a). The opposite was seen in measurements of NAPE-PLD activity. NAE formation was significantly decreased in glioblastoma tissue and meningioma tissue (64% and 83%, respectively; Fig. 5b). Degradation of NAE by FAAH was also significantly decreased in tumour tissue compared with non-tumour brain tissue, although it was more pronounced in glioblastoma tissue (61%) than in meningioma tissue (30%) as measured by the amount of ethanolamine released (Fig. 5c). From the *N*-acyltransferase assay using incubation conditions with radiolabelled phosphatidylcholine as substrate, another time-dependent product, unrelated to NAE metabolism, was identified as MAG on the TLC plates. The amount of MAG formed per minute per milligram of protein is depicted in Fig. 5(d). The enzymes degrading phosphatidylcholine to MAG were found to be significantly increased in meningioma tissue (fivefold), while the same enzymes in glioblastoma tissue were comparable with those of non-tumour brain tissue. In a separate experiment using membrane fractions from three meningioma samples and three glioblastoma samples, it was investigated whether increased phosphatidylcholine-hydroly-

Table 2 Acyl composition of NAE in resected human brain tissue

Acyl group	Non-tumour brain tissue	Non-tumour brain tissue	Glioblastoma	Meningioma	Non-tumour brain tissue	Glioblastoma	Meningioma
	pmol/g wet wt		pmol/μmol P			mol%	
16:0	142 \pm 22	7.6 \pm 1.2	49 \pm 23	8.5 \pm 1.7 ^b	50 \pm 2.1	37 \pm 2.1 ^a	41 \pm 1.8 ^a
18:0	63 \pm 6.0	4.0 \pm 1.0	37 \pm 16 ^a	5.3 \pm 1.0 ^b	24 \pm 2.3	28 \pm 2.4	26 \pm 1.1
18:1 n-9	34 \pm 4.4	1.8 \pm 0.3	22 \pm 13 ^a	3.1 \pm 0.7 ^b	12 \pm 0.7	13 \pm 1.4	15 \pm 1.2 ^a
18:1 n-7	28 \pm 3.4	1.7 \pm 0.5	11 \pm 1.7 ^a	1.7 \pm 0.3 ^b	10 \pm 0.6	18 \pm 4.6	8.9 \pm 1.0
18:2	5.5 \pm 1.0	0.3 \pm 0.1	4.4 \pm 2.9 ^a	1.1 \pm 0.2 ^a	2.1 \pm 0.3	2.3 \pm 0.3	5.7 \pm 0.6 ^{a,b}
20:4	4.3 \pm 0.8	0.3 \pm 0.1	4.6 \pm 3.1 ^a	0.6 \pm 0.2	1.6 \pm 0.2	2.1 \pm 0.4	3.0 \pm 0.4 ^a

^a $p < 0.05$ relative to non-tumour brain tissue; ^b $p < 0.05$ relative to glioblastomas. Measurements of individual acyl species of NAE in lipid extracts of tissue samples were carried out by GC-MS in the presence of deuterated internal standards, after determination of lipid phosphorus (P) in each sample. Values are means \pm SEM of non-tumour brain tissue ($n = 10$) and glioblastomas from the same patients ($n = 10$) and meningiomas from separate patients ($n = 10$).

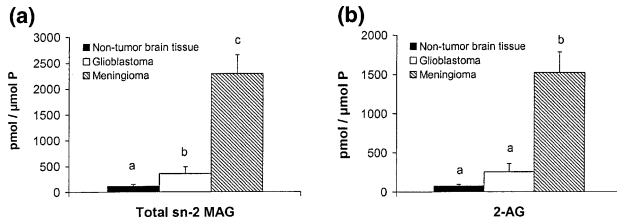


Fig. 4 Total *sn*-2 MAG (a) and 2-AG (b) in lipid extracts of tissue samples, measured by GC-MS in the presence of deuterated internal standards, after determination of lipid phosphorus (P) in each sample. Values are means \pm SEM of non-tumour brain tissue and glioblastomas from the same patients ($n = 9$) and meningiomas from separate patients ($n = 10$). Columns with a letter in common are not significantly different ($p = 0.05$).

sing PLD activity was implicated in the increased MAG formation in meningioma tissue. The resulting product of this PLD reaction, phosphatidylbutanol, amounted to 3.5 ± 1.2 pmol/min/mg protein in meningioma tissue compared with 3.9 ± 0.3 pmol/min/mg protein in glioblastoma tissue.

Discussion

In human glioblastomas, increased levels of individual NAPes and NAEs were found when compared with non-tumour brain tissue from the same patient. In contrast, NAPE and NAE levels in human meningiomas were not different from those of non-tumour brain tissue. Published information on NAPE and NAE levels in human tumours is scarce (Pagotto *et al.* 2001; Ligresti *et al.* 2003) except for a comparative survey (Schmid *et al.* 2002) and a study of human brain tissue (Maccarrone *et al.* 2001). Whereas different human cancers contained varying amounts of NAEs (Schmid *et al.* 2002), the NAE levels measured by Maccarrone *et al.* (2001) in both meningioma and normal brain were several orders of magnitude higher than those reported here or those expected from other studies (Hansen

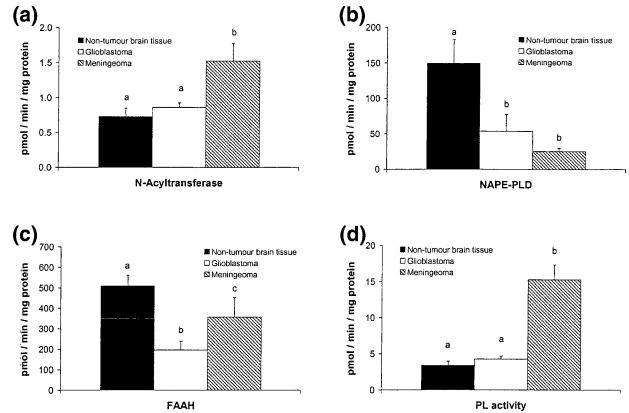


Fig. 5 Enzyme activities measured in membrane fractions of human non-tumour brain tissue, glioblastomas and human meningiomas. All assays were carried out in triplicate at four separate time points, including 0 min at 37°C, and the activity was expressed as the amount of product formed during a time-frame where formation was linear (usually including all time points). (a) *N*-Acyltransferase activity was measured as the amount of NAPE and NAE formed when using radiolabelled phosphatidylcholine as substrate. (b) NAPE-PLD activity was measured as the amount of NAE formed when using radiolabelled NAPE as substrate, and (c) FAAH activity was determined as the amount of ethanolamine formed when incubating samples of each membrane fraction with radiolabelled anandamide. (d) Formation of MAG via a phospholipid-hydrolysing activity (PL) was quantified on TLC plates in all samples from the *N*-acyltransferase assay after verification of the authenticity of the spot in separate experiments. Values are means \pm SEM of non-tumour brain tissue and glioblastomas from the same patients [n (a, b, c, d) = 10, 12, 12, 10] and meningiomas from separate patients ($n = 12$). Columns with a letter in common are not significantly different ($p = 0.05$).

et al. 2000; Schmid *et al.* 2002). They were also questionable on technical grounds (Schmid *et al.* 2002).

In the earlier study by Schmid *et al.* (2002), the levels and composition of NAEs and their precursor phospholipids in single specimens of human tumour tissue were reported. As a result of the low number of observations, no statistical

Table 3 Acyl composition of *sn*-2 of MAG in resected human brain tissue

Acyl group	Non-tumour brain tissue	Non-tumour brain tissue	Glioblastoma	Meningioma	Non-tumour brain tissue	Glioblastoma	Meningioma
	pmol/g wet wt		pmol/ μ mol P		mol%		
18:1 n-9	159 \pm 38	9.6 \pm 2.2	39 \pm 14 ^a	364 \pm 97 ^{a,b}	11 \pm 2.4	13 \pm 2.5	15 \pm 2.3
18:1 n-7	259 \pm 65	22 \pm 11	46 \pm 16 ^a	340 \pm 78 ^{a,b}	14 \pm 3.0	15 \pm 3.3	15 \pm 3.3
18:2	86 \pm 18	6.9 \pm 3.7	17 \pm 5.3 ^a	65 \pm 21 ^{a,b}	4.9 \pm 0.7	5.9 \pm 1.3	2.9 \pm 0.7
20:4	1131 \pm 99	75 \pm 22	255 \pm 106	1524 \pm 260 ^{a,b}	70 \pm 2.5	66 \pm 4.2	67 \pm 2.2

^a $p < 0.05$ relative to non-tumour brain tissue; ^b $p < 0.05$ relative to glioblastomas. Measurements of individual acyl species in the *sn*-2 position of MAG lipid extracts of tissue samples were carried out by GC-MS in the presence of deuterated internal standards, after determination of lipid phosphorus (P) in each sample. Values are means \pm SEM of non-tumour brain tissue ($n = 9$) and glioblastoma from the same patients ($n = 9$) and meningiomas from separate patients ($n = 10$).

analysis was presented. Nonetheless, when averaging data from the different tumours, there was a significant increase in total NAPE in tumour tissue compared with adjacent normal tissue ($p < 0.05$; paired t -test), supporting the same tendency seen in tissue samples from human glioblastomas in the present study. Total NAE measured per lipid phosphorus, on the other hand, was not changed from benign tissue to tumour tissue (Schmid *et al.* 2002). This lack of change in total NAE seen in the human meningioma tissue in the present study, in contrast to the significant increase seen in glioblastoma tissue, indicates marked differences in the metabolic changes induced by the two types of tumours.

A hallmark of the N -acyl composition of NAPE and NAE in brain tumour tissue is a change in mol% of specific amide-linked fatty acids, which includes a significant decrease in the most abundant fatty acid, palmitic acid, in both types of tumour tissue. When averaging data for NAPE species from the eight different human tumours with corresponding benign tissue, as presented in an earlier study (Schmid *et al.* 2002), a significant decrease in N -palmitoylethanolamine ($p < 0.05$; paired t -test) was revealed. In the present study, this is counteracted by a significant increase in the amide-linked stearic acid, linoleic acid, and arachidonic acid of NAPE. The same tendency, although not statistically significant, for redistribution is seen in NAE species. It is noteworthy that the change in mol% of NAPE and NAE species seen in glioblastoma tissue was mirrored in meningioma tissue in which there was no significant increase in the compounds compared with non-tumour brain tissue. This redistribution of fatty acids probably results from a relatively decreased availability of NAPE precursor phospholipids enriched in ester-linked palmitic acid in the sn -1 position, since fatty acids in this position are transferred by N -acyltransferase to become the amide-linked fatty acids of NAPE.

Twenty years ago it was reported that the fatty acid composition of all major phospholipid classes changed in meningioma tissue, resulting in a marked relative increase in unsaturated fatty acids (Riboni *et al.* 1984). Redistribution in phospholipid fatty acid composition of human gliomas compared with non-malignant brain tissue has also been reported, with a significant reduction in the levels of stearic acid and of the polyunsaturated fatty acid, docosahexaenoic acid (Martin *et al.* 1996). Another group of researchers reported significant increases in n -6 fatty acids (including linoleic acid and arachidonic acid) at the expense of n -3 fatty acids (including docosahexaenoic acid) in plasma membranes isolated from gliomas and meningioma tissue compared with normal human brain tissue (Kokoglu *et al.* 1998). In conclusion, the redistribution of amide-linked fatty acids of NAPE and NAE in tumour tissue may be a result of changes in substrate composition, i.e. in the sn -1 position of the phospholipids. Generally, a relative decrease in amide-linked palmitic acid was seen in tumour tissue and an increased utilization of linoleic acid for formation of other

amide-linked polyunsaturated fatty acids resulted in an increased degree of unsaturation of the fatty acids.

The most noteworthy lipid change in meningioma tissue was the massive increase of sn -2 MAG. In contrast, Maccarrone *et al.* (2001) detected no change in 2-AG in meningioma tissue compared with healthy human brain tissue (perilesional white matter surrounding the tumour area). However, the level of this metabolite as measured by Maccarrone *et al.* (2001) was more than 30-fold higher than the level reported in non-tumour brain tissue (predominantly white matter, not perilesional, from patients diagnosed with a glioblastoma) in the present study. The 20-fold increase in sn -2 MAGs observed in the present study fitted nicely with the fivefold increase in MAG formation detected in the enzyme assay using phosphatidylcholine as a substrate. The pathway from phosphatidylcholine to MAG does not seem to be due to increased phosphatidylcholine-hydrolysing phospholipase D in the meningioma tissue. This hypothesis was tested because phospholipase D was reported to be a critical regulator of cell proliferation, survival signalling, cell transformation and tumour progression (Foster and Xu 2003). Determination of the exact pathway involved in increased MAG formation must await further studies; these should include diacyl glycerol generation through phosphatidylcholine-selective phospholipase C activity (Di Marzo *et al.* 1996) and phosphatidylinositol turnover (Stella *et al.* 1997), since this is apparently increased in tumour tissue (Gunther *et al.* 2003; Newton 2004).

The activity of N -acyltransferase resulting in NAPE formation in meningioma tissue was increased, while NAPE-PLD activity and FAAH activity were both significantly decreased. These enzymatic changes do not explain the observed decrease in total NAPE. One hypothesis could be that there is a decrease in phospholipid substrate availability for N -acyltransferase that cannot be compensated for by up-regulation of the enzyme activity. To explain the increased levels of NAPE and NAE in glioblastoma tissue, the observed reduction in FAAH activity must be all-important compared with the reduction in NAPE-PLD activity. In support of these results, FAAH expression was found to be decreased in the more malignant cells of human colorectal carcinoma Caco-2 cells (Ligresti *et al.* 2003), as well as in a comparison of a range of human cancer cell line proteomes (Jessani *et al.* 2002).

Cannabinoid receptor stimulation promotes ERK1/2 activation in C6.9 glioma cells, astrocytoma cells and primary astrocytes, and there is strong evidence that the succeeding intracellular accumulation of ceramide contributes to the pro-apoptotic properties of cannabinoids in transformed cells (Sánchez *et al.* 1998a,b). In other cell lines, inhibition of tumour growth by cannabinoids may occur exclusively by inhibition of cell proliferation (De Petrocellis *et al.* 1998; Melck *et al.* 2000). A recent report, however, showed that THC can induce cancer cell proliferation via activation of

epidermal growth factor receptor and metalloproteases when applied in nanomolar concentrations to human cancer cell lines, while micromolar concentrations induced apoptosis in the same cells (Hart *et al.* 2004). Anandamide in micromolar concentrations produced anti-proliferative effects in C6 glioma cells via activation of CB receptors, as well as involvement of vanilloid receptor stimulation (Fowler *et al.* 2003). The increased level of anandamide in human glioblastoma tissue is here suggested to play a role as endogenous lipid mediator of tumour cell anti-proliferation. Interaction with CB2 receptors is particularly interesting as this receptor subtype is primarily located in peripheral tissues but is also expressed by some gliomas (Sánchez *et al.* 2001). Furthermore, selective activation of the CB2 receptor induced a regression of glioma cell growth *in vivo*, and the CB2 receptor expression on human astrocytomas appeared to be closely correlated with tumour grade and degree of malignancy (Sánchez *et al.* 2001). Stimulation of CB2 receptors by the increased level of the endocannabinoid, anandamide, is therefore a possible anti-proliferative as well as pro-apoptotic response that might take place in glioma cells *in vivo*.

A similar putative anti-proliferative response might take place in meningioma tissue mediated by the excessive amounts of 2-AG produced. The increased level of 2-AG could be responsible for stimulation of CB receptors as described earlier (De Petrocellis *et al.* 1998; Melck *et al.* 2000; Fowler *et al.* 2003), while no 2-AG-mediated activation of vanilloid receptors was observed (Jacobsson *et al.* 2001; Fowler *et al.* 2003). The 2-AG might thereby also act as an endogenous lipid mediator of anti-tumour effects. Further speculation, however, must await characterization of meningioma cells in terms of presence of cannabinoid receptors.

In relation to the malignant progression of tumours, evidence points to a decreased ceramide level with increased diagnostic grade of human glial tumours, and the level of ceramide has therefore been suggested as a marker of malignancy of this tumour type. Furthermore, a positive correlation between ceramide level in human glial tumours and survival was found (Riboni *et al.* 2002). It would be of interest to investigate whether lower levels of NAE and 2-AG could be used as a marker of increased tumour malignancy in glioma tissue and meningioma tissue, respectively, as the inverse relationship reported for ceramide level and malignant progression of human glial tumours. The endogenous level of ceramide might be increased by *N*-oleoylethanolamine. This monounsaturated NAE was earlier described as an effective inhibitor of ceramidase (Sugita *et al.* 1975) due to structural similarity to ceramide, and this structural similarity might mean that all NAEs produced in tissues are important inhibitors of ceramidase (Schmid 2000), indicating that in glioblastoma tissue the increased level of NAEs might be part of a natural defense

against tumour proliferation via their direct inhibition of ceramidase. In prostatic cancer cell lines, *N*-oleoylethanolamine has been shown to potentiate apoptotic/necrotic responses via inhibition of ceramidase induced by anandamide-stimulated CB1 receptor activation (Mimeault *et al.* 2003). However, there has also been a report of phosphatidic acid, the other product of PLD-catalysed NAPE hydrolysis, exhibiting the opposite effect as an inhibitor of ceramide-mediated responses (Kishikawa *et al.* 1999).

N-Stearoylethanolamine, which is significantly increased in human glioblastoma tissue, has been reported to exhibit pro-apoptotic activity in C6 glioma cells by regulation of nitric oxide in a way opposite to that reported for anandamide via non-CB1, non-CB2, non-vanilloid receptors (Maccarrone *et al.* 2002). Furthermore, *N*-palmitoylethanolamine enhances the anti-proliferative effect of anandamide mediated by vanilloid and cannabinoid receptors by inhibition of FAAH expression (Di Marzo *et al.* 2001; De Petrocellis *et al.* 2002). In human glioblastoma tissue, the non-significant increase in *N*-palmitoylethanolamine may, in conjunction with the overall increase in NAE, contribute to the same effect, i.e. inhibiting FAAH expression. A possible effect of *N*-oleoylethanolamine and *N*-palmitoylethanolamine as natural agonists for the peroxisome proliferator-activated receptor α (Fu *et al.* 2003; Lo Verme *et al.* 2005) in relation to anti-tumour effects should also be considered in further studies.

The use of a recently developed assay identifying proteome signatures through activity-based protein profiling (Liu *et al.* 1999) has shown that regulation of specific enzyme activities in tumour-derived cell lines differs significantly from the corresponding regulation of the same enzymes in the primary tumour from which they were derived, thereby emphasizing the importance of microenvironmental influence on tumour biology (Jessani *et al.* 2004). These findings strongly question the relevance of measuring tumour-related changes in cell cultures, thus underscoring the importance of the *in vivo* data presented here. Very recently, inhibition of FAAH as a strategy for inhibition of proliferation was shown to be effective in rat thyroid tumour xenografts induced in athymic mice (Bifulco *et al.* 2004). These findings strongly support the suggested hypothesis that endocannabinoids may exert a tonic inhibition of cancer cell growth that can be strengthened by inhibition of endocannabinoid breakdown. If NAEs in human brain tumour tissue exhibit a submaximal inhibition of cancer cell growth as suggested earlier in colorectal cancer cells (Ligresti *et al.* 2003), reinforcement of the tonic inhibition on FAAH enzyme activity by administration of specific FAAH inhibitors becomes an attractive new strategy for blocking tumour growth (Bifulco *et al.* 2004). So far, only preliminary results from one clinical study applying a strategy of local THC administration in patients with recurrent glioblastoma multiforme has been reported. Biopsies from two patients showed impairment of the vascular endothelial growth factor pathway following cannabinoid treatment, thereby stressing the

magnitude of blunting angiogenesis and tumorigenesis via *de novo*-synthesized ceramide (Blázquez *et al.* 2004).

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