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Review

N-Acylethanolamines and precursor phospholipids — relation to cell injury

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Abstract

The present review focuses on the relationship between formation of *N*-acylethanolamine phospholipids (NAPEs) and *N*-acylethanolamines (NAEs) catalyzed by *N*-acyltransferase and NAPE-hydrolyzing phospholipase D, respectively, and cell injury in tissues like brain, heart, and testis. A number of mechanisms are proposed by which these two groups of lipids may have cytoprotective properties. The mechanisms may involve activation of cannabinoid receptors, as well as non-receptor-mediated effects such as stabilization of membrane bilayers, antioxidant mechanisms, inhibition of calcium leakage from mitochondria, and direct inhibition of ceramidase. Anandamide (20:4-NAE) is formed as a minor component along with other NAEs during cell injury. Whether 20:4-NAE has a separate physiological role is at present not known, but some data suggest that 20:4-NAE may be formed, e.g. in the uterus, by a more selective mechanism without being accompanied by a vast majority of saturated and monounsaturated NAEs.

Keywords: *N*-Acylethanolamine; *N*-acylethanolamine phospholipid; anandamide; *N*-acylethanolamine phospholipid-hydrolyzing phospholipase D; *N*-acyltransferase; injury

Abbreviations: 2-AG, 2-arachidonoylglycerol; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; NAE, *N*-acylethanolamine; 16:0-NAE, *N*-palmitoylethanolamine; 18:0-NAE, *N*-stearoylethanolamine; 18:1-NAE, *N*-oleoylethanolamine; 18:2-NAE, *N*-linoleoylethanolamine; 20:4-NAE, *N*-arachidonoylethanolamine = anandamide; 22:5(n-6)-NAE, *N*-docosapentaenoylethanolamine; 22:6(n-3)-NAE, *N*-docosahexaenoylethanolamine; NAPE, *N*-acylethanolamine phospholipid; 16:0-NAPE, *N*-palmitoylethanolamine phospholipid; 18:0-NAPE, *N*-stearoylethanolamine phospholipid; 18:1-NAPE, *N*-oleoylethanolamine phospholipid; 20:4-NAPE, *N*-arachidonoylethanolamine phospholipid; 22:5(n-6)-NAPE, *N*-docosapentaenoylethanolamine phospholipid; NAPE-PLD, *N*-acylethanolamine phospholipid-hydrolyzing phospholipase D.

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1. Introduction

In 1990, Schmid and coworkers summarized their work on *N*-acylethanolamine phospholipids (NAPEs) and *N*-acylethanolamines (NAEs) within a comprehensive review. At that time the authors concluded that these lipids were not found in measurable amounts in mammalian tissues in vivo, but NAPEs and NAEs could be formed in response to cell injury. The present review will refer the reader to this excellent review for some of the older papers and concentrate on papers published after 1990. NAPE and NAE metabolism has also been reviewed in several previous papers (e.g. Schmid et al., 1996a; Hillard and Campbell, 1997; Di Marzo, 1998; Mechoulam et al., 1998), and it is discussed in detail by HHO Schmid in this issue. A renewed interest in the biochemistry and functions of NAPEs and NAEs appeared with the discovery of *N*-arachidonoylethanolamine (20:4-NAE; also called anandamide) as an endogenous ligand for the cannabinoid receptor type 1 (CB1) in 1992 (Devane et al., 1992). Since then, another lipid, 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995, 1996a; Kondo et al., 1998; Sugiura et al., 1999), has also been found to bind and activate cannabinoid receptors, both the CB1 and the cannabinoid receptor type 2 (CB2), and this has raised a discussion of whether one or both of these lipids, 2-AG and 20:4-NAE, are neurotransmitters or local hormones. The level of 2-AG in tissues is usually 2–3 orders of magnitude larger than that of 20:4-NAE (Berrendero et al., 1999; Bisogno et al., 1999a,b; Straiker et al., 1999) but a discussion of the biological role of 2-AG is beyond the scope of the present review.

It is still an open question whether 20:4-NAE is a neurotransmitter, but it is quite clear that several species of NAE, including a minor percentage of 20:4-NAE, accumulate in tissues as a response to tissue injury. Several reviews have focused on the possible biological role of 20:4-NAE more or less disregarding the other NAEs that may be formed along with 20:4-NAE. This review will focus on our current knowledge about how, where, and when NAEs and NAPEs are formed in mammalian tissues, and their possible biological functions with respect to cell injury.

2. Formation of NAPEs

In mammals, NAPEs are formed by a membrane-associated *N*-acyltransferase that transfers the *sn*-1 acyl group of a donor phospholipid to the amino group of ethanolamine phospholipids (Natarajan et al., 1982; Reddy et al., 1983a) (Fig. 1; Table 1). The enzyme does not seem to discriminate between the fatty acids in the *sn*-1 position, but it does seem to prefer phosphatidylcholine over phosphatidylethanolamine as exogenously added acyl donor (Sugiura et al., 1996b; Cadas et al., 1997; Moesgaard B and Hansen HS, unpublished). As opposed to the mammalian enzyme, the plant-derived *N*-acyltransferase, which has been purified, utilizes free fatty acids as acyl donors (Chapman and Moore, 1993; McAndrew and Chapman, 1998). The mammalian *N*-acyltransferase uses diacyl, alkenylacyl, and alkylacyl species of ethanolamine phospholipids equally well as acceptor substrates (Natarajan et al., 1982; Schmid et al., 1990; Moesgaard et al., 1999).

At present, very little is known about this enzyme (Table 1), and it has not been purified. It is membrane associated and is found in several fractions upon subcellular fractionation of tissues and cells (Natarajan et al., 1982; Schmid et al., 1990; Cadas et al., 1997). In neuronal cells in culture, there are some indications that NAPEs to a large extent may be found in the plasma membrane (Gulaya et al., 1989; Cadas et al., 1996a). *N*-Acyltransferase can be solubilized with detergent and an initial ion exchange purification has been reported (Cadas et al., 1997). The enzyme is activated by calcium ions (EC50 being around 0.2–0.5 mM) (Natarajan et al., 1982; Schmid et al., 1990) and calcium ionophores have been found to stimulate the enzyme in cell cultures (Di Marzo et al., 1994; Hansen et al., 1995; Cadas et al., 1996b). Protein kinase C does not seem to be involved in the regulation of NAPE formation whereas there is some controversy whether addition of cyclic AMP to cells will affect the activity of the enzyme (Cadas et al., 1996b; Hansen et al., 1997). Investigation of the tissue distribution of the enzyme activity in rats showed high levels especially in brain and testis but the activity was also found in several other tissues (Table 1). Dis-

tribution of activity in different regions of the brain has also been reported with highest activity in the brainstem (Cadas et al., 1997). It is noteworthy that high activity was found in canine heart whereas activity could not be detected in rat heart (Schmid et al., 1990) indicating that a marked species difference exists. Furthermore, in rat brain the activity of *N*-acyltransferase changes considerably during development being high in infant rats and several fold lower in adult rats (Natarajan et al., 1982; Schmid et al., 1990; Moesgaard et al., 2000).

3. Formation of NAEs

20:4-NAE can be formed in vitro by direct condensation of arachidonic acid and ethanolamine catalyzed by the fatty acid amidohydrolase using mM concentrations of ethanolamine and μM concentrations of arachidonic acid (Katayama et al., 1999). The fatty acid amidohydrolase is considered as one of several enzymes that degrades NAEs in vivo (Hillard and Campbell, 1997; Di Marzo, 1998; Childers and Breivogel, 1998; Mechoulam et al., 1998; Randall and Kendall, 1998; Ueda et al., 1999; Patricelli et al., 1999). Thus, the same enzyme that degrades these lipids may theoretically form both 20:4-NAE and other NAEs. However, it is considered unlikely that this is a major way of formation of NAEs in vivo due to the high concentrations required of fatty acid and ethanolamine. There has been a report of an anandamide synthase in murine uterus that apparently is different from the fatty acid amidohydrolase (Paria et al., 1996). It must await further studies to determine how important this enzyme is for the formation of 20:4-NAE and other NAEs in vivo.

The major way of NAE formation in vivo is considered to be catalyzed by a NAPE-hydrolyzing phospholipase D (NAPE-PLD; Fig. 1) (Schmid et al., 1983; Hillard and Campbell, 1997; Di Marzo, 1998; Childers and Breivogel, 1998; Hansen et al., 1998; Mechoulam et al., 1998; Randall and Kendall, 1998; Di Marzo, 1999). At the same time, phosphatidic acid is formed as a second product, and this lipid may have second messenger functions (Dhalla et al., 1997; Arneson et al., 1999; Daniel et al., 1999; Rizzo et al., 1999; Tool et al., 1999). NAPE-PLD has not been purified and all characterization has been performed with microsomal preparations (Table 2). The enzyme seems to be rather specific for NAPE (Schmid et al., 1983), but it does not seem to discriminate between the fatty acids of the *N*-acyl

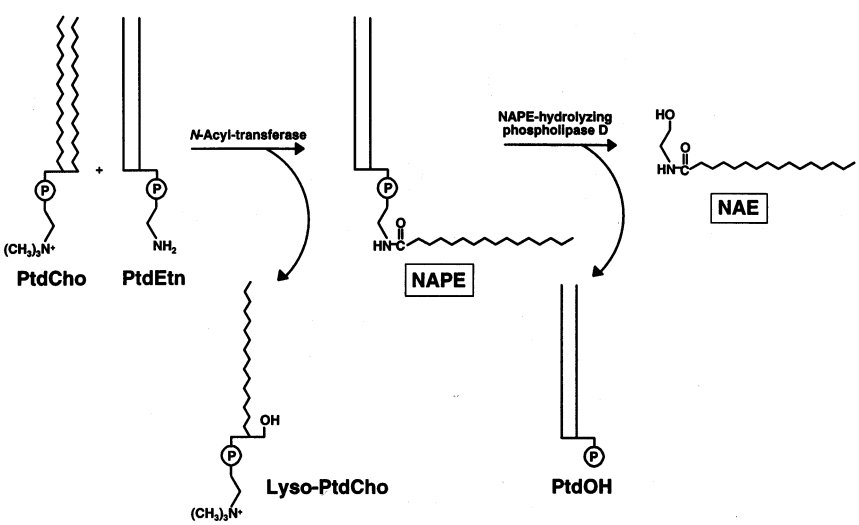

Fig. 1. Formation of NAPE and NAE by *N*-acyltranferase and NAPE-hydrolyzing phospholipase D, respectively. Phospholipids other than PtdCho may also serve as donor substrates.

Table 1
Properties of mammalian *N*-acyltransferase

Characteristics			References
Donor substrates	*sn*-1-Acyl of PC > 1-acyl-lysoPC, PE, cardiolipin		Natarajan et al., 1982, 1983; Reddy et al., 1983a,b, 1984; Sugiura et al., 1996a
Acyl-specificity of donor substrates	None		Cadas et al., 1997
Acceptor substrates	Plasmalogen-, alkylacyl-, diacyl-, and lyso-PE		Natarajan et al., 1981, 1982, 1983; Reddy et al., 1983b; Sugiura et al., 1996b; Moesgaard et al., 1999
Co-factors	Stimulated by Ca^{2+}, Sr^{2+}, Mn^{2+}, Ba^{2+}, and cystine reducing agents		Natarajan et al., 1982, 1983; Reddy et al., 1984; Natarajan et al., 1986; Cadas et al., 1997
Inhibitors	Sulfhydryl reagents, alkylating agents, several divalente and monovalente cations including Be^{2+}, Zn^{2+}, and Ag^{+}		Reddy et al., 1984; Cadas et al., 1997
pH optimum	Canine and infant rat brain:	10	Natarajan et al., 1983, 1986
	Rat brain:	7.0–7.5	Sugiura et al., 1996b
	Canine heart:	8.5–9.0	Reddy et al., 1984
	Canine heart:	9–10	Natarajan et al., 1982
	Bovine brain:	8.0–8.5	Moesgaard, B. and Hansen, H.S., unpublished
Subcellular localization (canine brain)	Microsomes > synaptosomes > mitochondria		Natarajan et al., 1983
Tissue distribution	Rat:	Brain > testis > muscle > lung, stomach, pancreas, heart, intestine, spleen, kidney, liver	Cadas et al., 1997
	Rat brain:	Brainstem > cortex, striatum, cerebellum, hippocampus, medulla > olfactory tubercle, thalamus, hypothalamus, olfactory bulb	Cadas et al., 1997

group (Sugiura et al., 1996a,b). Furthermore, it does not seem to discriminate between NAPE species with an *O*-acyl or an *O*-alkenyl in the *sn*-1 position (Schmid et al., 1983). Most mammalian phospholipase D enzymes can utilize an alcohol, e.g. butanol, instead of water in the cleavage of their phospholipid substrate thereby generating phosphatidylbutanol, a reaction termed transphosphatidylation (Yu et al., 1996; Steed et al., 1998; Frohman et al., 1999; Ogino et al., 1999; Waite, 1999). The transphosphatidylation reaction is very commonly used to detect and measure the activity of phospholipase D enzymes in cells and tissues. NAPE-PLD lacks this ability, suggesting that this enzyme is not structurally related to most of the other known phospholipase Ds (Petersen and Hansen, 1999).

A number of endogenous compounds that can activate other mammalian phospholipase D enzymes have been tested on NAPE-PLD and found to be without effect (Petersen and Hansen, 1999). At present it is not known whether NAPE-PLD can be activated in tissues. Studies with $H_2^{18}O$ in macrophages have indicated a rather fast basal turnover of the *N*-acyl group of NAPEs in rat peritoneal macrophages suggesting that NAPE-PLD have a certain measurable basal activity in these cells (Kuwae et al., 1999). In cortical neurons in primary culture, the formation of NAE increased in parallel with NAPE formation upon

exposure to glutamate, calcium ionophore, sodium azide, and hydrogen peroxide, suggesting that NAPE-PLD was constitutive active, and that it was the availability of the substrate NAPE that determined the rate of NAE formation (Hansen et al., 1995, 1997; Hansen et al. 1999b).

In rats, the highest activity was found in the heart, whereas in cows the highest activity was found in the brain while the bovine heart showed quite a low activity (Table 2). Apparently, there is some species variation with respect to the activity of cardiac NAPE-PLD. In rat brain, we have found that the activity of NAPE-PLD increases dramatically with development being very low at birth and increasing to a 15-fold higher level in adult rats (Moesgaard et al., 2000). This would seem to favor an efficient conversion of NAPEs to NAEs in the brain of adult rats as opposed to newborn rats. While the activity of NAPE-PLD in rat brain increases with development, the activity of the *N*-acyltransferase decreases within the same developmental period. This is in agreement with the observation that post mortem accumulation of NAPEs in rat brains is very high in infant rats as opposed to very low in adult rats (Moesgaard et al., 2000).

4. In vivo levels of NAPEs and NAEs

It is known that NAPEs and NAEs accumulate post mortem (Schmid et al., 1990, 1995, 1996b; Kempe et al., 1996; Moesgaard et al., 1999). Recently however, it has become apparent that these lipids are not just formed upon cell injury but can be detected in small amounts in normal mammalian tissues. By rapid freezing of tissues and by the use of new sensitive analytical methods, it has clearly been demonstrated that these lipids can also be found in non-injured tissues in vivo. The use of mass spectrometry in a number of different assays has improved the detection limits, and basal levels have been reported for different NAPE species including the anandamide

Table 2
Properties of mammalian NAPE-hydrolyzing phospholipase D

Characteristics			References
Substrates	Diacyl-[a], plasmalogen-, lyso-NAPE[a], *N*-acetyl-Pe[a], GP–NAE[a]		Schmid et al., 1983; Natarajan et al., 1984
N-Acyl-specificity of substrate	None		Sugiura et al., 1996a,b
Co-factors	No stimulatory effect of Ca^{2+}[b], Mg^{2+}, Zn^{2+}, PIP_2, oleate, α-synuclein, mastoparan		Schmid et al., 1983; Petersen and Hansen, 1999
Inhibitors	The sulfhydryl reagent p-CMPS		Sasaki and Chang, 1997
pH optimum	Rat heart:	7–8.5$\frac{1}{2}$	Schmid et al., 1983
	Canine brain:	8–9	Natarajan et al., 1984
K_m	Rat heart:	21 μM	Schmid et al., 1983
	Canine brain:	150 μM	Natarajan et al., 1984
Transphosphatidylation ability	Cannot catalyze this reaction		Petersen and Hansen, 1999
Subcellular localization (canine brain)	Microsomes > synaptosomes > mitochondria		Natarajan et al., 1984
Tissue distribution	Rat:	Heart > brain, testis, kidney > spleen, lung, liver	Schmid et al., 1983
	Bovine:	Brain > kidney, spleen, lung > heart, liver	(Petersen et al., 2000)

[a] Relative efficiency dependent on presence of detergent and pH.
[b] In rat heart. In rat brain and canine brain a slight stimulatory effect was seen with low Ca^{2+} concentrations.

Table 3
In vivo levels of NAPEs[a]

Tissue	Species	20:4-NAPE pmol/g tissue	Total NAPEs pmol/g tissue	References
Brain	Rat	50	12072	Sugiura et al., 1996b
		22	3666	Cadas et al., 1997
		50–190[b,c]	–	Yang et al., 1999
		59–359[c]	–	Bisogno et al., 1999a
		63[d]	–	Berrendero et al., 1999
		139–786[c]	–	González et al., 1999
		61	4248	Hansen, H.H., Ikonomidou, C., Bittigau, P., Hansen, S.H., Hansen, H.S., (unpublished)
Retina	Bovine	51	–	Bisogno et al., 1999b
Spinal cord	Rat	320[b]	–	Yang et al., 1999
Kidney	Rat	68	610	Deutsch et al., 1997a
Spleen	Rat	50[b]	–	Yang et al., 1999
Testis	Rat	274	2525	Sugiura et al., 1996a
		242	2887	Kondo et al., 1998
		470[b]	–	Yang et al., 1999
Uterus	Murine	27–81[e]	713–4095[e]	Schmid et al., 1997

[a] All NAPE data are from reports on normal (control) tissue levels. –, No other NAPEs determined.
[b] Calculations assume 100 mg protein per g tissue wet weight.
[c] In various brain regions.
[d] Determination from adult rats (>8 weeks) of developmental study.
[e] In various stages of pregnancy.

precursor, 20:4-NAPE (Table 3), and for 20:4-NAE as well as other NAEs (Table 4). Most studies have concentrated on measuring 20:4-NAE and 20:4-NAPE, but a few studies have also quantified most of the other molecular *N*-acyl species of NAPE and NAE. In cases where the *N*-acyl composition of NAPE has been determined, it typically involves hydrolyzing the NAPE molecules thereby generating NAE species, which are then analyzed. However, it is now possible to determine all acyl groups in NAPEs by electrospray mass spectrometry (Hansen et al., 1999a). We have elaborated on this method to analyze the *N*-acyl composition of NAPE in neocortical neurons in culture. We found eight different species including a species containing a putative trans-configured double bound in the *N*-arachidonoyl moiety. Sodium-azide exposure tripled the amount of NAPE while maintaining the same relative *N*-acyl composition. The anandamide precursor constituted 0.1% of all NAPEs detected in the neurons (Hansen et al., 2000). We are now applying this method on different animal models of neurodegeneration.

Total NAPE levels seem to be a few nmol/g tissue accounting for approximately 0.01% of the endogenous phospholipids, assuming 20 μmol lipid phosphorus per g tissue. Many different fatty acids can be found in the *N*-acyl group, and the three major *N*-acyl species of NAPE in rat brain were found to be 16:0-NAPE (70%, 84%), 18:1-NAPE (15%, 5%), and 18:0-NAPE (12%, 11%), while 20:4-NAPE accounted for only 0.4–0.6% of the analyzed NAPE species. The two percentages given in parentheses refer to the work of Sugiura et al. (1996b) and of Cadas et al. (1997), respectively.

In rat brain, 20:4-NAPE levels vary with a factor of 4–6 within the different regions of the brain, with the highest levels in striatum and brainstem and the lowest levels in cerebellum and cortex (Bisogno et al., 1999a; Yang et al., 1999). The other molecular species of NAPE was not measured in these two studies but by comparing the values with other reported NAPE values in rat brain (Table 3) it seems likely that 20:4-NAPE accounts for less than a few percentages of the

total NAPE species. We have found that 20:4-NAPE and total NAPE levels in the brain were about 2-fold higher in adult rats compared to infant rats (Hansen H.H., Ikonomidou C., Bittigau P., Hansen S.H., Hansen H.S., to be published). This is in agreement with the increase of 20:4-NAPE levels in adult rats compared to neonatal and infant rats reported by Berrendero et al. (Berrendero et al., 1999). In rat kidney, 16:0-NAPE (41%), 18:1-NAPE (18%), and 18:0-

Table 4
In vivo levels of NAEs[a]

Tissue and extracellular compartments	Species	20:4-NAE pmol/g tissue	Total NAEs pmol/g tissue	References
Brain	Human	25–148[b]	–	Felder et al., 1996
	Rat	20–29[b]	–	Felder et al., 1996
		4	597	Sugiura et al., 1996b
		12–35[b,c]	513–1397[b,c,d]	Koga et al., 1997
		11	136	Cadas et al., 1997
		10–87[b]	–	Bisogno et al., 1999a
		15[e]	–	Berrendero et al., 1999
		$\leq$25[b]	–	González et al., 1999
	Murine	10–15	–	Schmid et al., 1997
	Porcine	383	–	Devane et al., 1992
		17	2037	Schmid et al., 1995, 1996a
	Bovine	10	957	Schmid et al., 1995, 1996a
	Sheep	n.d.	835	Schmid et al., 1995, 1996a
Retina	Rat	n.d.	185[f]	Straiker et al., 1999
	Bovine	64	–	Bisogno et al., 1999b
Heart	Human	10	–	Felder et al., 1996
	Rat	21[c]	130[c,d]	Koga et al., 1997
Liver	Rat	31[c]	176[c,d]	Koga et al., 1997
Kidney	Rat	8	186	Deutsch et al., 1997a
Spleen	Human	15	–	Felder et al., 1996
	Rat	6	–	Felder et al., 1996
		77[c]	286[c,d]	Koga et al., 1997
Thymus	Rat	n.d.	210[c,d]	Koga et al., 1997
Testis	Rat	6	122	Sugiura et al., 1996a
		11[c]	109[c,d]	Koga et al., 1997
		5	97	Kondo et al., 1998
Uterus	Murine	2215–20982[g]	2719–22269[g]	Schmid et al., 1997
Skin	Rat	23	–	Felder et al., 1996
		49	741[h]	Calignano et al., 1998
ECF (brain)	Rat	1.5 pmol/300 μl[i]	3.7 pmol/300 μl[d,i]	Giuffrida et al., 1999
		2.8 fmol/15 μl[i]	–	Walker et al., 1999
CSF	Human	0.3 pmol/ml	3.0 pmol/ml[d]	Leweke et al., 1999
Plasma	Rat	5 pmol/ml	30 pmol/ml[d]	Giuffrida and Piomelli, 1998

[a] All NAE data are from reports on normal (control) tissue levels. –, no other NAEs determined; n.d., not detected; CSF, cerebrospinal fluid; ECF, extracellular fluid.
[b] In various brain regions.
[c] Determinations from 24-week-old rats of developmental study.
[d] Only 16:0-, 18:1- and 20:4-NAE determined.
[e] Determination from adult rats (>8 weeks) of developmental study.
[f] Only 16:0- and 18:1-NAE determined.
[g] In various stages of pregnancy.
[h] Only 16:0- and 20:4-NAE determined.
[i] Determined in microdialysis perfusion buffer.

NAPE (13%) also accounted for the three most abundant *N*-acyl species of NAPE, while 20:4-NAPE was reported to account for 11% (Deutsch et al., 1997a). Generally, the same composition of *N*-acyl species of NAPE was also seen in rat testis except that this tissue further contained 22:5(n-6)-NAPE (15% of total) (Kondo et al., 1998), being in agreement with the relative high level of esterified 22:5(n-6) in rat testis phospholipids (Bourre et al., 1990).

It is noteworthy that the level of total NAPEs in different tissues (Table 3) does not correlate with the activity of the enzyme that generates NAPEs, e.g. rat testis had more than a 40-fold higher activity of *N*-acyltransferase than rat kidney (Cadas et al., 1997), whereas the total NAPE levels are only 4-fold different (Table 3).

Already by the discovery of 20:4-NAE in extracts from porcine brain, it was found that this compound was just one of a group of unsaturated NAEs, which all with variable degree of potency could activate the CB1 receptor (Hanus et al., 1993; Pertwee et al., 1994; Barg et al., 1995). In fact, NAEs containing all the common mammalian C22 and C20 polyunsaturated fatty acids with 3–6 *cis* double bonds will have some stimulatory effects on the CB1 receptor although the structure of 20:4-NAE seems to be optimal (Martin et al., 1999). A few studies have determined total NAE levels, and they are usually reported to be in the range of 100–600 pmol/g tissue except for the uterus, which is special in having high levels of mainly 20:4-NAE (Table 4). 20:4-NAE accounts for a smaller part of these NAEs usually between 1–10%, again except for the uterus. Not all studies referred to in Table 4 have reported on rapid freezing of the tissue before analysis, and some of the higher values in the table may therefore to some degree be due to an artificial accumulation during sampling and preparation of the tissues (Kempe et al., 1996). While NAPE is a phospholipid found in membranes within the cells, NAEs can exit the cells (Di Marzo et al., 1994; Hansen et al., 1995) and probably bind to proteins in the aqueous environment. Thus, NAEs including 20:4-NAE have also been found in rat plasma (Giuffrida and Piomelli, 1998) and in human cerebrospinal fluid (Leweke et al., 1999). Furthermore, 20:4-NAE has been found in brain extracellular compartment by using a microdialysis probe (Giuffrida et al., 1999; Walker et al., 1999).

The composition of esterified fatty acids in the tissue is reflected in the NAE species of that particular tissue, e.g. brain and retina have relatively high levels of docosahexaenoic acid and in these tissues 22:6(n-3)-NAE has been found (Sugiura et al., 1996b; Bisogno et al., 1999b), while rat testis has relatively high levels of 22:5(n-6) and contains 22:5(n-6)-NAE (Kondo et al., 1998). In studies where both NAE and NAPE levels in tissues have been reported, it is noteworthy that NAPE levels are roughly an order of magnitude larger than NAE levels, except for the uterus.

5. Stimulated in vivo formation of NAPEs and NAEs

There have been different reports on increases in in vivo levels of NAPEs as well as NAEs, i.e. in ischemic canine hearts (Epps et al., 1979, 1980), in inflamed rat testis (Kondo et al., 1998), and in uterus of pregnant mice (Schmid et al., 1997). In the former two cases, increased formation was a response to toxic stimuli, in agreement with our experience that toxic stimuli will increase NAPE and NAE formation in primary neocortical neurons in culture (Hansen et al., 1995, 1997, 1999b,c).

In ischemic canine heart, NAPEs accumulated to 4–6% of lipid phosphorus while NAEs increased to a level of 400–500 nmol/g tissue 24 h after coronary artery ligation (Epps et al., 1979, 1980). Only the study of $CdCl_2$-induced inflammation of rat testis included an analysis of the *N*-acyl composition of NAPE before and during the injury. It was found that the composition changed, i.e. all *N*-acyl species increased (in pmol/g tissue) except for 20:4-NAPE and 22:5(n-6)-NAPE, which were constant or slightly decreasing and thus fell in percentage of total NAPE species. At the same time, total NAE species accumulated progressively up to 25-fold 9 h after $CdCl_2$-injection. However, 18:2-NAE, 20:4-NAE and 22:5(n-6)-NAE accumulated more slowly, e.g. 20:4-NAE

only accumulated 5-fold to around 2.5 nmol/g tissue within the same time period (Kondo et al., 1998). In murine uterus, total NAPE levels seem to be stimulated in relation to pregnancy, with a 6-fold increase at the embryo implant sites at day 7 of pregnancy relative to the level at day 1 of pregnancy. The *N*-acyl composition of NAPE in murine uterus resembles the one seen in other tissues, but upon pregnancy (day 7) it is changed at the implant site (2-fold higher percentage of 18:0-NAPE), and at the interimplant sites (3-fold higher percentage of 20:4-NAPE). There is a corresponding 3-fold increase in percentage of 18:0-NAE at the implant site at day 7 of pregnancy. Total NAE levels are overall approximately 10-fold higher than total NAPE levels primarily due to 20:4-NAE. Total NAE levels increase 4-fold at the embryo interimplant sites at day 7 relative to the level at day 1 of pregnancy. 20:4-NAE accounts for 81–96% of total NAEs throughout the different stages of early pregnancy (Schmid et al., 1997). It was therefore suggested that formation of 20:4-NAE may be hormonally stimulated in relation to early pregnancy, probably via stimulation of an anandamide synthase (Paria et al., 1996; Schmid et al., 1997). Such an anandamide synthase activity, which is not inhibited by the well-known fatty acid amidohydrolase inhibitor PMSF (Deutsch and Chin, 1993), has only been reported in murine uterus (Paria et al., 1996).

It could be speculated that 20:4-NAE (and 20:4-NAPE) during tissue injury would increase more than other NAEs (and NAPEs) due to the fact that arachidonic acid is more or less selectively released during tissue injury (Deutsch et al., 1997b). Such a high level of arachidonic acid may promote its incorporation into the *sn*-1 position of phospholipids (Chilton et al., 1996; Kuwae et al., 1997) that serves as donor substrates for NAPE formation. However, this was not the case in $CdCl_2$-induced inflammation in rat testis (Kondo et al., 1998). Preincubation of neocortical neurons in culture with 50 μM arachidonic acid increased the level of 20:4-NAPE 3-fold, from 0.1–0.3% of total NAPE species (Hansen et al., 2000). This suggests that the level of the anandamide precursor can only be modestly increased by high concentrations of free arachidonic acid.

In cerebrospinal fluid of schizophrenic patients, the levels of 20:4-NAE and 16:0-NAE were increased about 2-fold relative to that of controls (Leweke et al., 1999), but the stimulus for this increase is not known. Release of 20:4-NAE into the extracellular fluid of rat brain periaqueductal gray was increased 1.4-fold upon electrical stimulation of the same brain area, as well as upon subcutaneous injection of formalin into the hind paws. The electrical stimulation produced profound analgesia while injection of formalin caused prolonged pain behavior. 20:4-NAE was in both cases suggested to mediate an analgesic effect via CB1 receptor activation (Walker et al., 1999). No other NAEs were, however, analyzed in this study. Levels of 20:4-NAE in rat dorsal striatum, as measured in microdialysis perfusion buffer, have been shown to be selectively increased by activation of dopamine D_2-receptors (Giuffrida et al., 1999). Since there was no comparable increase in 16:0-NAE and 18:1-NAE upon activation of dopamine D_2-receptors, one may speculate that 20:4-NAE under these circumstances is formed by another biosynthetic pathway than the one depicted in Fig. 1, or that 20:4-NAE is formed along with other NAEs, but only 20:4-NAE is released from the cells. A carrier-mediated cellular uptake system with a selectivity for 20:4-NAE as opposed to 16:0-NAE has been described (Beltramo et al., 1997; Bisogno et al., 1997; Hillard et al., 1997; Piomelli et al., 1999). Whether such a system also accounts for a selective release of 20:4-NAE is at present not known.

Chronic treatment of adult rats with Δ^9-tetrahydrocannabinol has been shown to down regulate CB1 receptors. In these same rats anandamide levels in the brain was largely unaffected except in the limbic area where it was increased 4 fold. The level of the anandamide precursor 20:4-NAPE was not changed. The significance of this finding is at present unclear (Di Marzo et al., 2000). Chronic ethanol exposure of neuroblastoma cells has been reported to increase the levels of ^{3}H-20:4-NAE and ^{3}H-20:4-NAPE in cells prelabelled with the levels of ^{3}H-arachidonate (Basavarajappa and Hungund, 1999). We have not been able to see such an effect in our neocortical neurons (unpublished) and it is not known how alcohol abuse will effect NAPE and NAE levels in vivo.

6. Possible cytoprotective functions of NAPEs

The precursor for NAPEs, phosphatidylethanolamine, is characterized as a phospholipid favoring hexagonal H-II phase structures (Kinnunen et al., 1994), thereby being membrane-destabilizing. However, conversion of phosphatidylethanolamine to NAPEs with saturated *N*-acyl groups results in a phospholipid that in in vitro experiments have been found to form lamellar structures, thereby being membrane-stabilizing (Newman et al., 1986; Akoka et al., 1988; Domingo et al., 1994). NAPEs with saturated *N*-acyl groups account for the major part of NAPE species seen in the studies of stimulated in vivo formation of NAPEs (Epps et al., 1980; Schmid et al., 1997; Kondo et al., 1998). Whether such a membrane-stabilizing effect can be demonstrated in vivo is at present unknown. As NAPE is a precursor for NAE, and as the latter may have cytoprotective effects, the increased formation of NAPEs in inflamed testis (Kondo et al., 1998) and in ischemic canine heart (Epps et al., 1980) can be seen as a response for generating cytoprotective NAEs.

7. Possible cytoprotective functions of NAEs

By the finding of NAEs in ischemic canine heart tissue (Epps et al., 1979), it was speculated whether NAEs could have cytoprotective functions. Cytoprotective functions can be manifested in several ways, e.g. by inhibiting the process of necrosis in the individual injured cell, by stimulating the injured cell and/or adjacent cells to activate apoptotic mechanisms in order to stop spreading of necrosis, and by inhibiting release of mediators that promote necrosis and inflammation. Different species of NAE may promote putative cytoprotective effects by mechanisms involving cannabinoid receptors, e.g. 20:4-NAE and other highly unsaturated NAEs, or by non-cannabinoid-receptor-mediated mechanisms, e.g. saturated and monounsaturated NAEs.

At the level of the individual injured cell a variety of saturated and monounsaturated NAEs may stabilize injured mitochondria, thereby preventing Ca^{2+} leakage (Epps et al., 1982), and monounsaturated and saturated NAEs have also been reported to have antioxidant properties (Parinandi and Schmid, 1988; Gulaya et al., 1998). Mitochondrial dysfunction is an important aspect of glutamate-induced neurotoxicity (Atlante et al., 1999; Castilho et al., 1999). 16:0-NAE has been reported (Skaper et al., 1996a) to protect cerebellar granule neurons in culture against excitotoxic death by an unknown mechanism in a narrow time window following glutamate exposure. Whether effects on mitochondria or oxidative stress mechanisms mediate this protection is at present not known. Furthermore, 18:1-NAE is a well-known ceramidase inhibitor (Sugita et al., 1975; Meacci et al., 1996), and exogenous 18:1-NAE has by increasing the endogenous level of ceramide been shown to facilitate induction of apoptosis in a variety of cells in culture (Wiesner and Dawson, 1996a,b; Wiesner et al., 1997; Huwiler et al., 1999; Ping and Barrett, 1999; Spinedi et al., 1999). Since NAE can leave the cell where it is generated and be taken up by adjacent cells, NAE formation in an injured cell may facilitate induction of apoptosis in the injured cell itself, as well as in neighboring cells thereby inhibiting spreading of a necrotic event.

During ischemia in the brain, free fatty acids are released in an excessive amount which may be harmful (Deutsch et al., 1997b; Homayoun et al., 1997). If these fatty acids could be degraded by increased ketogenesis in astrocytes, one could interpret this as a cytoprotective mechanism. Furthermore, such ketone bodies may be an easily available fuel for injured neurons. Stimulated ketogenesis in astrocytes may be achieved by activation of carnitine palmitoyltransferase I by increased intracellular levels of ceramide (Blázquez et al., 1999). Ceramide levels in astrocytes can be increased via a CB1-mediated activation of a sphingomyelinase (Blázquez et al., 1999). If 18:1-NAE is formed along with 20:4-NAE during brain ischemia, both types of NAEs may stimulate ketogenesis in astrocytes via ceramide formation, i.e. 20:4-NAE via a CB1-mediated stimulation of sphingomyelinase, and 18:1-NAE via a direct inhibition of ceramidase. Activation of presynaptic CB1 receptors has been shown to

inhibit glutamate release thereby protecting neurons from excitotoxicity during ischemia in vitro and in vivo (Venance et al., 1997; Shen and Thayer 1998; Ameri et al., 1999; Nagayama et al., 1999; Shen and Thayer, 1999).

During cellular necrosis, there may be a release of inflammatory mediators. Saturated NAEs have been demonstrated to inhibit release of inflammatory mediators from activated mast cells, possibly via activation of a CB2-like receptor (Aloe et al., 1993; Facci et al., 1995; Mazzari et al., 1996). Activation of cerebral mast cells may be involved in neurodegenerative diseases (Eikelenboom et al., 1994), and their activation may promote astrocytes to produce neurotoxic nitric oxide, a mechanism that may be inhibited by the effect of saturated NAEs on mast cell activation (Skaper et al., 1996b). Furthermore, it has been reported that 20:4-NAE inhibits the production of tumor necrosis factor-α and nitric oxide from endotoxin-stimulated mouse cortical astrocytes (Molina-Holgado et al., 1997), as well as cytokine mRNA-expression in endotoxin-exposed microglial cells in culture, and this latter effect was not mediated by cannabinoid receptors (Puffenbarger et al., 2000). Whether the former effect is mediated by cannabinoid receptors is at present not known.

In conclusion, a number of putative cytoprotective mechanisms of NAPEs and NAEs have been obtained by in vitro experiments but very few in vivo data are available. However, it seems plausible that some of these mechanisms may be functioning in vivo, although many more data are needed in order to determine the exact role of NAPEs and NAEs in relation to cell injury.

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