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Review

N-Acylethanolamines in human reproductive fluids

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Abstract

N-Acylethanolamines (NAEs) are an important family of lipid-signaling molecules. Arachidonylethanolamide (anandamide) (AEA), palmitoylethanolamide (PEA), and oleoylethanolamide (OEA) are co-produced from similar phospholipid precursors when neurons are stimulated. AEA is an endogenous agonist (endocannabinoid) for cannabinoid receptors. It binds with higher affinity to type CB1 than to type CB2 cannabinoid receptors. PEA does not bind to CB1, while the hypothesis that it reacts with putative CB2-like receptors has been questioned. OEA does not activate currently known cannabinoid receptors, but it mimics the effects of AEA and cannabinoids in reducing the fertilizing capacity of sea urchin sperm. OEA and PEA also act as entourage compounds by inhibiting the hydrolysis of AEA by fatty acid amide hydrolase. Cannabinoid receptors and/or AEA are present in mammalian reproductive organs including the testis, epididymis, prostate, ovary, uterus, sperm, preimplantation embryo and placenta, as well as prostatic and mammary carcinomas. We now report that analysis by high-performance liquid chromatography/mass spectrometry (HPLC/MS) shows the presence of AEA, PEA, and OEA in human seminal plasma, mid-cycle oviductal fluid, follicular fluid, amniotic fluid, milk, and fluids from malignant ovarian cysts. Previous studies showed that AEA-signaling via cannabinoid receptors regulates capacitation and fertilizing potential of human sperm, early embryonic development and blastocyst implantation into the uterine mucosa of rodents, as well as proliferation of human mammary and prostatic carcinomas. Current results imply that NAEs also may modulate follicular maturation and ovulation, normal and pathological ovarian function, placental and fetal physiology, lactation, infant physiology, and behavior. Collectively, these findings suggest that NAEs in human reproductive fluids may help regulate multiple physiological and pathological processes in the reproductive system, and imply that exogenous cannabinoids delivered by marijuana smoke might impact these processes. This study has potential medical and public policy ramifications

Abbreviations: NAEs, *N*-Acylethanolamines; AEA, arachidonylethanolamide (anandamide); PEA, palmitoylethanolamide; OEA, oleoylethanolamide; 2-AG, 2-arachidonoyl glycerol; HPLC/MS, high-performance liquid chromatography/mass spectroscopy; THC, (–) Δ^9 -tetrahydrocannabinol; HA, hyperactivated sperm motility.

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because of the incidence of marijuana abuse by adolescents and adults in our society, previously documented reproductive effects of marijuana, and the ongoing debate about medicinal use of marijuana and cannabinoids.

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Keywords: Eicosanoids; Endocannabinoids; Reproduction; Fertilization; Seminal plasma; Oviductal fluid; Follicular fluid; Ovarian cysts; Amniotic fluid; Milk

1. Introduction

N-Acylethanolamines (NAEs) are lipid-signaling molecules that are widely distributed in plant, invertebrate, and mammalian tissues (Bachur et al., 1965; Berdyshev et al., 1996; Di Tomaso et al., 1996; Bisogno et al., 1997; Bezuglov et al., 1998; Piomelli et al., 1998; Berdyshev, 1999, 2000; Chapman, 2000; Hansen et al., 2000; Schmid, 2000; Stella and Piomelli, 2001). Arachidonylethanolamide (AEA), palmitoylethanolamide (PEA), and oleoylethanolamide (OEA) are enzymatically released together from membrane phospholipid precursors when cells are stimulated by depolarizing agents, neurotransmitters, and hormones (Di Marzo et al., 1994; Bisogno et al., 1998; Piomelli et al., 1998; Berdyshev, 2000; De Petrocellis et al., 2000; Schmid, 2000; Stella and Piomelli, 2001). Released NAEs are quickly eliminated by fatty acid amide hydrolase, indicative of possible roles in cell-signaling (Deutsch and Chin, 1993; Piomelli et al., 1998; Ueda et al., 2000; Giuffrida et al., 2001a,b,c). AEA, also known as anandamide, was the first endogenous agonist for cannabinoid receptors to be identified (Devane et al., 1992). It mimics many of the pharmacological effects of (–) Δ^9 -tetrahydrocannabinol (THC), the primary psychoactive substance in marijuana (Mechoulam and Hanus, 2000). Related NAEs such as dihomogamma-linolenylethanolamide, 7,10,13,16-docosatetraenylethanolamide, mead ethanolamide, etc. have been identified as endogenous agonists for cannabinoid receptors (Hanus et al., 1993; Priller et al., 1995; Mechoulam and Hanus, 2000; Schmid, 2000; Piomelli et al., 1998, 2000; Salzet et al., 2000). 2-Arachidonoylglycerol (2-AG) is also a potent endogenous ligand for cannabinoid receptors (Mechoulam et al., 1995; Stella et al., 1997; Mechoulam and Hanus, 2001; Sugiura et al., 1998; Sugiura and Waku, 2000). Since these NAEs and

2-AG are ligands for cannabinoid receptors, they are collectively known as endocannabinoids. It is possible that additional endocannabinoids remain to be discovered.

Two subtypes of cannabinoid receptors have been cloned and characterized; CB1 originally found in brain and CB2 originally found in spleen cells (Matsuda et al., 1990; Gerard et al., 1991; Munro et al., 1993; Matsuda, 1997). Additional cannabinoid receptor subtypes may exist (Pertwee, 1999; Kunos and Batkai, 2001). AEA and 2-AG have higher affinity for CB1 than for CB2 receptors (Khanolkar et al., 1996; Shohami et al., 1996), but differ in their efficacy to elicit a signal via these receptors (Breivogel and Childers, 2000; Gonsiorek et al., 2000; Hillard, 2000). PEA can act as an entourage compound by protecting other NAEs from enzymatic hydrolysis (Mechoulam et al., 1998; Lambert and Di Marzo, 1999; Lambert et al., 1999; Di Marzo et al., 2001b; Jonsson et al., 2001). PEA is a potent anti-inflammatory and neuroprotective agent (Kuehl et al., 1957; Schmid et al., 1990; Facci et al., 1995; Mazzari et al., 1996; Skaper et al., 1996; Conti et al., 2002). It was suggested that PEA elicits these effects via uncharacterized CB2-like receptors (Facci et al., 1995; Skaper et al., 1996; Calignano et al., 1998), but this hypothesis remains controversial. CB2 mRNA, but not CB1 mRNA, is expressed by RBL-2H3 (rat peritoneal basophilic leukemia) and rat peritoneal mast cells (Facci et al., 1995). Both PEA and AEA were reported to inhibit specific binding of the potent cannabinoid agonist [3 H]WIN55,212-2 to CB2 in membranes isolated from rat mast cells, IC_{50} 1.0 ± 0.6 and 33 ± 29 nM, respectively. PEA down-modulates mast cell activation to reduce inflammatory edema and pain (Facci et al., 1995; Mazzari et al., 1996; Conti et al., 2002). Cerebellar granule cells express mRNA for CB1 and CB2 (Skaper et al., 1996). Scatchard

plot of the specific binding of [³H]WIN55,212-2 to cerebellar granule cell membranes suggests the presence of two binding sites (K_{D1} 1.6 ± 1.0 nM and K_{D2} 11.0 ± 1.5 nM). The authors stated that "...AEA fully displaced bound radioligand, while PEA seemed to be only partially effective (data not shown)..." (Skaper et al., 1996). PEA, but not AEA, protects cerebellar granule neurons from glutamate-induced apoptosis. The anti-inflammatory and neuroprotective effects of PEA are antagonized by AEA (Facci et al., 1995; Skaper et al., 1996). However, other investigators found that PEA does not bind effectively to rat spleen cells and to cloned human CB2 (Sheskin et al., 1997; Lambert et al., 1999). Nevertheless, the anti-inflammatory and analgesic actions of PEA are blocked by the CB2-specific antagonist SR144528 (Piomelli et al., 1998; Conti et al., 2002). These findings suggest the possible existence of another target for both PEA and SR144528, different from currently known cannabinoid receptors (Calignano et al., 1998). Additional research is required to resolve these questions. OEA does not react with CB1 or CB2 (Bezuglov et al., 1998; Giuffrida et al., 2000), and its anorexic action in rats is not blocked by CB1- or CB2-specific antagonists (Rodriguez de Fonseca et al., 2001).

Cannabinoid receptors are linked to multiple inhibitory and stimulatory guanine nucleotide binding proteins, regulate signal transduction mechanisms in cells, modulate receptors for other neurotransmitters (acetylcholine, 5HT, dopamine, serotonin, opioids, etc.), and are widely distributed in neural and non-neural cells of the body (Berdyshv, 2000; Chaperon and Thiebot, 1999; Devane et al., 1988; Gerard et al., 1991; Munro et al., 1993; Galiegue et al., 1995; Deutsch et al., 1997; Glass and Felder, 1997; Matsuda, 1997; Kenney et al., 1999; Di Marzo et al., 1998; Lambert and Di Marzo, 1999; Paria and Dey, 2000; Hillard, 2000; Schmid, 2000; Schuel et al., 1999). With respect to reproductive organs, cannabinoid receptors and/or endocannabinoids have been detected in the pituitary gland, testis, Leydig cells, epididymis, prostate, sperm cells, ovary, uterus, oviduct, preimplantation embryo, placenta, embryo, fetus and neonates, as well as in prostatic and mammary carcinomas (Gerard et al., 1991; Galiegue et al.,

1995; Hansen et al., 2000; Koga et al., 1997; Buckley et al., 1998; Kenney et al., 1999; De Petrocellis et al., 2000; Sugiura and Waku, 2000; Pagotto et al., 2001; Wenger et al., 2001; Habayeb et al., 2002; Schuel et al., 2002). These factors may account, in part, for the effects of marijuana and THC on secretion of gonadotrophic hormones and gonadal steroids, sperm production, ovulation, mating behavior, sperm capacitation, fertilization, early embryonic development; implantation of blastocysts into the uterine endometrium, placental functions, fetal growth, number of pregnancies carried to term, postnatal development, tumor growth, etc. (Powell and Fuller, 1983; Maykut, 1985; Smith and Asch, 1987; Astley and Little, 1990; Murphy et al., 1994; Maccarrone et al., 2000; Fried and Smith, 2001; Paria and Dey, 2000; Mani et al., 2001; Paria et al., 2001; Schuel et al., 2002). Collectively, these observations suggest that endocannabinoids may be normal physiological constituents of human reproductive fluids.

Here we present evidence for the presence of AEA, PEA, and OEA in human seminal plasma, mid-cycle oviductal fluid, follicular fluid, amniotic fluid, milk, and fluids from malignant ovarian cysts. Preliminary accounts of this study have been reported previously (Giuffrida et al., 2001a,b).

2. Materials and methods

Protocols involving human subjects were performed in accordance with policies of the Institutional Review Board of the University at Buffalo and the University of California, Irvine.

2.1. Biological materials

Human semen samples were collected from healthy volunteer donors of proven fertility. Liquefied semen was centrifuged at $300 \times g$ for 5 min. The supernatant (seminal plasma) was collected by aspiration. Mid-cycle human oviductal fluid was obtained from volunteers during elective surgery for tubal ligation (Lippes et al., 1972). Oviductal fluids were also collected from rabbits and goats at the time of ovulation. Human follicular fluid was retained following oocyte

aspiration from women undergoing in vitro fertilization. Amniotic fluid was retained from women undergoing elective removal of conceptus. Fluids from malignant ovarian cysts were retained from women undergoing elective ovariectomy. Milk was collected from volunteer nursing mothers using breast pumps. Frozen samples were stored at -20°C .

2.2. High-performance liquid chromatography/mass spectrometry

The presence of NAEs in reproductive fluids was determined by high-performance liquid chromatography/mass spectrometry (HPLC/MS; Giuffrida et al., 2000). Thawed samples were centrifuged at $16,000 \times g$ for 10 min at room temperature. The supernatants were recovered and spiked with [$^2\text{H}_4$]-labeled AEA, PEA, and OEA (500 pmol each) to act as internal standards. Proteins were precipitated with cold acetone and removed by centrifugation at $1000 \times g$ for 10 min. Residual acetone in supernatants was evaporated under a stream of nitrogen. Lipids contained in the supernatants were extracted with methanol/chloroform (1:2, v/v). Chloroform phases were recovered, evaporated to dryness under N_2 , reconstituted in a mixture of chloroform/methanol (1:3, 80 μl) and injected into a HPLC/MS (HP 1100 Series, Hewlett Packard) equipped with a Hewlett Packard octadecyl-silica Hypersil column (100 mm \times 4.6 mm i.d., 5 μm). Fractionation of NAEs was carried out in reversed-phase using a gradient of methanol (B) in water (A) (25% A, 75% B for 2 min; 15% A, 85% B for 3 min; 5% A, 95% B for 20 min; 100% B for 5 min). Analyses were performed with an electrospray ion source set in the positive ionization mode. Quantitation of NAEs was carried out by isotope dilution, monitoring diagnostic ions (protonated molecule, $[\text{M}+\text{H}]^+$, and sodium adducts of the molecular ions, $[\text{M}+\text{Na}]^+$) in the selected ion monitoring mode.

2.3. Statistical analysis

Significance of data was evaluated by Student's *t*-test (Snedecor and Cochran, 1980). Data are

presented as mean values \pm S.E.M. for *N* number of trials.

3. Results and discussion

3.1. Reproductive tract fluids

Freshly ejaculated sperm from human or other mammals, bathed in male secretions comprising the seminal plasma, are not yet capable of fertilizing an egg (Yanagimachi, 1994). Sperm acquire the capacity to fertilize eggs following removal from seminal plasma and exposure for several hours to female reproductive tract fluids in vivo, or by incubation in appropriate culture medium in vitro (Burkman, 1990, 1995; Kopf et al., 1999; Meizel, 1985, 1997; Yanagimachi, 1994). "Capacitated" sperm exhibit vigorous hyperactivated motility required for fertilization, and can undergo physiological acrosome reactions at the egg's surface (Burkman, 1990, 1995; Wassarman, 1987; Yanagimachi, 1994). The acrosome is a secretory granule in the anterior region of the sperm head. Exocytosis of the acrosomal granule (acrosome reaction) is a ligand-stimulated event that enables sperm to penetrate the egg's investments (cumulus matrix and zona pellucida), and to fuse with the egg's plasma membrane during fertilization (Wassarman, 1987; Yanagimachi, 1994; Darszon et al., 1999). Mechanisms that modulate these processes within the female reproductive tract are poorly understood.

Studies with sea urchin gametes provided the first evidence that cannabinoids could directly affect fertilization (Schuel et al., 1987, 1991; Berdyshev, 1999). This is not surprising since echinoderm gametes have been an ideal model system to examine the effects of psychoactive drugs (morphine, cocaine, nicotine, alcohol, etc.) on fertilization since the late 19th century (Hertwig and Hertwig, 1887; Clark, 1936; Schuel, 1984). The potent cannabinoid agonist [^3H]CP-55,940 binds with high affinity (K_D 5.16 ± 1.02 nM) to sea urchin sperm (Chang et al., 1993). THC, other cannabinoids, and AEA inhibit fertilization by acting on sperm, not on eggs (Schuel et al., 1987, 1994). OEA and linolenylethanolamide, but not

PEA, mimic the effects AEA and THC on sperm fertility (Berdyshev, 1999). While AEA and THC enhance sea urchin sperm motility, they inhibit fertilization by blocking the egg jelly-stimulated acrosome reaction (Chang and Schuel, 1991; Schuel et al., 1991, 1994, 1999). The rank order of potency to inhibit specific binding of [³H]CP-55,940 to sperm and to inhibit the egg jelly-induced acrosome reaction is CP-55,940 > THC > (+)THC (Chang et al., 1993). In mammals, the acrosome reaction is stimulated by a specific glycoprotein (ZP3) in the egg's zona pelucida (Wassarman, 1987; Van Duin et al., 1994). The inhibitory effects of cannabinoid agonists on the acrosome reaction and sperm fertilizing capacity in sea urchins are reversible, and may be mediated by affecting signal transduction processes, e.g. opening of Ca²⁺ and K⁺ channels, activation of phospholipase A₂, etc. (Chang et al., 1991; Schuel et al., 1991, 1994, 1999). Sea urchin eggs have the capacity to produce AEA, PEA, and stearoylethanolamide during fertilization (Bisogno et al., 1997). Together, these results indicated that sea urchin eggs may release AEA after activation by the fertilizing sperm, and that released AEA reacts with sperm cannabinoid receptors to prevent other sperm in the vicinity from undergoing the acrosome reaction, thereby helping to prevent polyspermic fertilization (Schuel et al., 1994, 1999). This AEA-mediated regulatory process resembles those operating in mammalian brain where retrograde AEA and 2-AG signals from depolarized postsynaptic neurons inhibit neurotransmitter release at excitatory synapses (Elphick and Egertová, 2001; Wilson and Nicoll, 2001).

Transcripts of human brain CB1 are expressed in human testis (Gerard et al., 1991; Galiegue et al., 1995). This finding, together with our discovery of functional cannabinoid receptors in sea urchin sperm, led us to postulate that human sperm might contain cannabinoid receptors (Chang et al., 1993; Schuel et al., 1994). Subsequent studies on whole organs showed that AEA is synthesized in rodent testis, oviduct, and uterus (Paria et al., 1995; Sugiura et al., 1996; Schmid et al., 1997; Paria and Dey, 2000). The mouse uterus contains higher levels of AEA than any mammalian organ including the brain (Schmid et al.,

1997). Recent radioligand binding experiments using the potent cannabinoid agonist [³H]CP-55,940 (K_D 9.71 ± 1.04 nM) confirmed our hypothesis by showing that human sperm contain functional cannabinoid receptors similar to those in sea urchin sperm and mammalian somatic tissues (Schuel et al., 2002). Together, these findings suggested that NAEs may be normal physiological constituents of fluids within human reproductive tracts.

We detected AEA, OEA, and PEA in human seminal plasma, mid-cycle oviductal fluid, and follicular fluid analyzed by HPLC/MS (Table 1). The levels of PEA and OEA were significantly higher than AEA in seminal plasma, oviductal fluid, and follicular fluids (*P* < 0.005 to < 0.0001). OEA was the most abundant NAE in human follicular fluid (*P* < 0.005). It is interesting to note that these results are very different from those obtained with the whole mouse uterus, where AEA represents up to 95% of total NAEs (Schmid et al., 1997). Furthermore, AEA, PEA, and OEA were also detected in oviductal fluids collected from rabbits and goats at the time of ovulation (data not shown). NAEs in human follicular fluid may be produced by granulosa in ovarian follicles and by granulosa cells in the cumulus matrix surrounding ovulated eggs, as previously shown for progesterone and prostaglandin E (Meizel et al., 1990; Schaefer et al., 1998). Since cannabinoid receptors are present in the ovary (Galiegue et al., 1995) and THC inhibits cAMP accumulation by cultured rat granulosa cells (Treinen et al., 1993), NAE-signaling may help regulate follicular maturation and development. These factors may account, in part, for the adverse effects of marijuana and cannabinoids on ovulation (Powell and Fuller, 1983).

Sperm are sequentially exposed to seminal plasma, oviductal fluid, follicular fluid, and secretions of granulosa cells in the cumulus matrix surrounding ovulated eggs as they move from the vagina to the site of fertilization in the oviductal ampulla (Burkman, 1990; Mortimer, 1995; Yanagimachi, 1994). The presence of NAEs in these locations and the detection of cannabinoid receptors in human sperm (Schuel et al., 2002) imply that NAE-signaling may regulate sperm capacita-

Table 1
NAEs in human reproductive tract fluids

Source	AEA	PEA	OEA
Seminal plasma ($N = 15$)	12.1 ± 2.1	31.5 ± 7.3 ($P < 0.0001^*$)	32.9 ± 4.7 ($P < 0.0001^{**}$) ($P > 0.3^{***}$)
Mid-cycle oviductal fluid ($N = 15$)	10.7 ± 2.5	30.4 ± 6.9 ($P < 0.005^*$)	36.9 ± 7.5 ($P < 0.001^{**}$) ($P > 0.05^{***}$)
Follicular fluid ($N = 9$)	2.9 ± 0.9	11.3 ± 1.3 ($P = 0.0001^*$)	19.3 ± 2.9 ($P < 0.0001^{**}$) ($P < 0.005^{***}$)

Values are given in nM.

* AEA vs. PEA.

** AEA vs. OEA.

*** PEA vs. OEA.

tion and fertilizing potential within human reproductive tracts.

While the biological effects of PEA and OEA in reproductive tract fluids remain to be determined, evidence has been obtained suggesting that AEA-signaling via sperm cannabinoid receptors modulates sperm functions required for fertilization (Schuel et al., 2002). AEA is rapidly eliminated by carrier-mediated uptake into somatic cells (Beltramo et al., 1997), and hydrolyzed into arachidonic acid and ethanolamine by fatty acid ethanolamide amidase (Deutsch and Chin, 1993; Ueda et al., 2000; Giuffrida et al., 2001c).

R-Methanandamide (AM-356) is a metabolically stable substitute for AEA (Abadji et al., 1994; Khanolkar et al., 1996). The effects of AM-356 and THC on sperm capacitation and fertilizing potential were examined in vitro (Schuel et al., 2002). AM-356 elicited biphasic effects on the incidence of hyperactivated sperm motility (HA) between 1 and 6 h of culture: at 2.5 nM it inhibited HA, while at 0.25 nM it stimulated HA ($P < 0.05$; ANOVA). These data are consistent with results obtained by Burkman and colleagues on modulation of rabbit sperm HA in the oviduct that pointed to an unknown inhibitory substance in the isthmus which was negated upon 10-fold dilution (Johnson et al., 1981; Burkman et al., 1984). Factors in the isthmic environment provide regulatory control initially inducing sperm quiescence and, at the appropriate time, stimulating vigorous HA motility thus enabling sperm to escape and swim to the ampulla. There are regional differences in composition of fluids within the lumen of bovine and porcine oviducts (Anderson and Killian, 1994; Hunter, 1990). In addition,

bovine isthmic and ampullary fluids produce different effects on motility, acrosome reaction, zona binding, and fertility of bull sperm (Grippio et al., 1995; Topper et al., 1999).

Acquisition of acrosomal competence during capacitation represents an intermediate state preparing sperm to respond to physiological stimuli at the egg's surface (Lee et al., 1987b; Kligman et al., 1991; Kim et al., 2001a,b). This process appears to be correlated with alteration of acrosomal caps of human, bovine, and rodent sperm in vitro, and/or in vivo within the isthmus, ampulla, and cumulus (Hunter et al., 1991; Kligman et al., 1991; Kim et al., 2001a,b; Lee et al., 1987a,b; Nolan et al., 1992; Yanagimachi and Phillips, 1984). Here, membrane destabilization and localized transient fusion events expose acrosomal matrix proteins required for sperm-zona binding and acrosomal exocytosis. Both AM-356 and THC inhibited morphological alterations over acrosomal caps between 2 and 6 h (IC_{50} 5.9 ± 0.6 pM and 3.5 ± 1.5 nM, respectively), suggesting that they modulate this aspect of capacitation (Schuel et al., 2002).

Previous studies showed that AEA and cannabinoids reduce the fertilizing capacity of sea urchin sperm (Schuel et al., 1987, 1991, 1994; Chang et al., 1993; Berdyshev, 1999). Ethical concerns preclude similar experiments on live human eggs. Nevertheless, fertilizing potential of human sperm can be determined on the basis of tight binding of capacitated sperm to the zona pellucida in the hemizona assay (Burkman et al., 1988; Burkman, 1995; Franken et al., 1989). This assay is highly predictive of sperm fertility during human in vitro fertilization. In this assay, one-half of a bisected zona was inseminated with sperm that had been

capacitated in culture medium containing AM-356, and the matching half was inseminated with sperm incubated in medium containing vehicle to serve as an internal control. Tight binding of sperm was reduced 50% by 1 nM AM-356 under these conditions (Schuel et al., 2002). Our results provide the first evidence that endocannabinoids can directly modulate sperm capacitation and fertilization in human, and imply that exogenous cannabinoids delivered by marijuana smoke might impact these processes.

NAEs in reproductive tract fluids may also act via mechanisms that do not involve sperm cannabinoid receptors. For example, prostaglandins (derived from the seminal vesicles) regulate functions of epithelia and contractility of smooth muscle in the female reproductive tract via receptor-mediated mechanisms (Luke and Coffey, 1994). Further, semen is a potential vehicle for the introduction of infectious organisms into the female. As foreign cells, sperm are vulnerable to immunological attack in the female reproductive tract. Sperm are also very sensitive to damage by reactive oxygen radicals and lipid peroxidation (De Lamirande and Gagnon, 1999). PEA and OEA are potent anti-inflammatory, antioxidant, and antimicrobial agents (Schmid et al., 1990; Berdyshev et al., 1996; Mazzari et al., 1996; Calignano et al., 1998; Conti et al., 2002). These properties are shared with certain cannabinoids (Zimmerman et al., 1991; Hampson et al., 1998; Burstein, 1999; Cabral, 1999; Conti et al., 2002). These findings suggest that NAEs may perform multiple roles in reproductive tract fluids by modulating sperm capacitation, regulating reproductive tract function, protecting against infection, and maintaining sperm viability.

3.2. Amniotic fluid

Marijuana smoke and THC adversely affect fetal growth, development, and the number of pregnancies carried to term (Harbison et al., 1972; Maykut, 1985; Asch and Smith, 1986; Smith and Asch, 1987). Cannabinoid receptors are present in the uterus and early embryo (Paria and Dey, 2000). Localized differences in AEA levels within the uterus determine implantation sites, while

exogenous cannabinoid agonists such as THC and CP-55,940 inhibit embryonic development and implantation. In situ hybridization studies showed that mRNAs for CB1 and CB2 are expressed in the placental cone and smooth muscle of the uterus in rats, rodent embryo and fetus, and term human placenta, as well as in human BeWo choriocarcinoma cells (Buckley et al., 1998; Kenney et al., 1999). Together, these observations implied that human amniotic fluid might contain endocannabinoids.

Here we show that AEA, OEA, and PEA are constituents of human amniotic fluid (Table 2). The biological functions of these NAEs in amniotic fluids remain to be determined. However, previous studies using THC and other cannabinoids suggest functional roles for cannabinoid receptors in placental physiology, embryogenesis, and fetal development. THC crosses the placenta, accumulates in chorionic and amniotic fluids, and all fetal organs (Harbison et al., 1972; Martin et al., 1977; Asch and Smith, 1986; Bailey et al., 1987). Maternal plasma contains significant amounts of 11-nor-9-carboxy THC, a major metabolite of THC, but it is not detected in amniotic fluid or the fetus (Bailey et al., 1987). These data indicate that 11-nor-9-carboxy THC does not cross the placenta, and that the fetus cannot metabolize THC. The placenta of rhesus monkeys chronically exposed to THC during pregnancy shows gross morphological abnormalities and vascular infarctions (Sassenrath et al., 1979). Cannabinoid agonists such as THC and WIN55,212-2 inhibit uptake of amino acids (valine and alpha-amino isobutyric acid) by slices of term human placenta, and inhibit the serotonin transporter in human BeWo choriocarcinoma cells (Fisher et al., 1987; Kenney et al., 1999). These observations indicate that the human placenta is a

Table 2
NAEs in human amniotic fluid

AEA	PEA	OEA	Gestation (weeks)
7.9±4.9	8.8±2.9	10.8±4.7	16.2±0.8

Observed differences in NAE concentrations are not significant: $P > 0.2$ to > 0.9 ; $N = 9$; values are given in nM.

direct target for cannabinoids during pregnancy. Furthermore, the embryo and fetus contain cannabinoid receptors. Indeed, CB1 mRNA is expressed in the neural tube, as well as in the fetal central nervous system, retina, autonomic ganglia, enteric ganglia, thyroid, and adrenal glands (Buckley et al., 1998). CB2 mRNA is expressed exclusively in the liver as early as embryonic stage-E13. The region-specific expression of CB1 and CB2 mRNAs suggests functional roles for these receptors during embryogenesis. Collectively, these findings indicate that endocannabinoid-signaling may directly mediate diverse functions from early embryonic development to birth. All these processes are potential targets for exogenous cannabinoids, which may account for many adverse effects of marijuana and cannabinoids on pregnancy outcome.

3.3. Human milk

Milk provides complete nutritional support for infants. Previous investigators showed that bovine and human milk contain AEA, oleamide, 2-AG, 2-palmitoylglycerol, and linoleoylglycerol (Di Marzo et al., 1998; Fride et al., 2001). Working independently (Giuffrida et al., 2001a,b), we detected AEA, PEA, and OEA in human milk (Table 3). Human milk contains significantly higher levels of OEA than that of AEA and PEA ($P = 0.003$ and $P < 0.04$, respectively).

The presence of AEA, PEA, OEA, 2-AG, and oleamide in milk implies potential effects on nursing infants such as feeding and sleep. These possibilities are suggested by the use of *Cannabis* to stimulate appetite and induce sleep, properties that have been known since ancient times (Abel, 1980; Mechoulam, 1986; Mechoulam and Hanus,

2001; Ahokas, 2002). Recent studies showed that AEA and THC produce similar effects (Holister, 1999; Jamshide and Taylor, 1999; Tassinari et al., 1999; Williams and Kirkham, 1999; Hao et al., 2000; Budney et al., 2001; Di Marzo et al., 2001a; Mechoulam and Fride, 2001). Withdrawal from marijuana smoke or oral THC in human is associated with decreased appetite and sleep (Haney et al., 1999; Budney et al., 2001). The CB1 antagonist SR141716A decreases food uptake and body weight, indicative of regulation by endocannabinoid-signaling (Arnone et al., 1997; Rowland et al., 2001). SR141716A also reduces AEA-induced food intake and sleep (Columbo et al., 1998; Jamshide and Taylor, 1999; Williams and Kirkham, 1999; Murillo-Rodriguez et al., 2001). Administration of OEA to rats causes potent and persistent decrease in food intake (Rodriguez de Fonseca, 2001). PEA also produces anorexic effects but is less potent than OEA. The inhibitory effect of OEA on feeding is not blocked by CB1- and CB2-specific antagonists (SR141716 and SR144528, respectively), suggesting that it does not act via currently known cannabinoid receptors. Oleamide is a sleep inducing agent (Cravatt et al., 1995), is an effective appetite suppressant in rats (Rodriguez de Fonseca et al., 2001), exhibits some cannabimimetic activity, and binds with low affinity to CB1 and CB2 (Lambert and Di Marzo, 1999). SR141716A blocks the soporific action of oleamide suggesting a possible role for cannabinergic pathways (Mendelson and Basile, 2001). The physiological interactions of NAEs, 2-AG, and oleamide appear to be quite complex and are poorly understood. Since NAEs and oleamide are substrates for fatty acid amide hydrolase, substances such as PEA and OEA may function as entourage compounds that enhance the actions of

Table 3
NAEs in human milk

AEA	PEA	OEA	Lactation (days)
5.1±2.1	23.4±7.2 ($P < 0.02^*$)	66.7±18.4 ($P = 0.003^{**}$) ($P < 0.04^{***}$)	110±32.3

$N = 10$; values are given in nM.

* AEA vs. PEA.

** AEA vs. OEA.

*** PEA vs. OEA.

endocannabinoids by preventing their inactivation (Mechoulam et al., 1998; Lambert and Di Marzo, 1999; Di Marzo et al., 2001b). Furthermore, endocannabinoids and oleamide may affect appetite and sleep indirectly via interactions with other signaling systems, e.g. leptin, dopamine, serotonin acetylcholine, etc. (Chaperon and Thiebot, 1999; Kenney et al., 1999; Lambert and Di Marzo, 1999; Di Marzo et al., 2001a).

Endocannabinoid-signaling has a critical role in nursing (Fride et al., 2001). Injection of SR141716A into newly born mouse pups produces devastating effects on suckling and milk ingestion, followed by death within 4–8 days. Oral administration of AEA and 2-AG to mice can produce psychotropic effects, suggesting that these endocannabinoids reach the brain (Di Marzo et al., 1998). Co-administration of THC or 2-AG protects pups from the adverse effects of SR141716 on suckling and survival, suggesting the involvement of CB1 receptors (Fride et al., 2001). These findings indicate that milk-derived endocannabinoids may influence infant physiology and behavior, a notion supported by common parental experience that nursing babies get sleepy as they become satiated.

Studies on human, monkeys, and rodents showed that THC administered to nursing mothers accumulates in milk, and is transferred to their offspring (Chao et al., 1976; Dalterio and Bartke, 1979; Frischknecht et al., 1980; Perez-Reyes and Wall, 1982; Asch and Smith, 1986; Astley and Little, 1990). Dairy animals, grazing in regions where *Cannabis* is part of the natural vegetation, produce milk contaminated with cannabinoids that can be transferred to human infants (Ahmad and Ahmad, 1990). Postnatal growth, motor development, and behavior are adversely affected in babies fed THC-contaminated milk (Dalterio et al., 1984; Frischknecht et al., 1980; Astley and Little, 1990). Furthermore, THC inhibits suckling-induced milk ejection in lactating rats (Tyrey and Murphy, 1988). Collectively, these observations suggest that THC in milk can directly affect newborns, and that it also can produce secondary effects in infants by reducing maternal milk availability.

3.4. Malignant ovarian cysts

NAEs and cannabinoids inhibit proliferation of human prostatic and mammary carcinoma, as well as tumors of non-reproductive organs (De Petrocellis et al., 2000; Di Marzo et al., 2001b). THC induces apoptosis in glioma and prostatic carcinoma cells, by mechanisms that do not depend on currently known cannabinoid receptors (Ruiz et al., 1999; Sanchez et al., 1998). Significantly, the activity of the enzyme responsible for removal of NAEs (fatty acid amide hydrolase) is fivefold higher in human adenocarcinoma than in normal epithelial cells in a healthy uterus (Maccarrone et al., 2000). These results imply that NAE-signaling normally regulates cell division, differentiation, and survival. We detected AEA, OEA, and PEA in fluids collected from human malignant ovarian cysts (Table 4). The concentrations of PEA and OEA in fluids from malignant ovarian cysts are significantly lower than those in follicular fluid. The possible physiological significance of these data remains to be determined.

4. Conclusions

The discovery of functional cannabinoid receptors and their endogenous ligands has stimulated an explosive expansion of research during the past decade on their functions in reproduction and other physiological processes. Many obvious questions need to be answered. For example, are there other endogenous ligands? Do additional subtypes of cannabinoid receptors exist? Are there endocannabinoid receptors that do not react with classical cannabinoids? How does NAE-signaling affect other signal systems to modulate cellular responses to stimulation? How do exogenous cannabinoids delivered via smoking marijuana or by other modalities impact these processes? What are the medical consequences of marijuana abuse? Does marijuana smoke reduce human fertility significantly, and are the adverse effects of cannabinoids on reproductive functions reversible? Should public policies regulating the use of marijuana and synthetic cannabinoids be modified? Contradictory findings in the literature need

Table 4
NAEs in fluids from human malignant ovarian cysts and fluids from normal ovarian follicles

Source	AEA	PEA	OEA
Ovarian cysts ($N = 7$)	2.7 ± 0.9 ($P > 0.6$)	6.8 ± 0.6 ($P < 0.001$)	6.8 ± 2.2 ($P < 0.05$)
Follicular fluids ($N = 9$)	2.9 ± 0.9	11.3 ± 1.3	19.3 ± 2.9

P-values compare significance of levels for each NAE in malignant ovarian cysts fluids to those in follicular fluids; values are given in nM.

to be reconciled. Finally, the effects of orally administered cannabinoids or endocannabinoids need to be explained by demonstrating their absorption and tissue distribution. Do these compounds reach the brain and other target organs unchanged and in sufficient amounts? Additional research is required to resolve these issues.

Normal operation of an endogenous signaling system requires regulated rapid release and removal of endogenous agonists. A classic example of this phenomenon is the hydrolysis of acetylcholine by acetylcholine esterase immediately after its release at synaptic endings. A similar situation applies to signals mediated by AEA, other NAEs, 2-AG, and oleamide which are hydrolyzed by fatty acid amide hydrolase (Piomelli et al., 1998; Giuffrida et al., 2001c; Hillard, 2000). 2-AG is also degraded by monoacylglycerol lipase (Schmid, 2000). By contrast, drugs such as THC flood endocannabinoid signal systems because they are slowly metabolized, accumulate in fat stores, and produce persistent effects with potentially damaging consequences, especially in chronic marijuana smokers (Nahas et al., 2002). For example, AEA and CB1 receptors are found in the testis, suggesting possible roles for AEA-signaling in modulation of sperm production and maturation (Gerard et al., 1991; Galiegue et al., 1995; Sugiura et al., 1996; Wenger et al., 2001). These factors may account, at least in part, for the adverse effects of marijuana smoke and cannabinoids on sperm production in human and laboratory animals (see Schuel et al., 1999 and references cited therein).

Children born to parents who are chronic marijuana users are vulnerable to cumulative effects of cannabinoids. Exposure begins during maturation of eggs and sperm within their parents' gonads. It continues during sperm capacitation

and fertilization, preimplantation development of the embryo within the oviduct and uterus, and during pregnancy via THC transit across the placenta (Harbison et al., 1972; Martin et al., 1977; Asch and Smith, 1986; Paria and Dey, 2000; Paria et al., 2001; Schuel et al., 2002). Newborn humans, that had been exposed to marijuana prenatally, exhibit increased tremors, exaggerated responses to stimulation, and spend less time sleeping quietly (Fried and Smith, 2001). Nursing infants ingest THC in mother's milk (Astley and Little, 1990), and are also continuously exposed to secondary marijuana smoke at home. Together, these factors may be responsible for the adverse effects of marijuana and cannabinoids on growth and behavior in offspring of human and laboratory animals (Vardaris et al., 1976; Dalterio and Bartke, 1979; Frischknecht et al., 1980; Morrill et al., 1983; Dalterio et al., 1984; Asch and Smith, 1986; Fisher et al., 1987; Astley and Little, 1990; Ahmad and Ahmad, 1990; Fried and Smith, 2001).

Prior to the discovery of cannabinoid receptors and their endogenous ligands, it was generally believed that the peripheral pharmacological effects of cannabinoids resulted from their actions within the central nervous system. Direct drug-induced effects on non-neural cells outside the central nervous system were considered to be unlikely. These opinions reflected the primary focus of investigators on the psychoactive properties of marijuana, and its classification as a dangerous illicit substance. However, recent studies show that cannabinoid receptors are expressed by epithelial, smooth muscle, and immune cells in peripheral organs, as well as by sperm and preimplantation embryos (Deutsch et al., 1997; Schuel et al., 1999, 2002; Berdyshev, 2000; De Petrocellis et al., 2000; Paria et al., 2001).

Endocannabinoids are likewise produced locally by these cells. With the wisdom of hindsight, it is now clear that marijuana's peripheral effects were red flags announcing the presence of NAE-signaling systems outside the nervous system. Understanding these phenomena will expand our knowledge of human biology and pathology, and also may provide the basis for the future development of novel drugs for medicinal uses.

NAEs and/or cannabinoid receptors have been identified in mammals, fish, invertebrates, microorganisms, and higher plants (Schmid et al., 1990; Chang et al., 1993; Schuel et al., 1994, 1999; Bisogno et al., 1997; Di Tomaso et al., 1996; Chapman, 2000; De Petrocellis et al., 1999; Salzet et al., 2000; Schmid, 2000; Elphick, 2002). Furthermore, cannabinoids affect cyclic nucleotide metabolism in protozoa (Zimmerman et al., 1981). These findings suggest that cannabinoid receptors and their endogenous ligands have an ancient origin in evolutionary history, which may predate the origin of multicellular animals and plants (Chang et al., 1993; Schuel et al., 1994, 1999, 2002). Within this context, NAE-mediated signaling has far greater biological significance than the psychotropic properties of marijuana.

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