The Biodisposition and Metabolism of Anandamide in Mice

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ABSTRACT
The endogenous cannabinoid anandamide (AN) has been reported to produce pharmacological effects similar to those of Δ⁹-tetrahydrocannabinol but with a shorter duration of action. Also, AN is known to be metabolized to arachidonic acid. The purpose of this study was to examine the time course of distribution and metabolism of AN. Male mice were each administered 20 μCi ³H-AN and 50 mg/kg AN (i.v.). At 1, 5, 15 and 30 min after administration, the animals were sacrificed, and various tissues were removed, solubilized and counted to determine the distribution of ³H. Also, samples from brain, adrenal gland and plasma were extracted with ethyl acetate and analyzed by HPLC to separate ³H-labeled AN, arachidonic acid and other metabolites. AN was detectable in brain by 1 min after injection. At 1 min after injection, the rank order of radioactivity per milligram or microliter of tissue was adrenal > lung > kidney > plasma > heart > liver > diaphragm > brain > fat. Although the 1 and 5 min metabolic profiles of brain ²⁵H showed that AN was clearly present, most AN had already been transformed to arachidonic acid and other polar metabolites, and there were almost no detectable brain levels of AN at 15 and 30 min. In plasma and adrenal gland, AN was the predominant form at 1 and 5 min. Our experiments confirm that AN quickly reaches the brain and suggest that rapid metabolism of AN plays a key role in the time course of the pharmacological activity of this naturally occurring cannabinoid receptor ligand.

AN, or arachidonylethanolamide, the putative endogenous cannabinoid, is an ethanolamine derivative of the 20-carbon fatty acid AA. AN, first isolated from porcine brain (Devane et al., 1992), has been demonstrated to bind competitively to the cannabinoid receptor (Hilliard et al., 1995; Adams et al., 1995; Devane et al., 1992; Childers et al., 1994) and produce many of the pharmacological effects of (−)-Δ⁹-THC (Smith et al., 1994). Smith et al. (1994) have shown that AN produces antinociception, hypothermia, hypomotility and catalepsy in mice after i.v., i.p. or intrathecal administration. Others have also reported that AN decreases spontaneous motor activity and body temperature (Devane et al., 1992; Crawley et al., 1993; Fridé and Mechoulam, 1993). Behavioral studies have demonstrated that i.v. administration of AN induces immediate pharmacological effects. However, these effects, with the exception of antinociception, are almost completely dissipated by 30 min. In contrast, Δ⁹-THC produces a longer duration of action for hypoactivity and immobility (Smith et al., 1994).

Although AN and Δ⁹-THC have similar immediate pharmacological effects, these two compounds have few structural similarities. Thus it is not surprising that these two very different cannabinoid receptor agonists are metabolized through different biochemical pathways. Δ⁹-THC is metabolized via the P450 pathway (Aguirre et al., 1986), whereas AN has been reported to undergo rapid hydrolysis by a membrane-associated amidase (Deutsch and Chin, 1993). Furthermore, AN hydrolysis has also been shown to be blocked by the serine protease/esterase inhibitor PMSF, both in vivo and in vitro (Deutsch and Chin, 1993; Childers et al., 1994; Smith et al., 1994; Adams et al., 1995). The differences in duration of action of AN and Δ⁹-THC may be attributed to the differences in metabolism of the two compounds. The purpose of this study was to examine the time course of biodisposition and metabolism of AN in vivo. Such examination of the metabolism of AN may help explain the short duration of action seen in behavioral assays.

Materials and Methods
Male Institute of Cancer Research (ICR) mice (average wt. 30 g, Harlan, Dublin, VA) were administered an AN mixture of 20 μCi ³H-AN (160–249 Ci/mmol, Dupont-NEN, Boston, MA) and 50 mg/kg of unlabeled AN (Cayman Chemicals, Ann Arbor, MI). AN was dissolved in ethanol/Emulphor/saline (1:1:18). The total injection volume was 0.1 ml/10 g mouse, administered via the tail vein. This vehicle has been extensively used in previous studies of cannabinoids (Smith et al., 1994). The ethanol dose in the vehicle is relatively low for mice compared with humans and has been shown to produce, at best, additive but not synergistic effects with respect to THC (Esplin and Capek, 1976; Chait and Perry, 1994). At 1, 5, 15 and 30 min after injection with ³H-AN, animals were decapitated. The 50-mg/kg dose and the selected time points were chosen because previous behav-

ABBREVIATIONS: AN, anandamide; AA, arachidonic acid; PMSF, phenylmethyl-sulfonfyl fluoride; Δ⁹-THC, Δ⁹-tetrahydrocannabinol.
ioral studies of AN and Δ^2-THC in mice had utilized these parameters (Smith et al., 1994). Samples for metabolism and biodisposition studies were collected from various tissues as described below. Six animals were sacrificed at each time-point. Samples were routinely prepared using 0.1 M potassium phosphate (monobasic) buffer, pH 7.4, containing 1.5 mM PMSF (Sigma, St. Louis, MO) to inhibit amidases.

**Biodisposition Study**

For this study, plasma, brain, adrenal gland, heart, kidney, liver, lung, diaphragm and mesenteric fat samples were removed. Plasma, brain and adrenal gland were prepared as described under “Metabolism Study.” Other tissues were rinsed with saline, weighed and placed into individual sample tubes containing 2 ml of ice-cold phosphate buffer. Each tissue sample was homogenized using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) at a setting of 4.5 for 20 sec. The homogenizer was rinsed with 2 ml of phosphate buffer after tissue processing. Rinses were combined with the sample for a total homogenate volume of 4 ml. An aliquot of 250 μl from each tissue sample, as well as a plasma sample, was added to a scintillation vial containing 1 ml of TS-2 tissue solubilizer (RPI, Mount Prospect, IL), vortexed and allowed to remain covered overnight at room temperature. The following day, 30 μl of glacial acetic acid was added to each vial and vortexed. Samples were counted in 4 ml of scintillation cocktail (Budget-Solve, RPI, Mount Prospect, IL) using a Beckman LS 6000IC Scintillation Counter (Schaumburg, IL). Using the specific activity of the injected radiolabeled plus nonradiolabeled AN, the content of each sample was expressed as ng equivalents of AN plus metabolites/mg tissue wet wt. or μl plasma. Equivalents are reported, instead of radioactivity, in order to give an indication of the amount of exogenous compound that entered each tissue.

**Metabolism Study**

In order to examine the time course of AN metabolism in vivo, this study was performed on brain, plasma and adrenal gland from 24 ICR mice as described below.

**Plasma sample preparation.** With the aid of a funnel, blood was collected from decapitated mice into a centrifuge tube holding 1 ml of phosphate buffer containing 1.5 mM PMSF. The funnel was rinsed with 1 ml of heparinized saline, which was collected into the same centrifuge tube. The blood was centrifuged at 1000 × g, 4°C, for 20 min in a Beckman GPRK centrifuge (Schaumburg, IL) to separate plasma from blood cells. The plasma layer was removed to a second centrifuge tube, and a 250-μl aliquot was removed for determination of total radioactivity by scintillation spectroscopy. The remaining plasma sample was flushed with nitrogen, capped tightly and placed at −70°C until further analysis.

**Brain sample preparation.** Immediately after decapitation, the brain was removed, rinsed with saline and weighed. Brains were homogenized in 2 ml of ice-cold phosphate buffer containing 1.5 mM PMSF using a Polytron homogenizer at setting 4.5 for 20 sec. The homogenizer was rinsed with 2 ml of buffer, which was combined with the sample for a total homogenate volume of 4 ml. A 250-μl aliquot of brain homogenate was removed to a scintillation vial for distribution and recovery determination as described in “Biodisposition study.” The remaining brain homogenate was flushed with nitrogen, capped tightly and stored at −70°C until further analysis.

**Adrenal gland sample preparation.** Both adrenal glands were removed from each mouse and processed separately by the procedure described above for brain tissue. A 250-μl aliquot was taken from one adrenal gland homogenate and placed in a scintillation vial for distribution and recovery determination as described in “Biodisposition study.” The second adrenal gland homogenate was flushed with nitrogen, capped tightly and stored at −70°C until further analysis.

**Extraction procedure.** Remaining brain, plasma and adrenal gland samples were extracted by addition of 2 volumes of ethyl acetate/88% formic acid (100:0.2). The organic phase was removed and placed in a separate conical tube. The aqueous phase was re-extracted a total of four times with ethyl acetate, and organic phases from each extraction were combined. Duplicate 1-μl aliquots from the aqueous phases were removed with a Hamilton positive displacement microsyringe and counted to determine radioactivity remaining in this phase. The pooled organic phase from each sample was dried under nitrogen and resuspended in 1 ml of ethanol. Duplicate 1-μl aliquots from each sample were subjected to scintillation counting for recovery determination. Samples were stored under nitrogen at −70°C until reverse-phase HPLC analysis.

**Reverse-phase HPLC analysis.** A standard mixture of [3H]-AN and [3H]-AA (Dupont-NEN, Boston, MA) was separated using an isocratic solvent system of methanol/water/acetate acid [84:5:0.5 (v/v/v)] with an Absorosphere C8 5-micron column (Alltech, Deerfield, IL) at a flow rate of 1.0 ml/min for 30 min. Radioactivity was monitored by diverting 50% of the eluate through a flow-through radioactivity detector (RAMONA-LS, Raytest, IN/US, Fairfield, NJ). Once a standard elution profile was obtained, plasma, brain and adrenal gland extracts were evaporated under nitrogen and resuspended in methanol/dH2O (60:40, v/v) for a total injection volume of 500 μl. The elute from the column was collected in 30-sec fractions using an ISCO model 1850 fraction collector (Lincoln, Nebraska). A 350-μl aliquot from each fraction was counted by scintillation spectrometry. Radioactivity vs. fraction time (min) was plotted to obtain an elution profile for each sample. Radioactive peaks were identified by co-chromatography with authentic AN and AA. The radioactivities in the various peaks were totaled, and each individual peak was expressed as a percentage of the total. This percentage was then multiplied by the total amount of exogenous compound recovered in each tissue after the biodisposition study to yield nanograms per milligram of tissue or microliter of plasma.

**Results**

**Behavioral observation.** Mice were observed from the time of injection to the time of sacrifice. Immediately after the AN injection, mice exhibited a significant decrease in motor activity. The loss of motor activity was less profound by 5 min, and animals regained full mobility by 30 min. The behavioral changes observed were characteristic of those seen in studies performed by Smith et al. (1994).

**Biodisposition study.** The biodisposition of radioactivity in mice is shown in table 1. Examination of plasma and various tissues indicates that the levels of exogenous AN equivalents (ng/μl or mg tissue) vary among anatomical regions in the mouse. By 1 min after injection, radioactivity was found in all tissues examined, which indicates rapid distribution. Initially, AN equivalents in brain tissue at 1 min after injection were among the lowest of all tissues analyzed. However, the lowest AN equivalents were in fat at the 1-min time-point. Interestingly, the adrenal gland reveals the greatest AN equivalents/mg at 1 min. Although the equivalents/mg are high in adrenal, the actual total adrenal radioactivity is comparatively low. The equivalents/mg are high because the total radioactivity is distributed in only 2.5 mg, the total average weight of both adrenals.

Changes in the tissue radioactivity were monitored for 30 min after administration. In the brain, insignificant changes in the levels of radioactivity occurred between 1 min and 30 min. However, in some tissues, AN equivalents decreased between 1 and 30 min. The initial level of AN equivalents in heart tissue decreased significantly by 5 min. AN equivalents in heart tissue further decreased by 15 min but then remained constant through 30 min. Lung tissue also showed a
significant decrease in AN equivalents between 1 and 5 min after injection, and this decrease was sustained for the duration of the experiment. Diaphragm muscle had sustained levels between 1 min and 5 min, followed by a decrease at 15 through 30 min. Unlike other tissues, liver radioactivity increased over the 30-min time course. Some tissues, such as kidney, plasma and adrenal gland, demonstrated a biphasic pattern in radioactivity levels between 1 and 30 min. Adrenal gland tissue exhibited a decrease in AN equivalents between 1 and 15 min. Subsequently, an increase in adrenal gland AN equivalents was observed by 30 min. Plasma and kidney tissue followed a similar biphasic biodisposition pattern between 1 and 30 min.

Metabolism study. In order to understand better the time course of the pharmacological activity of AN in the brain, we studied the metabolic profile of radioactivity in brain homogenates from mice. After extraction of the brain homogenate, 80% of the radioactivity was present as free acids in the ethyl acetate extract, and 20% remained in the aqueous phase at 1 min after injection (data not shown). The aqueous phase is known to contain polar compounds, including phospholipids. After 30 min, however, 50% of the radioactivity was recovered in the free acid phase, and 50% remained in the aqueous phase, a result that suggests further metabolism or incorporation into more polar compounds.

Analysis of extracts of brain homogenates by reverse-phase HPLC (Table 2) indicates that AN is present in the brain at 1 min after injection. AA and other polar products are also observed at 1 min and are proportionally much larger than AN. Analysis of brain extracts from the 5-min-time point also reveals the existence of AN in the brain. However, by 15 and 30 min, only trace quantities of AN remained. These results may explain the time course of behavioral changes observed after i.v. injection of AN.

The metabolic profile of plasma at 1 min (Table 2) shows that, unlike in the brain, AN is the major form present in the circulation. There are also other major plasma polar metabolites at 1 min, which may suggest that AN is degraded differently in the blood than in tissues such as the brain. Total plasma-associated radioactivity falls dramatically at 5 and 15 min and then increases at 30 min, especially in the form of AA and other polar metabolites (also see Table 1). This may suggest that radioactivity that was initially incorporated into the various tissues is being metabolized and is re-entering the circulation for further biodistribution.

Table 2 shows the metabolic profile of the adrenal gland. Adrenal gland tissue was included in this metabolism study because the 1-min time-point of the biodisposition study indicated the adrenal gland had the highest AN equivalents/mg tissue among all the tissues. However, it should be noted that the combined weight of both adrenals is on average only 2.5 mg. At 1 min, there is a significant proportion of AN in the adrenal gland, as well as AA and more polar metabolites. At 5 min, AN equivalents were significantly decreased, and recovery of radioactivity at the 15- and 30-min time-points was too low to identify AA or AN reliably by HPLC.

**Discussion**

Previous behavioral experiments indicated that pharmacokinetic studies of AN would be helpful in achieving a better understanding of the time course of the pharmacological activity of AN in the brain. We and others have shown that behavioral responses to AN peak at 1 min after injection and then decrease to baseline levels by 30 min. This pattern of behavioral activity has been observed in a variety of species and in a number of different behavioral paradigms. The pharmacokinetic profile of AN in the brain from this study may help to explain the time course of behavioral changes observed after i.v. injection of AN.
understanding AN’s potency and duration of action, especially as contrasted to the behavioral effects of Δ⁹-THC. Our studies indicate that AN reaches the brain within 1 min of injection and is present in the brain even at 5 min after injection. Interestingly, levels of AN detected in the brain were relatively low compared with other anatomical regions. Assuming that the exogenous AN in the brain at 1 and 30 min after injection (table 2) is evenly distributed throughout the brain, the concentration at 1 and 30 min would be 5.6 μM and 0.4 μM, respectively. This level is probably consistent with, at best, a moderately potent neuropharmacological agent. Note, however, that because the exact cellular distribution of exogenous AN in the brain is uncertain, such estimates of potency are highly speculative.

The current findings are consistent with previous reports indicating that AN has a rapid onset of action. Behavioral studies by Smith et al. (1994) have shown the production of maximal antinociception immediately after administration of AN, which suggests that AN quickly reaches brain regions responsible for pain modulation. In addition, Smith et al. (1994) observed other cannabinoïd effects, such as immobility and hypothermia, very soon after i.p., i.v. and intrathecal AN administration. In our studies, reduced mobility was also observed immediately after injection of AN.

At 1 min after injection of ³H-AN, the level of brain ³H-AA plus other polar metabolites was 10 times greater than that of ³H-AN, a result that implies extremely rapid and efficient brain metabolism of AN. This is in distinct contrast to the plasma and adrenal gland, where ³H-AN is the major form of radioactivity at 1 min after injection. This strong contrast in the chemical form of the radioactivity in these three tissues at the same time-point reinforces the value of separating the chemical constituents, as opposed to only examining total radioactivity.

At 15 and 30 min, the AN level in the brain was significantly decreased compared with 1 and 5 min. The metabolic profiles in plasma and adrenal gland differed from brain over the time course of 30 min, which again suggests differential amidase activity in various tissues. The degree of brain hydrolysis of AN seen in table 2 undoubtedly plays an important role in the ability of AN to produce and sustain neuropharmacological activity. On the basis of physiological and behavioral measures, a number of investigators have independently shown that AN has a short duration of action in vivo (Smith et al., 1994; Crawley et al., 1993; Frise and Mechoulam, 1993). Our results imply that the short duration of action is due to hydrolysis or metabolism of AN to AA and other polar metabolites. Our results also suggest that the behavioral effects of injected ³H-AN are not due to conversion to and action of AA, because the brain’s ³H-AA content stayed relatively stable over the 30-min observation period.

It is possible to compare the time course of AN levels in brain detected in the present study to centrally mediated pharmacological effects of AN, because Smith et al. (1994) used a treatment regimen identical to that employed in the present study. As mentioned above, Smith et al. (1994) found maximal effects of AN 5 min after administration, an observation consistent with the current study. At 15 min, however, brain concentrations of AN had fallen by an order of magnitude, while robust pharmacological effects remained in the behavioral study. At 15 min, antinociception had decreased by only 15% and hypothermia by approximately 20%, and hypoactivity was near maximal. Immobility was not measured at 15 min. AN’s pharmacological effects even persisted at 30 min for antinociception (50% effect), hypothermia (~40% effect) and hypoactivity (~40% effect). Immobility had disappeared by 30 min, which is consistent with AN brain concentrations. The discordance between AN brain levels and some pharmacological effects suggest several possibilities. AN may stimulate the release of endogenous substances that are responsible for effects that continue beyond the time when AN is present. Although AN-activation of biological systems persists in the absence of injected ³H-AN, this scenario may seem less plausible because other cannabinoids do not act in this fashion.

We cannot rule out the possibility that degradation of AN results in the formation of secondary products that have pharmacological effects. HPLC profiles from the current study reveal the existence of metabolites that are more polar than AN and AA. These polar metabolites may be responsible for a host of pharmacological activities. Previous studies from our laboratory have indicated that AN and Δ⁹-THC are arachidyl dilators when applied topically to the cortical surface of rabbit brain. Furthermore, we have reported that this AN- and Δ⁹-THC-induced vasodilation of cerebral arterioles is blocked by the cyclooxygenase inhibitor indomethacin, which suggests prostaglandin involvement in the dilator response to AN (Ellis et al., 1995). Our recent studies in rat astrocytes further confirm a possible role for AA metabolites in the response to AN and Δ⁹-THC, because both AN and Δ⁹-THC stimulate receptor-dependent release of AA from astrocytes prelabeled with ³H-AA (Shivachar et al., 1996).

In comparison with Δ⁹-THC, AN has been observed to be considerably less potent in some pharmacological tests after i.v. administration to mice (Smith et al., 1994). The shorter duration of action of AN could be explained by the dissimilarities in metabolism of the two cannabinoid receptor agonists. The primary metabolism of Δ⁹-THC is via the P450 pathway (Agurell et al., 1986), whereas AN has been reported to be hydrolyzed by amidases in both liver and brain (Hilliard et al., 1995). If degradation of AN is responsible for the less profound effects and shorter duration of action in hypoactivity and immobility, the relatively long duration of antinociception induced by AN may be explained by other mechanisms of action, such as differential metabolism of AN in different brain regions.

Studies by Hilliard et al. (1995) have shown that AN is catabolized in rat forebrain to AA and ethanolamine by an enzyme that is almost identical to N-acylethanolamide amidohydrolase, identified in rat liver and dog brain (Schmid et al., 1985; Natarajan et al., 1984). N-Acylethanolamide amidohydrolase hydrolyzes N-acylethanolamide to fatty acids and ethanolamine. This enzyme is inhibited by fatty acids, and its activity is not calcium-dependent. Therefore, the hydrolysis of AN may account for its inactivation, because the metabolic products of AN do not bind to the cannabinoid receptor (Hilliard et al., 1995).

Others have shown that the hydrolysis activity responsible for the degradation of AN can be inhibited by a nonspecific esterase and amidase inhibitor, PMSF (Deutsch and Chin, 1993; Childers et al., 1994). In receptor binding studies AN binding was shown to be enhanced in the presence of PMSF (Childers et al., 1994). Furthermore, Hilliard et al. (1995) have reported that AN hydrolyase activity is not homoge-
neously distributed in rat brain and that AN hydrolase activity correlates with the distribution of cannabinoid receptor binding sites.

Anandamide has been shown to be rapidly taken up by neurons and glia (Deutsch and Chin, 1993; Di Marzo et al., 1994). Also, Di Marzo et al. (1994) have reported the presence of AN amidase in primary embryonic neuronal cultures. Our initial studies show that cultured rat astrocytes do not metabolize 3H-AN during a 20-min incubation (Shivachar et al., 1996), which suggests that brain metabolism of AN is by neurons or other nonastrocytic cells.

In summary, our studies show that AN, administered i.v., rapidly moves into body tissues at different rates. We have shown that AN quickly reaches the brain and is more rapidly hydrolyzed there, to AA and more polar metabolic products, than in other tissues. Finally, this study suggests that AN-induced pharmacological effects may not be due solely to concurrent AN brain levels. AN may be metabolized to active metabolites, stimulate the release of other endogenous substances or activate biochemical pathways that are sustained beyond the presence of a rapidly metabolized AN.

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References


