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Microsomal omega-hydroxylated metabolites of *N*-arachidonoyl dopamine are active at recombinant human TRPV₁ receptors

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Abstract

N-arachidonoyl dopamine (NADA) is an endogenous lipid that modulates signal transduction in neuronal and immune pathways. NADA activates the non-selective cation channel, transient receptor potential vanilloid type 1 (TRPV₁) and cannabinoid receptor 1. That NADA is comprised of an arachidonic acid (AA) backbone suggests that it may be metabolized through many of the enzymes that act upon AA such as the other AA-derived signaling lipids, the endogenous cannabinoids. To investigate the metabolism of NADA through the cytochrome P450 (CYP450) metabolic pathway, we studied the *in-vitro* rat liver microsomal production of hydroxylated metabolites and their activity at recombinant human TRPV₁ receptors. We showed that following microsomal activation in the presence of NADA, omega and (omega -1) hydroxylated metabolites (19-, and 20-HETE-DA) were formed. These metabolites were active at recombinant human TRPV₁ receptors, inducing a dose-dependent calcium influx. Both metabolites exhibited lower potency compared to NADA. We conclude that CYP450 enzymes are capable of metabolizing this signaling lipid forming a larger family of potential neuromodulators.

INTRODUCTION

N-acyl dopamines are conjugates of fatty acids with dopamine via an amide bond. The synthesis and partial pharmacological characterization of *N*-acyl dopamines began prior to the realization that this class of compounds occurs in mammalian tissues^{1–5}. To date, four endogenous *N*-acyl dopamines, the dopamides of arachidonic (NADA), oleic (OLDA), palmitic (PALDA) and stearic (STEARDA) acids, have been identified^{5–7}. Among these, NADA has received the most attention. NADA is present in the mammalian nervous system^{5,8}, binds cannabinoid receptor 1 (CB₁)⁴, and is a potent activator of the non-selective cation channel, transient receptor potential vanilloid type 1 (TRPV₁)⁵.

In accordance with the activation of two molecular targets with differential anatomical distributions⁹, NADA was shown to modulate nociceptive pathways with opposing sensory outcomes^{2,3,5,10,11}. While NADA administered intraperitoneally was shown to induce

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analgesia in the 'hot plate' test², intradermal application induced TRPV₁-mediated hyperalgesia and enhanced spontaneous and heat-evoked activity in spinal nociceptive neurons^{5,12}. Similarly, topical NADA application in primates produced thermal allodynia with exposure to non-noxious warm water stimulation¹³. Conversely, intraplantar injection of NADA was shown to cause inhibition of neuronal responses to mechanical stimulation¹⁰.

NADA is also a potent modulator of other physiological systems. NADA acts as a CB₁ and TRPV₁-mediated anti-emetic in a ferret model of emesis¹⁴. It also induces guinea pig bronchi contractions in a TRPV₁-sensitive manner¹⁵. However, not all of NADA's effects can be explained by activation of known molecular targets. For example, NADA was shown to modulate calcium levels and neurotransmitter release in hippocampal synaptosomes in a CB₁ and TRPV₁-independent manner where the involvement of cyclooxygenase-2 (COX-2) metabolites, or amidase products, were ruled out¹⁶. In the immune system, NADA potentially inhibited *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine-induced migration of human neutrophils via an unknown target¹⁷. NADA was also shown to inhibit T cell proliferation and produce immunosuppressive effects via the inhibition of IL-2 and TNF alpha gene transcription¹⁸. These immunosuppressive effects were mediated through a yet unknown target. Finally, Goswami and colleagues¹⁹ reported that exposure to NADA induced calcium influx in growth cones and neurites, and caused a delayed collapse of growth cones, and an attenuated growth of neurites in dorsal root ganglion explants. The signaling mechanisms leading to delayed outcomes are not fully understood, and in most of these studies effects produced by degradation products of NADA have not been ruled out.

Two pathways for the biosynthesis of NADA have been proposed. 1) Condensation of dopamine and arachidonic acid (AA) and 2) enzymatic conversion of *N*-arachidonoyl tyrosine⁵. Much less is known about the inactivation or further metabolism of NADA. One mechanism of inactivation is through the hydrolysis of NADA to arachidonic acid and dopamine⁵. Another proposed mechanism is through the enzyme catechol-*O*-methyltransferase which contributes to the partial inactivation of NADA by producing a less potent methylated compound, *N*-arachidonoyl-homovanillyl-amine⁵. Other pathways for NADA metabolism have not been explored.

Advances in lipid analysis revealed that the metabolism of endocannabinoid-like compounds generates a larger family of signaling molecules^{11, 20}. These are produced by the action of metabolic enzymes such as lipoxygenases (LOX), COX and cytochrome P450 (CYP450) on lipid substrates^{21–25}. The formation and activity of NADA metabolites, excluding *N*-arachidonoyl-homovanillyl-amine, has not been explored. Here, we investigated the hypothesis that NADA is metabolized by CYP450 enzymes. We report that NADA is metabolized by rat liver microsomes yielding omega and (omega-1)-hydroxylated metabolites. These metabolites were synthesized and their activity was characterized at recombinant human TRPV1 receptors.

MATERIALS AND METHODS

Chemistry

All air or moisture sensitive reactions were carried out in oven-dried glassware under a dry argon atmosphere. AA was purchased from Nu-Check-Prep, Inc. ¹H NMR spectra were recorded on a Gem 300 MHz nuclear magnetic resonance spectrometer. Flash column chromatography was performed using 60 Å, 230–400 mesh silica gels. The 18-, 19-, and 20-hydroxyeicosa-5(Z),8(Z),11(Z),14(Z)-tetraenoic acids (18-, 19-, and 20-HETEs) were prepared using a modification of the procedure of^{26,27}. The spectroscopic data of the synthesized compounds were found to be identical to the published spectroscopic data. In this synthesis the oxidation of arachidonic acid to 14,15-epoxyarachidonic acid was accomplished by an alternate method versus the procedure described by^{27–29}. The 50 or 90% H₂O₂ used as oxidant

in this reaction are hazardous or are no longer commercially available. Therefore, a stable urea- H_2O_2 adduct³⁰ was used as an oxidant in this reaction as it was more easily handled. This step gave a 60% yield after methylation by diazomethane.

18-, 19-, and 20-HETEs were derivatized to 18-, 19-, and 20-*N*-(3, 4-dihydroxyphenethyl) hydroxyeicosa-5(*Z*), 8(*Z*), 11(*Z*), 14(*Z*)-tetraenamide (18-, 19-, and 20-HETEs-Dopamine) as outlined in Scheme 1. The hydroxyl of 18-, 19-, and 20-HETEs was protected with TBSCl (Corey and Venkateswrlu 1972; a). Each TBS protected HETEs was converted to an acid chloride with oxalyl chloride then after drying treated with dopamine-HCl^{4,5,27} b). Finally, they were deprotected with TBAF to get 18-, 19-, and 20-HETEs-DA³¹.

a) General synthetic procedure for TBS-protected 18-, 19-, and 20-HETEs

In a small vial, a mixture of 18, 19, or 20-HETE, TBSCl (1.5 eqv.) and imidazole (2.5 eqv.) in anhydrous DMF (0.1 ml) was stirred at r. t. under argon for 18 h. The mixture was diluted with ethyl acetate and was washed with 10% HCl, water and saturated NaCl solution. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. Purification by flash chromatography (10% ethyl acetate/hexane) gave the TBS protected 18-, 19-, and 20-HETE as a colorless oil.

TBS protected 18-HETE—Yield = 19 mg (76%) was obtained from 19 mg of **18-HETE**. $R_f = 0.78$ (ethyl acetate: hexane = 1:1), $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 5.44–5.29 (m, 8H), 3.62 (quintet, $J = 5.63$ Hz, 1H), 2.88–2.70 (m, 6H), 2.36 (t, $J = 7.34$ Hz, 2H), 2.17–2.08 (m, 4H), 1.72 (quintet, $J = 7.55$ Hz, 2H), 1.50–1.40 (m, 4H), 0.90–0.84 (m, 12H), 0.06 (s, 6H).

TBS protected 19-HETE—Yield = 25 mg (78%) was obtained from 24 mg of **19-HETE**. $R_f = 0.78$ (ethyl acetate: hexane = 1:1), $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 5.45–5.30 (m, 8H), 3.78 (sextet, $J = 5.90$ Hz, 1H), 2.88–2.70 (m, 6H), 2.36 (t, $J = 7.42$ Hz, 2H), 2.18–2.00 (m, 4H), 1.72 (quintet, $J = 7.42$ Hz, 2H), 1.50–1.38 (m, 4H), 1.15 (d, $J = 6.18$ Hz, 3H) 0.89 (s, 9H), 0.06 (s, 6H).

TBS protected 20-HETE—Yield = 24 mg (83%) was obtained from 22 mg of **20-HETE**. $R_f = 0.80$ (ethyl acetate: hexane = 1:1), $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 5.45–5.31 (m, 8H), 3.61 (t, $J = 6.52$ Hz, 2H), 2.85–2.69 (m, 6H), 2.36 (t, $J = 7.42$ Hz, 2H), 2.19–1.98 (m, 4H), 1.72 (quintet, $J = 7.35$ Hz, 2H), 1.52 (quintet, $J = 6.66$ Hz, 2H), 1.42–1.29 (m, 4H), 0.89 (s, 9H), 0.06 (s, 6H).

b) General synthesis procedure for TBS-protected 18-, 19-, and 20-HETE-DA

To a solution of TBS protected 18, 19, or 20-HETE in anhydrous benzene (1 ml) cooled to 0° C was added oxalyl chloride (2 eqv.). The mixture was stirred overnight allowing it to come to r. t. under argon. The solvent was removed under reduced pressure followed by the addition of benzene (2 ml) and was repeatedly evaporated *in vacuo* to remove traces of oxalyl chloride. ² To a solution of acid chloride in anhydrous DMF (0.8 ml), dopamine-HCl (1.2 eqv.) followed by DIEA (2.2 eqv.) was added at 0° C. The mixture was stirred at below 10° C for 18 h. Water was added and the solution was extracted with ethyl acetate. The organic layer was washed with saturated NaCl and was dried over Na_2SO_4 . Removal of solvent *in vacuo* gave brown oil. Purification by flash chromatography (20 to 50% ethyl acetate/hexane) gave the TBS protected 18, 19, and 20-HETE-Dopamine as a colorless oil.

TBS-protected 18-HETE-DA—Yield = 8 mg (34%) was obtained from 19 mg of TBS protected 18-HETE. $R_f = 0.27$ (ethyl acetate: hexane = 1:1), $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 6.81 (d, $J = 7.96$ Hz, 1H), 6.73 (br, s, 1H), 6.56 (d, $J = 7.90$ Hz, 1H), 5.51 (br, s, 1H, N-H) 5.45–5.27 (m, 8H), 3.61 (quintet, $J = 5.77$ Hz, 1H), 3.48 (q, $J = 6.86$ Hz, 2H), 2.86–2.72 (m,

6H), 2.68 (t, $J = 6.87$ Hz, 2H), 2.18–2.00 (m, 6H), 1.73 (quintet, $J = 7.48$ Hz, 2H), 1.52–1.42 (m, 4H), 0.90–0.84 (m, 12H), 0.06 (s, 6H).

TBS-protected 19-HETE-DA—Yield = 10 mg (37%) was obtained from 21 mg of TBS protected 19-HETE. $R_f = 0.27$ (Ethyl acetate: Hexane = 1:1), ^1H NMR (CDCl_3 , 300 MHz) δ 6.80 (d, $J = 7.97$ Hz, 1H), 6.73 (br, s, 1H), 6.56 (d, $J = 7.82$ Hz, 1H), 5.60 (br, s, 1H, N-H) 5.45–5.28 (m, 8H), 3.85 (sextet, $J = 5.63$ Hz, 1H), 3.48 (q, $J = 6.32$ Hz, 2H), 2.88–2.70 (m, 6H), 2.68 (t, $J = 6.73$ Hz, 2H), 2.18–1.96 (m, 6H), 1.69 (quintet, $J = 7.42$ Hz, 2H), 1.50–1.38 (m, 4H), 1.15 (d, $J = 6.18$ Hz, 3H) 0.88 (s, 9H), 0.06 (s, 6H).

TBS-protected 20-HETE-DA—Yield = 15 mg (48%) was obtained from 24 mg of TBS protected 20-HETE. $R_f = 0.28$ (ethyl acetate: hexane = 1:1), ^1H NMR (CDCl_3 , 300 MHz) δ 6.80 (d, $J = 7.97$ Hz, 1H), 6.73 (br, s, 1H), 6.57 (d, $J = 8.03$ Hz, 1H), 5.58 (br, s, 1H, N-H) 5.43–5.28 (m, 8H), 3.62 (t, $J = 6.59$ Hz, 2H), 3.48 (q, $J = 6.87$ Hz, 2H), 2.88–2.74 (m, 6H), 2.69 (t, $J = 7.00$ Hz, 2H), 2.20–2.00 (m, 6H), 1.76–1.46 (m, 4H), 1.42–1.30 (m, 4H) 0.89 (s, 9H), 0.06 (s, 6H).

c) General synthesis procedure for 18-, 19-, and 20-HETE-DA

To a solution of TBS protected either 18, 19, or 20-HETE-Dopamine in anhydrous THF (1 ml) was added TBAF (2 eqv.). The mixture was stirred at r. t. under argon for 15 h and was diluted with ethyl acetate. The organic layer was washed with water, saturated NaCl and was dried over Na_2SO_4 . The solvent was removed *in vacuo*. Purification by flash chromatography (50% ethyl acetate/hexane with 5% methanol) gave 18-, 19-, and 20-HETE-DA as a colorless oil.

18-HETE-DA—Yield = 4 mg (67%) was obtained from 8 mg of TBS protected 18-HETE-Dopamine. $R_f = 0.38$ (ethyl acetate: hexane: methanol = 1:1:0.1), ^1H NMR (CDCl_3 , 300 MHz) δ 6.80 (d, $J = 7.97$ Hz, 1H), 6.73 (br, s, 1H), 6.56 (d, $J = 7.88$ Hz, 1H), 5.54 (br, s, 1H, N-H) 5.45–5.28 (m, 8H), 3.60 (quintet, $J = 5.72$ Hz, 1H), 3.48 (q, $J = 6.87$ Hz, 2H), 2.88–2.74 (m, 6H), 2.68 (t, $J = 6.87$ Hz, 2H), 2.19–2.01 (m, 6H), 1.72 (quintet, $J = 7.48$ Hz, 2H), 1.52–1.42 (m, 4H), 0.96 (t, $J = 7.42$ Hz, 3H).

19-HETE-DA—Yield = 5 mg (63%) was obtained from 10 mg of TBS protected 19-HETE-Dopamine. $R_f = 0.38$ (ethyl acetate: hexane: methanol = 1:1:0.1), ^1H NMR (CDCl_3 , 300 MHz) δ 6.80 (d, $J = 7.97$ Hz, 1H), 6.73 (br, s, 1H), 6.56 (d, $J = 7.82$ Hz, 1H), 5.59 (br, s, 1H, N-H) 5.46–5.27 (m, 8H), 3.84 (sextet, $J = 5.97$ Hz, 1H), 3.48 (q, $J = 6.32$ Hz, 2H), 2.87–2.74 (m, 6H), 2.69 (t, $J = 6.73$ Hz, 2H), 2.19–1.99 (m, 6H), 1.68 (quintet, $J = 7.48$ Hz, 2H), 1.50–1.32 (m, 4H), 1.22 (d, $J = 6.18$ Hz, 3H).

20-HETE-DA—Yield = 7 mg (79%) was obtained from 11 mg of TBS protected 20-HETE-Dopamine. $R_f = 0.40$ (ethyl acetate: hexane: methanol = 1:1:0.1), ^1H NMR (CDCl_3 , 300 MHz) δ 6.80 (d, $J = 7.82$ Hz, 1H), 6.73 (br, s, 1H), 6.56 (d, $J = 8.24$ Hz, 1H), 5.63 (br, s, 1H, N-H) 5.44–5.26 (m, 8H), 3.66 (t, $J = 6.46$ Hz, 2H), 3.48 (q, $J = 6.78$ Hz, 2H), 2.87–2.74 (m, 6H), 2.69 (t, $J = 6.59$ Hz, 2H), 2.18–2.02 (m, 6H), 1.72–1.52 (m, $J = 4$ Hz), 1.43–1.33 (m, 4H).

Cell culture—The human TRPV₁ HEK 293 stably transfected cells were a kind gift from Merck Research Laboratories (Whitehouse Station, NJ). The HEK 293 TRPV₁ cell line was cultured in minimal essential medium, Eagle, modified with non essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine and 1.5 g/L sodium bicarbonate (ATCC, Manassas, VA), containing 1% penicillin-streptomycin, and 10% fetal bovine serum. The cells were passaged three times a week using Trypsin- EDTA 1X (Invitrogen, Carlsbad, CA) and grown under 5% CO₂ at 37°C.

Calcium imaging—Fura-2 AM was purchased from molecular probes (Eugene, Oregon). 5'-iodoresiniferatoxin (I-RTX) was purchased from Tocris Cookson (Ellisville, MO). NADA and AA for calcium imaging were purchased from Cayman Chemical (Ann Arbor, Michigan). Calcium influx was measured using a 96 well plate reader (FlexStation II, Molecular Devices, Union City, CA). HEK 293 cells were plated 24 hours before imaging on CellBIND 96 well, flat, clear bottom, black polystyrene microplates (Corning, NY). Cells were loaded with 3 μ M Fura 2 AM in 0.05 % W/V Pluronic 127 (Molecular Probes, Eugene, Oregon) in HEPES-Tyrode buffer. The cells were then washed twice with HEPES-Tyrode buffer and imaged in the same media. The FlexStation II was programmed with the following settings: initial volume: 177.5 μ l, volume added: 75 μ l at rate 2 (52 μ l/sec), pipette height: 200 μ l, sensitivity: 7 High, triturate settings: 1 $\times$ 100 h:200. For IRTX experiments the cells were pretreated with vehicle (DMSO) or I-RTX prior to drug application.

Enzymatic reaction—Rat liver microsomal fractions were purchased from In Vitro Technologies (Baltimore, MD), and used according to the manufacturers' instructions. Briefly, an NADPH regenerating system (NRS) containing 1.7 mg/ml b-NADP, 7.8 mg/ml glucose-6 phosphate, and 6 units/ml glucose 6-phosphate dehydrogenase (Sigma Aldrich, St. Louis, MO) was prepared in 2% NaHCO₃. The final 1 ml reaction mixture contained: 0.5 mg/ml microsomal protein, 50 mM Tris buffer (740 μ l), NADA (10 μ l; or arachidonic acid as a positive control), and NRS (250 μ l). The NRS was added to initiate the reaction. A time course was achieved by removing 100 μ l aliquots from the mixture reaction at different time points.

Partial purification of hydroxylated metabolites—Aliquots of 100 μ l were removed from the reaction tube at 0, 5, 10, 15, 20, 30, 60, and 210 min after the initiation of the reaction, and extracted to determine the initial rate of the reaction. Each 100 μ l aliquot was quenched by the addition of 2 ml HPLC grade methanol. The microsomal metabolites of NADA were partially purified from methanolic extracts on C18 500 mg solid phase extraction cartridges as previously described⁸. The 1.5 ml 90 % methanol elution was used to quantify NADA metabolites. Metabolites were identified by HPLC/quadrupole time-of-flight mass spectrometry and quantified using tandem mass spectrometry and compared to synthesized standards (see scheme 1).

HPLC Quadrupole-TOF Analysis of NADA metabolites (lipid identification method)—Exact mass measurements and structural characterization of NADA metabolites from rat liver microsomes were performed with a hybrid quadrupole time-of-flight (QqTOF) mass spectrometer (QSTAR Pulsar, Applied Biosystems-MDS Sciex; Foster City, CA) equipped with an electrospray ionization turbo ion source. The 90 % methanol eluants were combined and dried under a stream of argon. The sample was immediately reconstituted in 100% methanol and chromatographed on two linearly coupled Zorbax eclipse XDB reverse phase columns, a C8 2.1 $\times$ 50 mm, and a C18 2.1 $\times$ 150 mm. Gradient elution (200 μ l/min) was formed under pressure on a pair of Shimadzu (Columbia, Maryland) 10AdVP pumps. Chromatographic gradients began with 100% mobile phase A (85% water, 15% methanol, 1 mM ammonium acetate). Between 6.9 and 7 min mobile phase B (100% methanol, 1 mM ammonium acetate) was ramped from 0%–35%. Between 7 – 50 min, mobile phase B was ramped from 35%–100%, and was held at 100 % between 50–60 min. The ESI voltage was –4500V generating negatively-charged [M-H][–] molecular and fragment ions.

Mass spectrometry (lipid quantification method)—Rapid separation of analytes was obtained using 10 μ l injections of analyte (Agilent 1100 series autosampler, Wilmington, DE) onto a Zorbax eclipse XDB 2.1 $\times$ 50 mm reversed phase column. Gradient elution (200 μ l/min) was formed under pressure on a pair of Shimadzu (Columbia, Maryland) 10AdVP pumps. Mass spectrometric analysis was performed with an Applied Biosystems/MDS Sciex (Foster

City, CA) API 3000 triple quadrupole mass spectrometer equipped with an electrospray ionization turbo ion source. Levels of each compound were analyzed by multiple reactions monitoring (MRM) on the LC/MS/MS system. Mass spectrometric conditions were optimized for each compound using direct flow injection of synthetic standards of each compound. 90 % methanol eluants were tested for levels of 18-, 19- and 20-HETE-DA using MRM tandem mass spectrometric methods with parent and fragment ions as follows: in negative ion mode, 18-HETE-DA 454.3→396.4, 19-HETE-DA 454.3→410.3 and 20-HETE-DA 454.3→424.2. Positive control experiments were performed using AA as substrate, the levels of the metabolite 20-HETE were monitored using the following parent and fragment ions in negative ion mode: 319.2→289.3³².

Date analysis—The data was fitted to the Michaelis-Menten model to determine the Vmax (maximum enzyme velocity) and the Km (the concentration of substrate needed to get half-maximal velocity, using Graphpad Prism (San Diego, CA). The quantitation of analytes was achieved using Analyst software (Applied Biosystems-MDS Sciex; Framingham MA), which quantifies the amount of analyte in the sample based upon linear regression of known concentrations of synthetic standards. FlexStation data was analyzed using the formula: (peak fura ratio-mean baseline fura ration) in SoftMax pro5 (Molecular Devices, Union City, CA). All results were analyzed using One-Way ANOVA with post-hoc Fisher's LSD or Bonferroni (depending on the number of comparisons), using a 95% confidence interval for the mean (SPSS software, Chicago, IL). Data are presented as mean ± standard error of the mean where $p \leq 0.05$ was considered statistically significant. The mole per mole % conversion was calculated using the following formula: (The number of total measured moles of product in 1 ml reaction at 15 min of incubation)/(The total moles of substrate)*100. For 19-, and 20-HETE-DA the % conversion calculation was based on incubation with 50 μM NADA (n=3). For 20-HETE production the % conversion calculation was based on incubation with 16 μM AA (n=1).

RESULTS

Mass spectrometric identification of oxygenated NADA metabolites in liver microsomes

The metabolism of NADA to the hydroxylated metabolites 18-, 19-, and 20-HETE-DA was investigated in rat microsomal fractions enriched with cytochrome P450 enzymes. To identify novel oxygenated metabolites of NADA, lipid extractions and mass spectrometric conditions were optimized to the synthesized standards of 18, 19 and 20-HETE-DA. Identification of 19- and 20-HETE-DA from the microsomal reaction was accomplished by a partially purified lipid extract that was analyzed by HPLC-quadrupole/time-of-flight mass spectrometry (HPLC/QqTOF MS; Fig. 2) and by chromatographic matching using LC/MS/MS (Fig. 1). HPLC/QqTOF MS isolated a lipid compound within the microsomal extract with an exact mass of 454.2958 and which is 4 parts per million of the expected mass of 20-HETE-DA (454.2962). Additionally, the fragmentation pattern of the 454.2958 mass was the same as that produced by the synthetic standard (Fig. 2A, C), which is considered a mass fingerprint. HPLC/QqTOF MS isolated a second lipid compounds within the microsomal extract with an exact mass of 454.2958 and which is 4 parts per million of the expected mass of 19-HETE DA (454.2962; note at this level of resolution has the same exact mass as for 20-HETE-DA). The fragmentation pattern of the 454.2958 mass was the same as that produced by the synthetic standard (Fig. 2B, C). Likewise, with LC/MS/MS using a technique that filters for the parent ion (454.3 amu) and pairs it with a predicted fragment (424.2 amu for 20-HETE-DA and 410.3 amu for 19-HETE-DA; Fig. 1C) revealed a chromatographic match of a compound in the microsomal extract with the 20-, and 19-HETE-DA, respectively (Fig. 1A, B). 18-HETE-DA, if produced, was below our detection limit. Together, these data suggest that 19- and 20-HETE-DA are produced by microsomal enzymatic reactions and are, therefore, likely to be produced endogenously.

The Kinetics of 19-, and 20-HETE-DA formation

Following the chemical synthesis of three potential NADA metabolites (18-, 19-, and 20-HETE-DA), and the identification of the production of 19- and 20-HETE-DA by means of HPLC/MS/MS in activated rat liver microsomes, we investigated the kinetics of the enzymatic reaction. We used AA (16 μ M, 120 μ M, and 300 μ M) as a positive control, and monitored the production of 20-hydroxy eicosatetraenoic acid (20-HETE) 15 min post microsomal activation using LC/MS/MS. As previously reported³³, 20-HETE production by rat liver microsomes was saturable at approximately 100 μ M concentration of AA (data not shown). NADA (5–1200 μ M) was incubated for 15 min with NRS at 37° Celsius and data was fitted to the Michaelis-Menten equation. We observed that 19-, and 20-HETE-DA had equivalent K_m values, but different V_{max} values (V_{MAX} = 45 pmol/mg/min; K_M = 99 μ M; R^2 =0.99 for 20-HETE-DA, and V_{MAX} = 3.6 pmol/mg/min; K_M = 98 μ M, R^2 = 0.98 for 19-HETE-DA; Figure 3A, B). The mole per mole % conversion of 50 μ M NADA to 19- and 20-HETE-DA at 15 min post microsomal activation was calculated as $0.09\% \pm 0.01$ and $5.4\% \pm 1.9$, respectively (means $\pm$ s.d. of $n=3$). The mole per mole % conversion of 16 μ M AA under the same conditions was 6.3 % ($n=1$).

Activity of 18-, 19-, and 20-HETE-dopamines at recombinant human TRPV1 receptors

We further tested whether NADA metabolites evoke calcium influx in HEK 293 cells expressing recombinant human TRPV₁ receptors. We observed that all three hydroxylated metabolites evoked calcium influx in hTRPV₁ HEK 293 expressing cells (Fig. 4A). However, all metabolites exhibited lower potency compared with NADA. To verify the activation of calcium influx via the recombinant human TRPV₁ receptor, hTRPV₁ HEK 293 expressing cells were pre-incubated with the TRPV₁ antagonist I-RTX (Fig. 4B), and likewise challenged with the NADA metabolites. The effect of capsaicin, NADA, 18-, 19- and 20-HETE-DA was significantly attenuated by pretreatment with I-RTX (50 nM). Dopamine was used as a negative control and showed no effect on calcium influx.

DISCUSSION

NADA is a potent endocannabinoid/endovanilloid agonist with a myriad of effects on biological systems. However, the wide variability of NADA's effects cannot be explained solely by activation of CB₁ and TRPV₁. One possible explanation for this variability would be activation of different systems through NADA metabolites. We show here that NADA is metabolized into at least two active metabolites in the microsomal fraction of rat liver.

Microsomal fractions are rich in CYP450 enzymes, which are capable of metabolizing xenobiotics such as the plant-derived cannabinoids, delta 9-tetrahydrocannabinol, and cannabinol²⁴. CYP450 were also shown to metabolize several endogenous mammalian molecules that bear structural similarities to NADA. For example, CYP4A11 and CYP4F2, metabolize AA to the hydroxyl-eicosatetraenoic acid, 20-HETE^{34,35}, turning the common fatty acid to a potent vasoactive compound. Furthermore, CYP450 enzymes can metabolize fatty acid-containing lipids such as the glycerol ester, 2-arachidonoyl glycerol (2-AG)³⁶ and the acyl amide, *N*-arachidonoyl ethanolamine^{37,38}. The metabolism of fatty-acid containing lipids can lead to the formation of a larger family of signaling lipids, acting via novel targets. For example, 2-AG was shown to be efficiently metabolized by COX-2²¹ and to produce the nociceptive prostaglandin metabolite PGE₂-glycerol 25. Our work reveals two novel lipid metabolites of NADA most likely through CYP450 mediated metabolism, and characterizes their activity at recombinant human TRPV₁.

Several studies defined the structure–activity relationship of TRPV₁ receptors with its ligands^{39–42}. Aside from the potent, naturally occurring, plant-derived agonists, capsaicin and

RTX^{39, 43}, TRPV₁ can be activated by the endogenous mammalian lipids NADA and OLDA^{5,6}. In addition, LOX metabolites of AA including the hydroxylated metabolites, 5- and 15-(S)-hydroxy eicosatetraenoic acids, have been shown to activate TRPV₁ receptors *in-vitro*⁴⁴. Hence, it is not surprising that the structurally similar 19-, and 20-, HETE-DA modulate the activity of this receptor.

In the current study we show that hydroxylation of NADA at the omega-, (omega-1), and (omega-2) position activates human TRPV₁ receptors with much lower potency compared to NADA. Considering the endogenous availability and variety of CYP450 enzymes it is possible that this is one mechanism of NADA inactivation. This finding should be interpreted with caution since it has been shown that in different host cell expression systems (HEK293 versus 1321N1) the potency and efficacy of the endogenous receptor agonists is altered⁴⁵. Additionally, the production of NADA metabolites may be governed by other stimuli including, hormones, age, pathology and xenobiotics as has been shown for omega and (omega-1) hydroxylates of AA³⁴. Furthermore, CYP450 enzymes that metabolize AA are also present in the central nervous system. For example, the AA (omega-1) hydroxylase, CYP2J9, is predominantly expressed in the brain including the striatum and hippocampus which are areas rich in endogenous NADA⁴⁶. It is therefore possible that the metabolism of NADA is more effective in certain anatomical regions and that it may activate additional molecular targets.

The cellular localization of NADA (plasma membrane Vs. intracellular membranes; lipid rafts Vs. non-lipid rafts) and its mode of signaling (paracrine Vs. autocrine) is yet unknown. The first NADA metabolite, *N*-arachidonoyl-homovanillyl-amine, was reported by Huang and colleagues⁵. *N*-arachidonoyl-homovanillyl-amine is produced by the methylation of the dopamine moiety by catechol-*O*-methyl-transferase from rat liver cytosol (~2 pmol/mg/min). Here we report the identification and kinetic characterization of two additional metabolites using liver microsomes, offering another metabolic mechanism for NADA inactivation. All three known metabolites have been identified in *in-vitro* preparations. Additional investigations will be needed in order to investigate the metabolism of NADA by other enzymes in additional cellular locations including COX and LOX; these investigations will help determine which enzymatic mechanism for degradation of NADA predominates *in-vivo*. We conclude that NADA metabolism via the CYP450 metabolic pathway may serve as an endogenous mechanism for partial inactivation of NADA, reducing its potency at TRPV₁ receptors. In addition, NADA metabolites may be responsible for activating other as yet unknown molecular targets with greater potency.

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Figure 1. Chromatographic analysis of NADA metabolites

The synthesized NADA metabolites co-eluted with metabolites that were extracted from activated liver microsomes in the presence of NADA. The levels of each compound were analyzed by multiple reactions monitoring (MRM) on an LC/MS/MS system. **A.** the 20-HETE-DA standard co-eluted with the microsomal extraction. The following parent and fragment ions were monitored in negative ion mode 454.3→424.2. **B.** the 19-HETE-DA standard co-eluted with the microsomal extraction. The following parent and fragment ions were monitored in negative ion mode: 454.3→410.3. **C.** A scheme of the predicted diagnostic molecular and fragment ions for 19-, and 20-HETE DA (410.2700 and 424.2857, respectively). Also shown is the dopamine ion fragment (123.0451) that is present for both metabolites. In the negative

mode of electrospray ionization, the molecular and fragment ions are negatively charged due to a loss of a proton and is depicted by the $-H^+$ on the outside of the brackets for each molecular and fragment ion because it is unclear where the loss of the proton occurs.

Figure 2. Mass spectrometric analysis of NADA metabolites

A. Product ion scan mass spectrum of the negatively charged molecular ion of the 20-HETE-DA synthetic standard (grey), and the proposed molecular and fragment ions in the microsomal extract (black). The arrows point to the molecular and fragment ions and labeled with the calculated exact mass. **B.** Product ion scan mass spectrum of the negatively charged molecular ion of the 19-HETE-DA synthetic standard (grey). The arrows point to the molecular and fragment ions and labeled with the calculated exact mass. Product ion scan mass spectrum of the microsomal lipid extract tuned to the negatively charged 19-HETE-DA molecular ion (Black). Arrows point to the peaks of the proposed molecular and fragment ions. **C.** Mass measurements of $[M-H]^-$ and product ions and proposed structural assignments from the microsomal extract determined using HPLC/QqTOF MS.

Figure 3.

Kinetic analysis of NADA metabolite formation by rat liver microsomes. NADA concentration was varied from 5 μM to 1.2 mM and incubated for 15 min with NRS at 37° Celsius. A plot of reaction velocity by NADA concentration was fitted to a Michaelis-Menten equation **A**. $V_{MAX}= 45$; $K_M= 99$; $R^2=0.99$ for NADA's omega-hydroxylation, yielding 20-HETE-DA. **B**. $V_{MAX}= 3.6$; $K_M= 98$, $R^2= 0.98$ for NADA's (omega-1)-hydroxylation, yielding 19-HETE-DA.

Figure 4.

The effects of NADA and its metabolites (18-, 19-, and 20- HETE-DA) on calcium influx in HEK 293 cells stably expressing human TRPV1 receptors. Calcium influx was measured using a 96 well plate reader (FlexStation II, Molecular Devices, Union City, CA). **A.** dose-response curves for NADA, $EC_{50} = 512$ nM, $R^2 = 0.98$, HILLSLOPE = 0.41; 18-HETE-DA, $EC_{50} = 1.6$ μ M, $R^2 = 0.95$, HILLSLOPE = 0.17; 19-HETE-DA, $EC_{50} = 1.7$ μ M, $R^2 = 0.97$, HILLSLOPE = 0.16; 20-HETE-DA, $EC_{50} = 1.5$ μ M, $R^2 = 0.97$, HILLSLOPE = 0.22; $n = 3-15$ wells per condition. **B.** Calcium influx evoked by NADA (1 μ M), capsaicin (100 nM) or 18-, 19, and 20- HETE-DA (10 μ M) in the presence of vehicle (DMSO), was significantly attenuated by the TRPV₁ antagonist I-RTX (50 nM). *, $p < 0.0001$; One-Way ANOVA, Bonferroni post hoc. All error bars indicate S.E.M.

Scheme 1.

Reagents and Conditions: a) TBSCl, Imidazole, DMF, r. t., 18 h; b) Oxalyl chloride, C_6H_6 , r. t., 15 h, Dopamine·HCl, DIEA, DMF, $< 10^\circ \text{C}$, 18 h; c) $n\text{-Bu}_4\text{NF}$, r. t., 15 h