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Microsomal omega-hydroxylated metabolites of *N*-arachidonoyl dopamine are active at recombinant human $TRPV_1$ 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 ($TRPV_1$) 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 $TRPV_1$ 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 $TRPV_1$ 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.

1. 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 (CB_1) [4], and is a potent activator of the non-selective cation channel, transient receptor potential vanilloid type 1 ($TRPV_1$) [5].

In accordance with the activation of two molecular targets with differential anatomical distributions [9], NADA was shown to modulate nociceptive pathways with opposing sensory outcomes [2,3,5,10,11]. While NADA administered intraperitoneally was shown to induce analgesia in the 'hot plate' test [2], intradermal application induced $TRPV_1$-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 stimulation [13]. Conversely, intraplantar injection of NADA was shown to cause inhibition of neuronal responses to mechanical stimulation [10].

NADA is also a potent modulator of other physiological systems. NADA acts as a CB_1 and $TRPV_1$-mediated anti-emetic in a ferret model of emesis [14]. It also induces guinea pig bronchi contractions in a $TRPV_1$-sensitive manner [15]. 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 CB_1, and $TRPV_1$-independent manner where the involvement of cyclooxygenase-2 (COX-2) metabolites, or amidase products, were ruled out [16]. In the immune system, NADA potently inhibited *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine-induced migration of human neutrophils via an unknown target [17]. NADA was also shown to inhibit T cell proliferation and produce immunosuppressive effects via the inhibition of IL-2 and TNF alpha gene transcription [18]. These immunosuppressive effects were mediated through a yet unknown target. Finally,

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Goswami and colleagues [19] 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 tyrosine [5]. 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 dopamine [5]. Another proposed mechanism is through the enzyme catechol-*O*-methyl-transferase which contributes to the partial inactivation of NADA by producing a less potent methylated compound, *N*-arachidonoyl-homovanillyl-amine [5]. 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 lipooxygenases (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 $TRPV_1$ receptors.

2. Materials and methods

2.1. 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. ^{1}H 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% H_2O_2 used as oxidant in this reaction are hazardous or are no longer commercially available. Therefore, a stable urea–H_2O_2 adduct [30] 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 [31]. (a) Each TBS protected HETEs were 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-DA [31].

2.1.1. 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 equiv.) and imidazole (2.5 equiv.) 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.

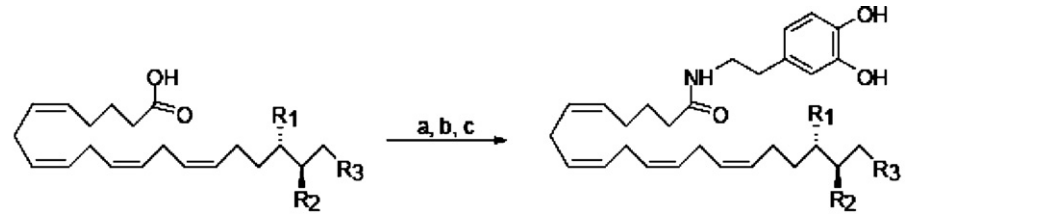
2.1.1.1. TBS protected 18-HETE. Yield = 19 mg (76%) was obtained from 19 mg of *18-HETE*. R_f = 0.78 (ethyl acetate: hexane = 1: 1), ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.44–5.29 (m, 8H), 3.62 (quintet, *J* = 5.63 Hz, 1H), 2.88–2.70 (m, 6H), 2.36 (t, *J* = 7.34 Hz, 2H), 2.17–2.08 (m, 4H), 1.72 (quintet,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

2.1.1.2. 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}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).

2.1.1.3. 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}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).

2.1.2. 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 equiv.). 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



18-HETE: R_1 = OH, R_2 = R_3 = H
19-HETE: R_1 = H, R_2 = OH, R_3 = H
20-HETE: R_1 = H, R_2 = H, R_3 = OH

18-HETE-DA: R_1 = OH, R_2 = R_3 = H
19-HETE-DA: R_1 = H, R_2 = OH, R_3 = H
20-HETE-DA: R_1 = H, R_2 = H, R_3 = OH

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 °C, 18 h; (c) *n*-Bu_4NF, r.t., 15 h.