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Original Contribution

6-Methylnitroarachidonate: A novel esterified nitroalkene that potently inhibits platelet aggregation and exerts cGMP-mediated vascular relaxation

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ABSTRACT

Nitro-fatty acids represent endogenously occurring products of oxidant-induced nitration reactions. We have previously synthesized a mixture of four isomers of nitroarachidonic acid, a novel anti-inflammatory signaling mediator. In this study, we synthesized and chemically and biologically characterized for the first time an esterified nitroalkene derived from the nitration of methylarachidonate (AAMet): 6-methylnitroarachidonate (6-AAMetNO$_2$). Synthesis was performed by reacting AAMet with sodium nitrite under acidic conditions. Analysis by mass spectrometry (positive-ion ESI-MS) showed an $[M+H]^+$ ion of *m/z* 364, characteristic of AAMetNO$_2$. Fragmentation of this ion yielded a daughter ion at *m/z* 317, corresponding to the neutral loss of the nitro group ($[M+H-HNO_2]^+$). Furthermore, IR signal at 1378 cm^{-1} and NMR data confirmed the structure of a 6-nitro-positional isomer. This novel esterified nitroalkene was capable of promoting vascular protective actions including: (a) the induction of vasorelaxation via endothelium-independent mechanisms, associated with an increase in smooth muscle cell cGMP levels, and (b) a potent dose-dependent inhibition of human platelet aggregation. We postulate that 6-AAMetNO$_2$ could be a potential drug for the prevention of vascular and inflammatory diseases, and the presence of the methyl group may increase its pharmacological potential.

Nitric oxide ($^{\bullet}NO$)[1] and $^{\bullet}NO$-derived species have the ability to oxidize, nitrosate, and nitrate biomolecules, and their reactions with unsaturated fatty acids yield a variety of oxidized and nitrated products [1–7]. Oxidative modifications of fatty acids are probably involved in proinflammatory events such as those derived from the interactions between oxidized low-density lipoprotein and endothelial, smooth muscle, and macrophage cells at the arterial wall. These events have been pointed out as responsible for the development of chronic inflammatory diseases such as atherosclerosis [8]. In contrast, an anti-inflammatory scenario may be the result of the interactions of cells with products derived from fatty acid nitration. Nitro-fatty acids have been shown to prevent inflammatory and atherogenic responses in endothelial cells [9] as well as other cell types [6,10–13]. Key reported effects of nitro-fatty acids include modulation of neutrophil and macrophage inflammatory responses (inhibition of cytokines, chemokines, and inducible enzymes involved in oxidative response) [10–14], inhibition of platelet aggregation [15], induction of antioxidant response (heme-oxygenase-1) [16,17], and increase in endothelium-independent vascular relaxation [11,18,19]. Molecular mechanisms associated with these protective actions are based on the electrophilic nature of nitroalkenes, which allows them to bind to nuclear receptors (i.e., peroxisome proliferator-activated receptor-γ) and to form adducts with proinflammatory transcription factors (i.e., NF-κB), modulating cell signaling [11,20–22]. In addition some nitroalkene effects (i.e.,

Abbreviations: NO, nitric oxide; AAMet, methylarachidonic acid; AAMetNO$_2$, methylnitroarachidonate; 6-AAMetNO$_2$, 6-methylarachidonate; AA, arachidonic acid; MS, mass spectrometry; IR, infrared radiation; NMR, nuclear magnetic resonance; cGMP, cyclic guanosine monophosphate; AA(OH)NO$_2$, nitrohydroxyarachidonate; AANO$_2$, nitroarachidonate; COX, cyclooxygenase; ODQ, 1*H*-[1,2,4]oxadiazole[4,3-*a*]quinoxalin-1-one; L-NAME, N^G-nitro-L-arginine methyl ester; SNP, sodium nitroprusside; Nor, norepinephrine; ACh, acetylcholine; IBMX, 3-isobutyl-1-methylxanthine; HPLC-MS, high-pressure liquid chromatography tandem mass spectrometry; oxyHb, oxyhemoglobin; MeOH, methanol; EPR, electron paramagnetic resonance; DAF, 4,5-diaminofluorescein diacetate; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline oxyl-3-oxide; sGC, soluble guanylate cyclase; ELISA, enzyme-linked immunosorbent assay; SMC, smooth muscle cell; ESI-MS, electrospray ionization tandem mass spectrometry; NOS1, endothelial nitric oxide synthase; PGI2, prostacyclin; cAMP, cyclic adenosine monophosphate; COSY, homonuclear correlation spectroscopy; HMBC, heteronuclear multiple bond coherence; HMQC, heteronuclear multiple quantum coherence.

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vasorelaxation) have been associated with the release of $^{\bullet}NO/^{\bullet}NO$-like species during their decomposition in an aqueous milieu [23]. Recent data demonstrate mitochondrial generation of nitroalkenes and mitochondrial protein adduction [24,25]. Moreover, in vivo experiments further support endogenous generation and protective effects of nitro-fatty acids in cardiac ischemia/reperfusion [25] and preconditioning [26] models as well as by a clinically significant outcome: inhibition of neointimal hyperplasia induced by arterial injury [27].

Among other polyunsaturated fatty acids, arachidonic acid (AA), a 20-carbon fatty acid with four *cis* double bounds ((5*Z*,8*Z*,11*Z*,14*Z*)-icosa-5,8,11,14-tetraenoic acid), is a precursor of potent signaling molecules such as prostaglandins, leukotrienes, thromboxanes, and isoprostanes, via enzymatic and nonenzymatic oxidative pathways [3,18,28–31]. Nitration of AA by nitrogen dioxide ($^{\bullet}NO_2$) is kinetic and thermodynamically possible, the *trans* isomer of AA being the major oxidized product formed in biological membranes [18]. In fact, *trans*-AA affects the rigidity, asymmetry, and permeability of membranes, contributing to some pathologies [18,28,32]. In addition, nitrohydroxyarachidonic acid ($AA(OH)NO_2$) and nitroarachidonic acid ($AANO_2$) are also formed in vivo but to lower extents [2,3]. We have recently reported key anti-inflammatory properties exhibited by the four major synthetic isomers of $AANO_2$ (9-, 12-, 14-, and 15-$AANO_2$), including their ability to release $^{\bullet}NO$, induce endothelium-independent vasorelaxation, and modulate macrophage activation by interfering with nitric oxide synthase 2 expression [2,13]. We postulate that the addition of a methyl group into the $AANO_2$ structure could keep its biological properties and improve its biodisposal for in vivo administration as a potential vascular protective drug. The incorporation of the methyl moiety could increase the lipophilicity of $AANO_2$ and presumably facilitate its storage in lipidic matrices while reducing its elimination. In this regard, we hypothesized that nitration of methyl arachidonate (AAMet) could be a source of novel nitro-positional isomers derived from esterified AA, keeping the key anti-inflammatory properties already reported for $AANO_2$. In this study, we synthesized and chemically and biologically characterized a novel esterified nitroalkene: 6-methylnitroarachidonate (6-$AAMetNO_2$).

Materials and methods

This investigation was done in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Comisión Honoraria de Experimentación Animal, Universidad de la República (Montevideo, Uruguay).

Materials

Arachidonic acid and AAMet were purchased from Nu-Check Prep (Elysian, MN, USA). Silica-gel HF thin-layer chromatographic plates were obtained from Analtech. (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (NOC-18) was from Dojindo (Kumamoto, Japan). The solvents used in synthesis were HPLC grade; solvents for mass spectrometry were obtained from Pharmco (Brookfield, CT, USA). 1 *H*-[1,2,4]Oxadiazole[4,3-*a*]quinoxalin-1-one (ODQ), N^G-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), norepinephrine (Nor), acetylcholine (ACh), indomethacin, 3-isobutyl-1-methylxanthine (IBMX), thrombin, oxyhemoglobin (oxyHb), 4,5-diaminofluorescein diacetate (DAF), and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-oxyl-3-oxide (carboxy-PTIO) were obtained from Sigma Chemical (St. Louis, MO, USA).

Synthesis of $AAMetNO_2$

Nitration of AAMet was performed as previously described [2,33,34], with modifications. A solution of AAMet in hexane (1.50 mmol, 3.0 ml) was placed in a round-bottom flask at 0 °C and purged with anhydrous N_2 for 20 min. Then, 1% sulfuric acid (3.0 ml) was added after sodium nitrite ($NaNO_2$, 1.50 mmol in two portions). The biphasic system was kept under vigorous stirring and inert atmosphere at room temperature for 3 days. The reaction mixture was treated with brine (6.0 ml) and extracted with ethyl acetate (3×4.0 ml). The organic layers were mixed and dried over sodium sulfate. The solvent was evaporated in vacuo yielding a yellow oil and the residue was purified by flash chromatography (Sigma Chemical) using a hexane–ethyl ether gradient for elution. For comparative purposes, $AANO_2$ was synthesized as before [2].

Structural characterization of $AAMetNO_2$

The presence and localization of the nitro group in AAMet were determined by ^1H NMR (COSY), HMBC, HMQC, IR, and HPLC-MS. NMR spectra were recorded on a Bruker DPX-400 spectrometer using $CDCl_3$ as solvent. The chemical shift values are expressed in ppm relative to tetramethylsilane as the internal standard. Infrared spectra were recorded on a PerkinElmer 1310 apparatus using potassium bromide tablets; the frequencies are expressed per centimeter. Electrospray ionization tandem mass spectrometry (ESI-MS) was performed for qualitative analysis of $AAMetNO_2$ using a hybrid triple-quadrupole/linear ion trap mass spectrometer (QTRAP 2000; Applied Biosystems/MDS SCIEX). Reaction mixtures or purified fractions were injected in methanol (MeOH) at a flow rate of 10 µl/min with the instrument operating in the positive ionization mode. The skimmer potential was +50 V and desolvation temperature set to 350 °C; for daughter ion analysis, the collision energy was 20 eV. Identification of $AAMetNO_2$ was performed following the transition *m/z* 364/317, corresponding to the neutral loss of the nitro group ($[M+HNO_2]^+$) [2,11].

Platelet aggregation assay

Whole blood was collected from healthy volunteers who were free from drugs that affect platelet functions for at least 15 days. Platelet-rich plasma (PLP) was prepared by centrifuging whole blood collected into sodium citrate (blood:citrate, 9:1, v/v) at 250 *g*, 10 min, at room temperature and washed platelets were prepared from PLP by centrifuging at 900 *g*, 10 min. The platelet pellet was resuspended in 10 ml Tyrode's buffer (in mM: 134 NaCl, 12 $NaHCO_3$, 2.9 KCl, 0.34 Na_2HPO_4, 1.0 $MgCl_2$, 10 Hepes, 5 glucose), pH 7.4, calcium free with sodium citrate (9:1, v/v), and centrifuged at 800 *g*, 10 min. Finally, platelets were resuspended in 2 ml Tyrode's buffer. Platelet aggregation was performed under continuous stirring in the presence of 1 mM Ca^{2+} with 0.5×10^8 platelets/ml at 37 °C. Aggregation was initiated by the addition of thrombin (0.05 U/ml). In the nitroalkenes experiments, platelets were preincubated 1 min with either 6-$AAMetNO_2$ or $AANO_2$ (1 and 5 µM) before thrombin addition. Platelet aggregation was recorded as light transmission at 700 nm for 10 min and results were expressed relative to thrombin condition, which was considered a 100% aggregation response. Control experiments were performed with AAMet and AA, both at 10 µM.

Vasorelaxation assay

Vasorelaxation was evaluated as before [2,35]. Briefly, male Wistar Kyoto rats (250–300 g) were heparinized (100 U/ml, ip) and after 20 min anesthetized with pentobarbital (40 mg/kg, ip). The descending thoracic aorta was carefully removed and freed of connective tissue. Aortic rings (~4 mm in length) were cut and mounted in a Radnoti four-chamber tissue–organ bath system. Each chamber, thermostated at 37 °C, contained 30 ml of Krebs–Henseleit solution (in mM: 20 $NaHCO_3$, 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 1.2 $CaCl_2$, 5.6 glucose), pH 7.4, and bubbled with 95% O_2–5% CO_2. The aortic rings were stretched at