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Manuscript Draft

Title: 6-METHYL-NITROARACHIDONATE:A NOVEL ESTERIFIED NITROALKENE WHICH 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 four isomers mixture of nitroarachidonic acid, a novel anti-inflammatory signaling mediator. Herein, we synthesized, chemically and biologically characterized for a first time an esterified nitroalkene derived from the nitration of methyl-arachidonate (AAMet): 6-methyl-nitroarachidonate (6-AAMetNO2). Synthesis was performed by AAMet reaction with sodium nitrite in acidic conditions. Analysis by mass spectrometry (positive ion ESI-MS) showed a [M+H]+ ion of m/z 364, characteristic of AAMetNO2. Fragmentation of this ion yielded a daughter ion at m/z 317, corresponding to the neutral loss of the nitro group ([M+H-HNO2]+). Furthermore, IR signal at 1378 cm-1 and NMR data confirmed the structure of a 6-nitro positional isomer. This novel esterified nitroalkene showed to be capable of promoting vascular protective actions including: a) the induction of vasorelaxation via endothelium-independent mechanisms, associated with an increase of smooth muscle cells cGMP levels and b) a potent dose-dependent inhibition of human platelet aggregation. We postulate that 6-AAMetNO2 could be a potential drug for prevention of vascular and inflammatory diseases, where the presence of the methyl group may increase its pharmacological potential.

Universidad de la República

Facultad de Medicina

Departamento de Bioquímica

November 22, 2010

Harry Ischiropoulos, Ph D
Associate Editor
Free Radical Biology & Medicine

Dear Dr. Ischiropoulos,

On behalf of my co-authors, I would like to thank you and the reviewers for the insightful and helpful critique of our manuscript No.: FRBM-D-10-00311 entitled "6-Methyl-Nitroarachidonate: A novel esterified nitroalkene which potently inhibits platelet aggregation and exerts cGMP mediated vascular relaxation". We have taken into consideration all the reviewers' comments, clarifying key issues in the text. Changes, additions, new figures and new data in the revised manuscript are in red. As separate document, you will find our point-by point responses to the reviewers' comments.

Once again we thank the reviewers for their efforts and their thoughtful critical comments. We think the manuscript has been significantly improved and we have satisfactory dealt with all raised issues to make it acceptable for the Free Radical Biology and Medicine.

For my co-authors,

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Reviewer I:

1) Is there a difference in kinetics of relaxation in response to AAMetNO2 vs AANO2? Yes, there are some differences in the kinetics of vasorelaxation response that were incorporated in this revised version: 6-AAMetNO_2 caused a greater decrease on active tension of precontracted aortic rings than $AANO_2$ at the time of addition (see new figure 3S in supporting information), which makes sense in virtue of its high stability. However, the final level of tension at end points was similar in both cases (new figure 3S and also new figure 3c). These new findings were included in the revised version (page 14 and 15). We agree that an ideal study would be to determine circulating half times in an appropriate in vivo model, which is under investigation but out of the scope of this manuscript.

2) To answer the reviewer we expand the time for evaluation of $AANO_2$ decay in aqueous solution (see new figure 1S): $AANO_2$ decay with a first order decay as well as $AAMetNO_2$. However, its decay is faster as shown by the observed rate constants. While at the beginning both nitroalkenes have similar kinetics of decay, after 2 minutes the non methylated nitroalkene continue to decay in a faster way. The half life for $AANO_2$ is 17 min which is in accordance to previous results for other nitroalkenes where it has been established that when the number of carbons and double bonds increase, the half life in buffer decrease. This result suggests that the blockade of the carboxyl moiety of the fatty acid increases nitroalkene stability in aqueous conditions.
The extent of aqueous stability of $AAMetNO_2$ should be important since preventing its rapid decay could preserve the observed and reported $AANO_2$ biological properties.

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Reviewer II:

Data of platelet aggregation reported in the original submission was obtained in different experimental conditions that in the revised version. We improved the experimental conditions using washed platelets instead plasma reach platelet. In fact, in the original version 6-AAMetNO$_2$ (20 µM) was used as inductor of platelet aggregation in plasma reach platelet and considered 100% platelet aggregation its response to AA (20 µM). In that case, we didn't use thrombin as inductor of platelet aggregation. In the new experiments reported in this revised version, we used thrombin to induce platelet aggregation and nitrolipids were incubated with washed platelets before thrombin addition. So, the inhibitory effect of free as well as esterified nitroarachidonic acid on thrombin-mediated platelet aggregation is now reported (figure 2). In these experimental conditions, AANO$_2$ or 6-AAMetNO$_2$ (tested up to 50 µM) were unable to induce platelet aggregation (not shown, see page 13). Importantly, all the protective biological effects of these nitroalkenes including inhibition of platelet aggregation were observed al low concentrations (up to 10 µM), which may be the upper security level.

Reviewer III:

1) As suggested by the reviewer we performed a dose-response analysis of NO production comparing methylated and non-methylated AANO$_2$ in presence of BAEC. At the range of concentration tested (up to 20 µM) we didn`t observed statistically differences in the magnitude of induced fluorescence between both compounds (see page 14).

2) The observation of reviewer about c-PTIO could be quenching DAF fluorescence was right. The quenching of DAF by c-PTIO was 10-14% (n=4), whereas the reduction of fluorescence of DAF in presence of c-PTIO generated by nitrolipids was between 20 and 30% at higher concentration of 20 µM. Therefore, considering quenching by c-PTIO, we only were able to detect fluorescence, which was inhibited by c-PTIO, at concentrations equal o higher than 20 µM. This information is now included in the manuscript (page 14-15).

3) BAEC's were incubated with 6-AAMetNO$_2$, AANO$_2$ and control conditions (NOC-18, MeOH, AA and AAMet) for 15 min in culture media, in the presence or absence of L-NAME or c-PTIO. Afterwards, DAF was added and the fluorescence was monitored immediately (time 0) and after 40, 90, 150, 210 and 270 minutes consecutively.These details are now included in the manuscript (page 9 and legend of Figure 5).

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6-METHYL-NITROARACHIDONATE: A NOVEL ESTERIFIED

NITROALKENE WHICH 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 four isomers mixture of nitroarachidonic acid, a novel anti-inflammatory signalling mediator. Herein, we synthesized, chemically and biologically characterized for a first time an esterified nitroalkene derived from the nitration of methyl-arachidonate (AAMet): 6-methyl-nitroarachidonate (6-AAMetNO$_2$). Synthesis was performed by AAMet reaction with sodium nitrite in acidic conditions. Analysis by mass spectrometry (positive ion ESI-MS) showed a [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 showed to be capable of promoting vascular protective actions including: a) the induction of vasorelaxation *via* endothelium-independent mechanisms, associated with an increase of smooth muscle cells 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 prevention of vascular and inflammatory diseases, where the presence of the methyl group may increase its pharmacological potential.

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Introduction

Nitric oxide (·NO) and ·NO-derived species have the ability to oxidize, nitrosate and nitrate biomolecules, where reactions with unsaturated fatty acids yield a variety of oxidized and nitrated products [1-7]. Oxidative modifications of fatty acids are likely involved in pro-inflammatory events as those derived from the interaction between oxidized low-density lipoprotein (LDL) with 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 interaction of cells with products derived from fatty acid nitration. Nitro-fatty acids have been shown to prevent inflammatory and atherogenic responses in endothelial [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] as well as 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 allow 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. vasorelaxation) have been associated with the release of ·NO/·NO-like species during their decomposition in aqueous milieu [23]. Recent data demonstrate mitochondrial generation of nitroalkenes and mitochondrial protein adduction [24, 25]. Moreover, *in vivo* experiments further supports endogenous generation and protective

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effects of nitro-fatty acids in cardiac ischaemia/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 ((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid) is a precursor of potent signaling molecules as prostaglandins, leukotrienes, thromboxanes and isoprostanes, *via* enzymatic and non-enzymatic oxidative pathways [3, 18, 28-31]. Nitration of AA by nitrogen dioxide ($\cdot NO_2$) is kinetic and thermodynamically possible, being the *trans* isomer of AA 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]. Besides, nitrohydroxyarachidonic acid ($AA(OH)NO_2$) and nitroarachidonic acid ($AANO_2$) are also formed *in vivo* but at 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 $\cdot NO$, 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 bio-disposal 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 reduce 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 $AANO_2$. Herein, we synthesized,

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chemically and biologically characterized a novel esterified nitroalkene: 6-methyl-nitroarachidonate (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 US 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). Silica gel HF thin-layer chromatographic (TLC) plates were obtained from Analtech. NOC-18 was from Dojindo (Kumamoto, Japan). The solvents used in synthesis were HPLC graded; solvents for mass spectrometry were obtained from Pharmco (Brookfield, CT). 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), NOC-18 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).

Synthesis of AAMetNO₂- 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 0ºC, and purged with anhydrous N_2 during 20 min. Then, 1% sulfuric acid (3.0 mL) was added following sodium nitrite (NaNO₂, 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

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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 was determined by ^{1}H-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 shifts values are expressed in ppm relative to tetramethylsilane as internal standard. Infrared spectra were recorded on a Perkin-Elmer 1310 apparatus using potassium bromide tablets; the frequencies are expressed in cm^{-1}. Electrospray ionization tandem mass spectrometry (ESI-MS/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 MeOH at a flow rate of 10 µL/min with the instrument operated in the positive ionization mode. The skimmer potential was +50 V and desolvation temperature set to 350ºC; 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 x g, 10 min at room temperature (RT) and washed platelets prepared from PLP by centrifuging at 900 x g, 10 min. The platelet pellet was

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resuspended in 10 mL Tyrodes 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 x g, 10 min Finally, platelets were resuspended in 2 mL Tyrodes buffer. Platelet aggregation was performed under continuous stirring in the presence of 1 mM Ca^{+2} with 0.5 $x10^8$ platelets/mL at 37ºC. 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 as 100% aggregation response. Controls 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, i.p) and after 20 min anesthetized with pentobarbital (40 mg/kg, i.p). 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, thermostatized 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 an optimal passive tension of 2 g and allowed to equilibrate for 1h. After this period, isometric tension was induced by addition of 1 μM norepinephrine (Nor). When tension reached a plateau, aliquots of 6-AAMetNO$_2$ and AANO$_2$ (1-30 μM) were added. The mechanisms involved in the effects of 6-AAMetNO$_2$ were evaluated by incubation of the aortic rings the nitric oxide synthase (NOS) inhibitor L-NAME (30 μM) [36], added 30 min before

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6-AAMetNO$_2$ or the ·NO scavenger OxyHb (10 µM), added 10 min before 6-AAMetNO$_2$; alternatively, the soluble guanylate cyclase (sGC) inhibitor ODQ (30 μM) in DMSO (5mg/mL, <0,1%) [2, 3] or the cyclooxygenase inhibitor indomethacin (10 µM) in MiliQ water, [3, 30] were added to the bath 15 min before 6-AAMetNO$_2$. In all cases, following preincubations aortic rings were contracted with Nor. For experiments with endothelium-denuded arteries, aorta preparations were rubbed in their internal surface with a rough glass capillary; this process was confirmed by the lack of vasorelaxation in response to 1 µM ACh. Control experiments were performed with AAMet and drug vehicle methanol (MeOH) at comparable concentrations.

Nitric oxide releasing activity of 6-AAMetNO$_2$- The capacity of 6-AAMetNO$_2$ to release ·NO was evaluated by electron paramagnetic resonance (EPR), using the specific ·NO scavenger carboxy-PTIO ($k= 10^4$ M^{-1} s^{-1}) as before [2] and DAF as ·NO probe. For EPR, the Bruker EMX spectrometer was tuned as follows: frequency, 9.76 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; receiver gain, 2.83 x 10^3; time constant, 1.28 ms; sweep time, 5.24 s; center field, 3479 G; sweep width, 80.64 G; power, 19.97 mW; scan parameter, 16 scans. Experiments using DAF were performed by incubating confluent bovine aortic endothelial cells (BAECs) with 6-AAMetNO$_2$, AAMet (both at 10, 20 and 50 μM), NOC-18 (5, 10 and 20 μM) or vehicle (MeOH <0,1%, control) in M199 medium for 15 min. In parallel, incubations were performed in the presence of L-NAME (5 mM) or carboxy-PTIO (600 µM) to evaluate at which extent DAF fluorescence response was mediated by ·NO. Next, DAF was added to cell culture at 10 µM and fluorescence (λ_{exc} 485nm, λ_{em} 520nm) was monitored immediately (time 0) and after 90, 150, 210 and 270 min. For comparison, incubations of 6-AAMetNO$_2$ with DAF were carried out in the absence of endothelial cells.

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Cyclic GMP assay- Aortic rings with and without endothelium were prepared as described above. Rings were placed in 10 mL oxygenated Krebs-Henseleit solution and incubated during 30 min with 100 μM IBMX. The induction of cGMP in aortic rings by 6-AAMetNO$_2$ or AANO$_2$ was also analyzed using their corresponding fatty acid precursors. A positive control of sGC activation was performed using SNP or NOC-18. Incubations were carried out for 10 min, the time needed by 6-AAMetNO$_2$ to mediate aortic ring vasorelaxation. In addition, spontaneous cGMP generation was evaluated by incubating aortic rings with buffer under same experimental conditions. For sGC inhibition experiments, rings were preincubated during 15 min in the presence of ODQ (30 μM) before adding the stimuli (6-AAMetNO$_2$ or controls). For cGMP extraction, after incubation rings were frozen in liquid N$_2$, macerated, homogenized in ice with 5% trichloroacetic acid, centrifuged at 15000 x g for 10 min and then supernatants were collected. Trichloroacetic acid was removed by extracting 5 times with 5 volumes of H$_2$O-saturated ether and heating at 70ºC for 5 min. Then samples were bubbled with N$_2$ for 1 hour to remove solvent. Extracts were reconstituted with 50 mM sodium acetate, pH 6.2 and cGMP levels were quantified by ELISA using an inmunoassay kit (Cayman Chemical). cGMP concentrations were normalized to protein content, determined by Bradfors' method using serum albumin as standard.

Data and Statistical Analysis- The relaxation response was quantified by the decrease in vascular tension and expressed as percentage of Nor-induced vascular tension. The cGMP level in vascular smooth muscle was expressed (mean±SD) relative to the value obtained for the positive control SNP, which was considered 100%. Statistical analyses were performed using Newman-Keuls test for platelet aggregation, Mann-Withney test

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for vasorelaxation and multiple comparisons test for cGMP data. p values below 0.05 were considered as statistically significant.

Results

Nitration of AAMet was performed by its reaction with $NaNO_2$ under acidic conditions (see Materials and Methods), the reaction mixture was fractioned and the main nitrated product was characterized. From the chromatographic column four fractions were isolated: the fraction having higher R_f (F1) identified as *trans* AAMet followed by the main product of nitration (5%, F2), and two secondary nitrated products (F3 and F4) exhibiting lower R_f, both near 1% yield (see in supporting information). The positive ion ESI-MS analysis of the main nitrated product (F2) showed the ion with *m/z* 364, characteristic of $[M+H]^+$ ion for $AAMetNO_2$. Fragmentation of the *m/z* 364 ion yielded a *m/z* 317 daughter ion, corresponding to the neutral loss of the nitro ($-NO_2$) group ($[M+H-HNO_2]^+$ Fig. 1a). In addition, the IR analysis of this product confirmed the presence of a $-NO_2$ group attached to the carbon chain of the fatty acid. In accordance to previous reports by us and others [2, 5, 11], the IR spectrum of the product obtained under our experimental conditions showed characteristic absorption bands at 1524 and 1378 cm^{-1}, which corresponds to the nitro N=O bonds (data not shown). Further structural information was obtained from NMR ^1H, COSY, HMQC and HMBC experiments (exemplified in Fig. 1b, see also supporting information). New protons and carbons, respect to AA spectra, appear in the region near to 7 ppm and 130 and 150 ppm, respectively, corresponding to the new system $CH=CNO_2$ (7.10 for proton, and 135.0 and 150.0 for carbons). Besides, changes in the olefinic protons integrations were observed. On the other hand, with respect to allylic protons, changes in the chemical shifts and multiplicities were found. COSY, HMQC and HMBC-correlations with carbons at the 130 and 150 ppm regions unequivocal protons and carbons assignments were done. Besides, for head's and tail's protons, COSY experiment allow us to assign the rest of protons and carbons signals. The complete spectroscopic analysis confirmed

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that nitration occurred at carbon C6 being the product obtained the 6-AAMetNO$_2$ isomer. For fractions F3 and F4 we were unable to determine the exact chemical structures with the same spectroscopic tools. However, according to ESI-MS spectrometry, IR spectroscopy and NMR experiments (see in supporting information) we could claim that nitro-olefination processes took place. In accordance with the chemical characterization of AAMet nitration products, we choose fraction F2 for biological studies and referred to it as 6-AAMetNO$_2$.

In order to compare the stability of 6-AAMetNO$_2$ and AANO$_2$ in aqueous conditions, both nitroalkenes at 10 μM were incubated in 50 mM phosphate buffer, pH 7.4, 100 μM dtpa at 37ºC (see supporting information, Fig. 1S). The decay of both products was analyzed spectrophotometrically for 30 min following the appearance of a peak at 320 nm, in accordance to [23]. 6-AAMetNO$_2$ exhibited greater stability than AANO$_2$ since decayed significantly slowly in aqueous solution.

Next, the effects of 6-AAMetNO$_2$ on human platelet aggregation were evaluated. Addition of 1 and 5 μM 6-AAMetNO$_2$ to washed platelets lead to 50 and 80% inhibition of thrombin- induced aggregation, respectively (Fig.2). Control experiments were performed with AAMet, AANO$_2$ or 6-AAMetNO$_2$ (tested up to 50 μM) which were unable to induce platelet aggregation in these experimental conditions (not shown). Of note, the observed inhibition of platelet aggregation was greater in the presence of 6-AAMetNO$_2$ compared with AANO$_2$ reaching statistically significance at 5 μM (Fig. 2).

The vasorelaxating properties of 6-AAMetNO$_2$ were also evaluated. Similarly to AANO$_2$ [2], at micromolar concentrations 6-AAMetNO$_2$ showed a dose-dependent vasodilator effect on precontracted aortic rings (Fig 3a). The magnitude of vasorelaxation exerted by 10 μM 6-AAMetNO$_2$ was due to the presence of the nitroalkene, since neither the precursor (AAMet, 10 μM) nor the vehicle (MeOH, final

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concentration <0.1%) had any effect on the active tension of contracted aortic rings (40±13.8%, 0.8±1.1% and 1.1±1.5%, respectively; Fig. 3b). Furthermore, the vasorelaxating properties of 6-AAMetNO$_2$ were found to be similar to those exerted by AANO$_2$ (Fig 3c). However, a detailed kinetic analysis showed that 6-AAMetNO$_2$ (10 μM) caused a greater decrease on active tension of precontracted aortic rings than AANO$_2$ (10 μM) at the time of addition. Anyway, the final level of tension at end points was similar in both cases (Fig. 3S). Next, the involvement of endothelium was analyzed: 6-AAMetNO$_2$ induced-vasorelaxation was endothelium independent, since similar extents of vasorelaxation were obtained using denuded aortic rings (Fig. 4). Moreover, the observed effects were independent of endothelial nitric oxide synthase (NOS1) and cyclooxygenase (COX) activities since preincubation of aortic rings with L-NAME or indomethacin did not significantly reduced the vasorelaxing capacity of 6-AAMetNO$_2$ (Fig. 4). On the other hand, the involvement of sGC in our system was determined due to the significant decrease on 6-AAMetNO$_2$ induced-vasorelaxation observed on aortic rings preincubated with the sGC inhibitor ODQ (Fig. 4).

Since sGC activation is triggered by ˙NO, and nitroalkenes -including AANO$_2$- have shown to be capable of releasing ˙NO in aqueous media, we evaluated the potential ability of 6-AAMetNO$_2$ to release ˙NO. In contrast to AANO$_2$, we were unable to detect ˙NO by EPR during 6-AAMetNO$_2$ incubation in aqueous solution (at 300 μM each one, Fig. 5a). Similarly, when 6-AAMetNO$_2$ was incubated in cell-free conditions, DAF fluorescence was not detected (data not shown). Nevertheless, in the presence of endothelial cells, 6-AAMetNO$_2$ -but not its precursor AAMet- leads to a dose-dependent fluorescence response (Fig. 5b). This dose-response was similar in magnitude when using AANO$_2$ or 6-AAMetNO$_2$ (data not shown). 6-AAMetNO$_2$-induced fluorescence was not inhibited by L-NAME (not shown) but was partially inhibited by carboxy-PTIO

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(Fig. 5b). Importantly, this inhibition (20-30% at 20 μM) could only be associated with

·NO capture when nitroalkenes were assayed at concentrations $\geq$ 20μM as the presence

of carboxy-PTIO caused fluorescence quenching (10-14%, evaluated in cuadruplicates

in two independent experiments). As positive control, NOC-18 promoted similar DAF

fluorescence response than 6-AAMetNO$_2$ at comparable low μM concentrations; as a

difference, this was completely inhibited by carboxy-PTIO (data not shown). Taken

together, these suggest that 6-AAMetNO$_2$ was capable of generating modest amounts of

·NO/·NO like species in a cellular environment. This is also in accordance with the fact

that the vasorelaxation capacity of 6-AAMetNO$_2$ was partially reduced (from 31% to

23%, n=2) when aortic rings were preincubated with 10 μM oxyHb (data not shown).

Furthermore, to confirm the involvement of sGC activation on 6-AAMetNO$_2$-mediated

vasorelaxation, we determined the generation of cGMP during AANO$_2$ or 6-AAMetNO$_2$

(both at 10 μM) incubation with aortic rings. Results showed that both nitroalkenes

were capable of inducing cGMP formation (Fig. 6a). The extents of cGMP were similar

for AANO$_2$, 6-AAMetNO$_2$ and SNP and significantly greater than those caused by AA

or AAMet. This response was independent of the presence of endothelium, since similar

results were observed using endothelium-free aortic rings (Fig. 6b). The increase on

cGMP levels generated by 6-AAMetNO$_2$ or SNP was abolished by preincubation of

rings with ODQ (Figs. 6a and 6b), further supporting the involvement of sGC in

nitroalkene induced-cGMP generation.

Discussion

Herein, we described the synthesis of a novel nitroalkene from an esterified fatty acid, AAMet, which exerted relevant vascular protective actions. Furthermore, since we have previously demonstrated the formation of mononitrated-alkenes from AA nitration [2], we compared the products obtained from AAMet and AA nitration. Similarly to the results achieved from AA nitration, the *cis*-alkene isomerization of AAMet was the main reaction (90%) occurring during the incubation with $NaNO_2$, and the nitrated products were obtained in lower yields (between 1 to 5%). The presence of a $-NO_2$ group in the carbon chain of AAMet was confirmed in terms of IR data, with absorbance at 1524 and 1378 cm^{-1} corresponding to N=O moiety [2, 5, 11]. ^{1}H-NMR spectra confirmed this structure, due to new signals assigned to both new olefinic-proton and new olefinic-carbon, i.e. 7.10 ppm and 150.0 ppm corresponding to C$\underline{H}$=C-NO_2 and C=$\underline{C}$-NO_2, respectively (Fig. 1b). However, using AAMet as the fatty acid precursor the main product obtained is the result of the substitution of the alkenic proton 6 by the $-NO_2$ moiety instead of alkenic protons 9-, 12-, 14- or 15-substitutions as occurred with AA [2]. This observation is based on IR and NMR studies, since HMQC, HMBC and COSY experiments offered additional information with respect to allylic protons, changes in the chemical shifts and olefinic protons, and correlations with carbons in the 130-150 ppm regions. The observed differential reactivities between AA and the corresponding methyl ester can be explained by a) the difference in the 5-alkene-electronic density when the ester is present, and b) how the ester derivative of AA it is folded in the milieu and, consequently, how the 5-alkene is exposed to reactive species.

6-AAMetNO$_2$ was able to inhibit thrombin-induced platelet aggregation in a dose-dependent manner, exerting more potent effects than AANO$_2$ (Fig. 2). On the other

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hand, AANO$_2$ and 6-AAMetNO$_2$ promoted comparable extents of vasorelaxation on precontracted aortic rings that were inhibited by the presence of ODQ, suggesting sGC activation (Fig. 4). This was confirmed by the fact that both 6-AAMetNO$_2$ and AANO$_2$ showed similar ability to induce cGMP formation in vascular smooth muscle cells (SMCs) (Figs. 6a and 6b), as detected in intact and denuded aortic rings. Moreover, vasorelaxation and cGMP formation induced by 6-AAMetNO$_2$ were endothelium-independent, suggesting that its interaction with the endothelium did not contribute to generate ˙NO/˙NO-like species responsible for sGC activation (Figs. 4 and 6b). This is consistent with the fact that NOS1 was not involved in 6-AAMetNO$_2$-mediated vasorelaxation since the percentage of vasorelaxation was similar to that obtained in the presence of L-NAME (Fig. 4). To explore the involvement of ˙NO on 6-AAMetNO$_2$ - mediated sGC activation [37], we evaluated ˙NO release in aqueous solution (cell-free conditions) as well as during 6-AAMetNO$_2$ incubation with cells. In contrast with AANO$_2$, 6-AAMetNO$_2$ was not able to release ˙NO in cell-free conditions (Fig. 5a) but, in a cell environment, 6-AAMetNO$_2$ led to generation of ˙NO /NO-like species. This is based on the fact that 6-AAMetNO$_2$, but not AAMet, induced a DAF fluorescence response during incubation with endothelial cells which was partially inhibited by cPTIO, but not by L-NAME (data not shown). So far, there have been reports on several and controversial mechanisms by which nitroalkenes release ˙NO spontaneously [2, 3, 5, 19, 23, 38]. Differences between the ability of AANO$_2$ and 6-AAMetNO$_2$ to release ˙NO in an aqueous milieu could be explained by the lower solubility of the ester compared with the free fatty acid. Alternatively, the position of the -NO$_2$ group (closer to the ester moiety) could decrease the alkene-capability to act as Michael acceptor preventing the formation of an intermediary nitronate for ˙NO release (Nef mechanism, ref. [39]). It is known that, under neutral aqueous conditions, nitroalkene equilibrium

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with vicinal nitrohydroxy derivatives will facilitate formation of the nitronate anion and, following this event, the resulting nitroso intermediate provides a pathway that yields $^\cdot$NO *via* a reaction facilitated by reductants. This nitroso intermediate has a weak C-N bond that yields $^\cdot$NO and a carbon-centered radical product stabilized by conjugation with both the alkene and the OH group [21]. Nitric oxide detection during 6-AAMetNO$_2$ incubation with endothelial cells (Fig. 5b) suggests that 6-AAMetNO$_2$ could be converted to AANO$_2$ upon hydrolytic release by cell lipases and esterases followed by $^\cdot$NO release. If this is true, 6-AAMetNO$_2$ may be relevant as an esterified hydrolyzable reserve of AANO$_2$ in hydrophobic compartments that could contribute to modulate vascular tone through sGC activation.

The involvement of $^\cdot$NO in the vasorelaxating properties of 6-AAMetNO$_2$ is in agreement with the fact that $^\cdot$NO scavengenging by oxyHb led to a reduction of its vasorelaxating activity. However, since this reduction was partial, activation of sGC by other mechanisms cannot be ruled out. Arachidonic metabolites have been described as modulators of vasorelaxation; in particular prostacyclin (PGI2) [40, 41] together with hyperpolarizing K$^+$ fluxes triggered by epoxyeicosatrienoic acids can trigger relaxation of vascular SMCs [42]. Thus, we analyzed whether the generation of COX-derived metabolites of 6-AAMetNO$_2$ could contribute to its ability to promote vasorelaxation. However, this was not the case since vascular relaxation induced by 6-AAMetNO$_2$ was not affected by indomethacin (Fig. 4). This is in agreement with results showing that 6-AAMetNO$_2$ is not a good substrate for prostaglandin endoperoxide H synthase and did not interfere with the normal AA metabolic pathway evaluated in terms of PGI2 production, adenylate cyclase activation and cAMP levels [43, 44] (unpublished data). Alternatively, other cGMP-dependent signaling actions of 6-AAMetNO$_2$ on SMCs may be involved in the modulation of vascular tone [3, 14, 16]. Endothelium was not critical

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for mediating the vasoactive action of 6-AAMetNO$_2$, since inhibition of NOS1 as well as denudation of endothelium revealed similar extents of vessel relaxation (Fig. 4). Overall, our results suggest that 6-AAMetNO$_2$-mediated vasorelaxation involves sGC activation, leading to an increase in cGMP in SMCs. As reported for AA(OH)NO$_2$ [3], 6-AAMetNO$_2$ metabolization by vascular SMCs is likely needed, at least for the $^\cdot$NO-mediated effects. Similarly, metabolism of 6-AAMetNO$_2$ by platelets may regulate the bioavailability of intracellular Ca^{2+} and signaling cascades involving AMPc or GMPc mechanisms.

In conclusion, 6-AAMetNO$_2$ synthesized and characterized for a first time here could be considered as a potential novel vascular protective agent, where the presence of the methyl group serves to improve the bio-disposal of the parent molecule (AANO$_2$). While most of the signaling activities described for nitroalkenes require low concentrations, our data suggest an important pharmacological potential for 6-AAMetNO$_2$, since local concentrations in the nanomolar range would be enough to activate sGC and enhance cGMP-mediated vasorelaxation. Current studies are focusing on the administration of AANO$_2$ and 6-AAMetNO$_2$ in animal models of inflammatory diseases to effectively demonstrate if the presence of this methyl group increases its pharmacological potential *in vivo*.

Acknowledgments

List of Abbreviations

·NO, Nitric oxide

AAMet, methyl-arachidonic acid

AAMetNO$_2$, methylnitroarachidonate

6-AAMetNO$_2$, 6-methyl-nitroarachidonate

AA, arachidonic acid

m/z, mass to charge ratio

MS, mass spectrometry

IR, infrared radiation

NMR, nuclear magnetic resonance

cGMP, cyclic guanosine monophosphate

NOS2, inducible nitric oxide synthase

PPARγ, peroxisome proliferator-activated receptor-γ

·NO$_2$, nitrogen dioxide

AA(OH)NO$_2$, nitrohydroxyarachidonate

AANO$_2$, nitroarachidonate

COX, cyclooxygenase

TLC, thin-layer chromatography

HPLC, high pressure liquid chromatography

ODQ, 1*H*-[1,2,4]oxadiazole[4,3-*a*]quinoxalin-1-one

L-NAME, N^G-nitro-L-arginine methyl ester

SNP, sodium nitropruside

Nor, norepinephrine

Ach, acetylcholine

IBMX, 3-isobutyl-1-methylxanthine

HPLC-MS, HPLC tandem mass spectromerty

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

SMCs, smooth muscle cells

NaNO$_2$, sodium nitrate

ESI-MS/MS, Electrospray ionization tandem mass spectrometry

NOS1, endothelial nitric oxide synthase

PGI2, prostacycline

cAMP, cyclic adenosine monophosphate

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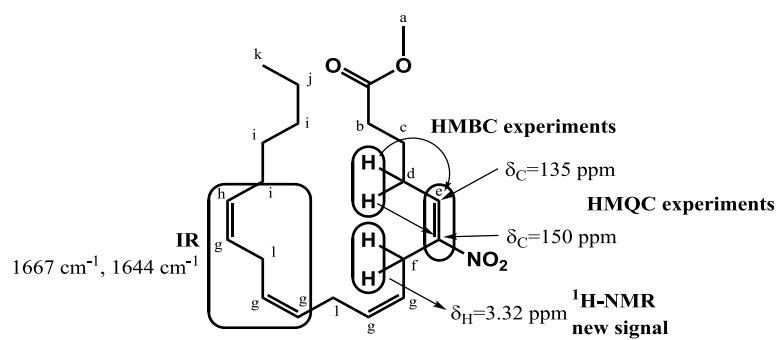
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Figure 1-

a)

b)

^{1}H-RMN (CDCl₃): δ(ppm)= 3.65(Ha), 2.30(Hb), 1.70(Hc), 2.20(Hd), 7.10(He), 2.95-2.35(Hf), 5.42(Hg), 5.54(Hh), 2.02(Hi), 1.32(Hj), 0.93 (Hk), 3.30-3.40(Hl).
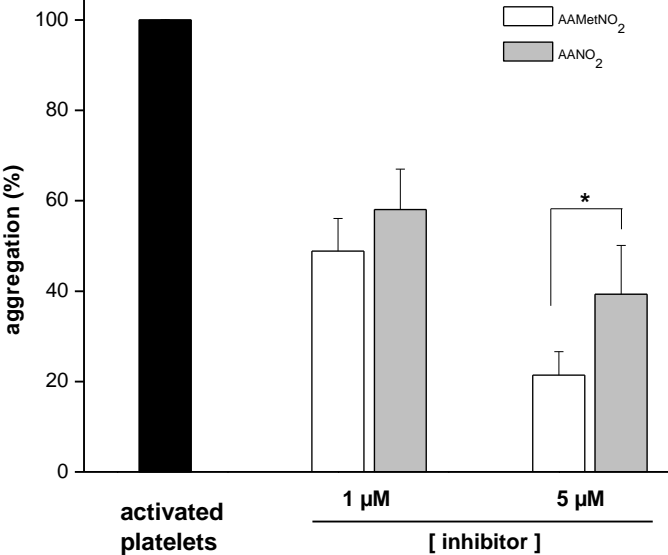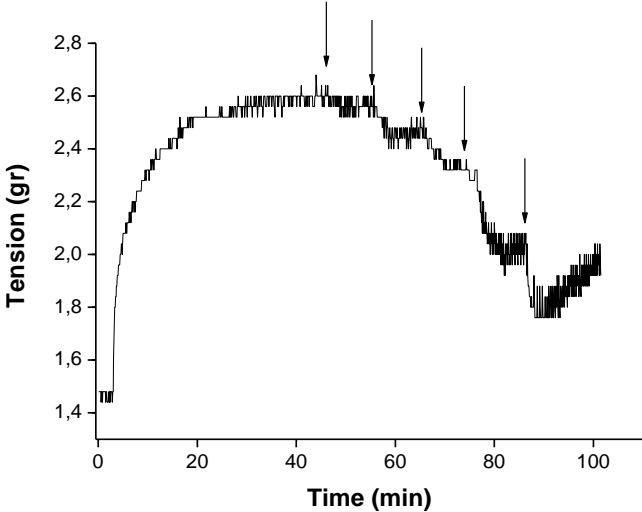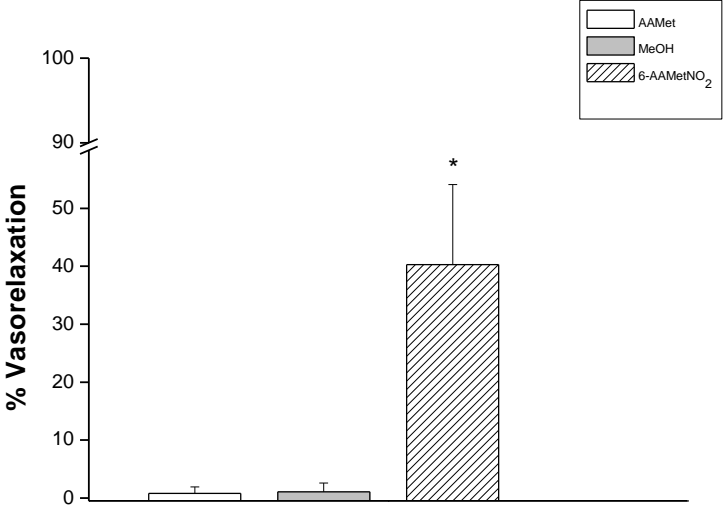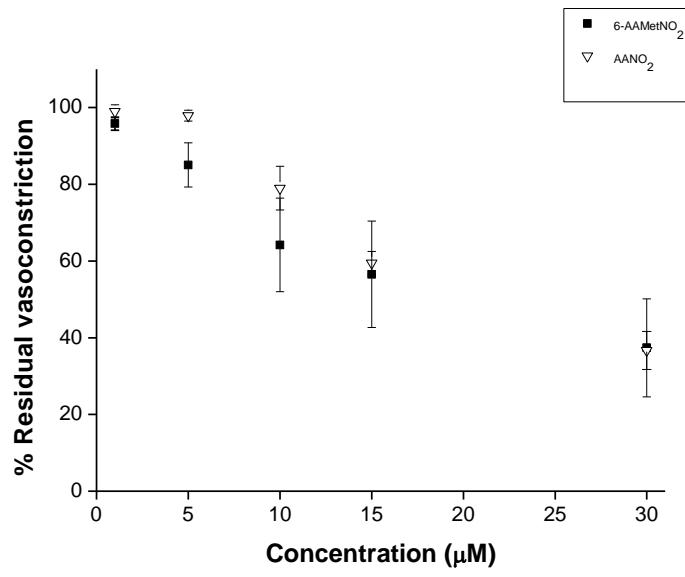
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Figure 2-

Figure 3-

a)

b)

28

c)

Figure 4-

Figure 5-

a)

b)

31

Figure 6-

a)

b)

Figure legends

Figure 1. Chemical characterization of 6-AAMetNO$_2$.

a) ESI-MS analysis of AAMetNO$_2$. Product analysis was performed by MS in the positive ion mode as explained in Material and Methods, following transitions consistent with the neutral loss of the nitro group (*m/z* 364/317). **b)** Summary of NMR and IR analysis of 6-AAMetNO$_2$. ^{1}H NMR spectroscopy in addition to 2D-COSY, HBMC and HMQC analysis were performed to confirm the structure of 6-AAMetNO$_2$.

Figure 2. 6-AAMetNO$_2$ is a powerful inhibitor of platelet aggregation.

Washed platelets (0.5 x 10^8 cells/mL) were preincubated for 2 min at 37°C in Tyrodes buffer with Ca^{+2} (1 mM) in absence (black bar) or presence of either AAMetNO$_2$ (white bar) or AANO$_2$ (gray bar). In all cases, aggregation was initiated by thrombin addition (0.05 U/mL). Light transmission was recorded at 700 nm for 10 min. Data correspond to mean±SEM, n=4, * p< 0.05. AAMet up to 10 μM did not inhibit platelet aggregation (data not shown).

Figure 3. 6-AAMetNO$_2$ induces a doses-dependent vasorelaxation.

a) Temporary course of tension by intact aortic rings contracted with 1 μM Nor. Arrows indicates 6-AAMetNO$_2$ addition to reach final concentrations of 1, 5, 10, 15 and 30 μM. **b)** Percentages of vasorelaxation induced by 10 μM AAMet, MeOH (<0.1%) and 10 μM 6-AAMetNO2 in contracted intact aortic rings added to bath after reaching the contraction plateau generated by 1 μM Nor. Data are represented as mean±SD, n=5, * p<0.05 compared to control conditions. **c)** Percentages of residual vasoconstriction induced by 1, 5, 10, 15 and 30 μM of 6-AAMetNO$_2$ and AANO$_2$ in contracted intact aortic rings. Data are represented as mean±SD, n=3.

Figure 4. 6-AAMetNO$_2$ induces endothelium-independent vasorelaxation.

Vessels with or without endothelium were contracted with 1 μM Nor and then exposed to 10 μM 6-AAMetNO$_2$. In parallel, experiments were done with 6-AAMetNO$_2$ in the presence of 30 μM L-NAME or 10 μM indomethacin. Results are expressed as the mean±SD, n=5. In addition, aortic rings were preincubated with 30 μM ODQ prior to 6-AAMetNO$_2$ addition. Significant differences between the ODQ+6-AAMetNO$_2$ and 6-AAMetNO$_2$ conditions were observed. Results are expressed as the mean±SD, n=5, * p<0.05.

Figure 5. 6-AAMetNO$_2$ releases $^.$NO or $^.$NO like species in the presence of cells.

a) Nitric oxide release by AAMetNO$_2$ or AANO$_2$ (300 μM each) was determined by EPR as explained in "Materials and Methods"; control was performed using AAMet (300 μM). 6-AAMetNO2 was unable to release $^.$NO in aqueous solution. **b)** Confluent bovine aortic endothelial cells were incubated during 15 min with AAMetNO$_2$ or AAMet (both at 20 μM) before adding 10 μM DAF, and fluorescence (λ_{exc} 485nm, λ_{em} 520nm) was recorded along time (0, 40, 90, 150, 210 and 270 minutes). Experiments were carried out in medium M199 (full square for 6-AAMetNO$_2$, open circle for AANO$_2$ and full triangle for MeOH) or in the presence of 600 μM cPTIO (full diamond for 6-AAMetNO$_2$, open star for AANO$_2$ and open square for MeOH). Controls with AAMet and AA were done and the results were similar to obtained with MeOH. Results are expressed as mean±SD of two independent assays.

Figure 6. 6-AAMetNO$_2$ induces cGMP formation.

Aortic rings with (**a**) and without (**b**) endothelium were exposed 10 min to 10 μM of the following compounds: AA, AANO$_2$, AAMet, 6-AAMetNO$_2$ or SNP. Under similar conditions, rings were incubated with ODQ (30 μM, 15 min) before adding 6-

AAMetNO$_2$. Then cGMP levels were determined using an immunoELISA kit as described in Materials and Methods. Data is expressed as cGMP levels (pmol/mg protein) relative to the maximum response obtained by incubation with SNP, and expressed as mean±SD, with at least n=3. *p<0.05; **p<0.01compared to control, AAMet and ODQ+AAMetNO$_2$ conditions.

6-METHYL-NITROARACHIDONATE: A NOVEL ESTERIFIED

NITROALKENE WHICH 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 four isomers mixture of nitroarachidonic acid, a novel anti-inflammatory signalling mediator. Herein, we synthesized, chemically and biologically characterized for a first time an esterified nitroalkene derived from the nitration of methyl-arachidonate (AAMet): 6-methyl-nitroarachidonate (6-AAMetNO$_2$). Synthesis was performed by AAMet reaction with sodium nitrite in acidic conditions. Analysis by mass spectrometry (positive ion ESI-MS) showed a [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 showed to be capable of promoting vascular protective actions including: a) the induction of vasorelaxation *via* endothelium-independent mechanisms, associated with an increase of smooth muscle cells 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 prevention of vascular and inflammatory diseases, where the presence of the methyl group may increase its pharmacological potential.

Keywords: nitric oxide**,** lipid nitration, nitro-fatty acids, nitroarachidonic acid, methyl-nitroarachidonate, cGMP, inflammation, vasorelaxation

Introduction

Nitric oxide (·NO) and ·NO-derived species have the ability to oxidize, nitrosate and nitrate biomolecules, where reactions with unsaturated fatty acids yield a variety of oxidized and nitrated products [1-7]. Oxidative modifications of fatty acids are likely involved in pro-inflammatory events as those derived from the interaction between oxidized low-density lipoprotein (LDL) with 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 interaction of cells with products derived from fatty acid nitration. Nitro-fatty acids have been shown to prevent inflammatory and atherogenic responses in endothelial [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] as well as 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 allow 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. vasorelaxation) have been associated with the release of ·NO/·NO-like species during their decomposition in aqueous milieu [23]. Recent data demonstrate mitochondrial generation of nitroalkenes and mitochondrial protein adduction [24, 25]. Moreover, *in vivo* experiments further supports endogenous generation and protective

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effects of nitro-fatty acids in cardiac ischaemia/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 ((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid) is a precursor of potent signaling molecules as prostaglandins, leukotrienes, thromboxanes and isoprostanes, *via* enzymatic and non-enzymatic oxidative pathways [3, 18, 28-31]. Nitration of AA by nitrogen dioxide ($\cdot NO_2$) is kinetic and thermodynamically possible, being the *trans* isomer of AA 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]. Besides, nitrohydroxyarachidonic acid ($AA(OH)NO_2$) and nitroarachidonic acid ($AANO_2$) are also formed *in vivo* but at 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 $\cdot NO$, 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 bio-disposal 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 reduce 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 $AANO_2$. Herein, we synthesized,

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chemically and biologically characterized a novel esterified nitroalkene: 6-methyl-nitroarachidonate (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 US 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). Silica gel HF thin-layer chromatographic (TLC) plates were obtained from Analtech. NOC-18 was from Dojindo (Kumamoto, Japan). The solvents used in synthesis were HPLC graded; solvents for mass spectrometry were obtained from Pharmco (Brookfield, CT). 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), N^G-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), NOC-18 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).

Synthesis of AAMetNO₂- 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 0ºC, and purged with anhydrous N_2 during 20 min. Then, 1% sulfuric acid (3.0 mL) was added following 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

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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 was determined by ^{1}H-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 shifts values are expressed in ppm relative to tetramethylsilane as internal standard. Infrared spectra were recorded on a Perkin-Elmer 1310 apparatus using potassium bromide tablets; the frequencies are expressed in cm^{-1}. Electrospray ionization tandem mass spectrometry (ESI-MS/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 MeOH at a flow rate of 10 μL/min with the instrument operated in the positive ionization mode. The skimmer potential was +50 V and desolvation temperature set to 350ºC; 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 x g, 10 min at room temperature (RT) and washed platelets prepared from PLP by centrifuging at 900 x g, 10 min. The platelet pellet was

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resuspended in 10 mL Tyrodes buffer (in mM: 134 NaCl, 12 NaHCO$_3$, 2.9 KCl, 0.34 Na$_2$HPO$_4$, 1.0 MgCl$_2$, 10 HEPES, 5 glucose) pH 7.4, calcium free with sodium citrate (9:1, v/v) and centrifuged at 800 x g, 10 min Finally, platelets were resuspended in 2 mL Tyrodes buffer. Platelet aggregation was performed under continuous stirring in the presence of 1 mM Ca^{+2} with 0.5 x10^8 platelets/mL at 37ºC. 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 as 100% aggregation response. Controls 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, i.p) and after 20 min anesthetized with pentobarbital (40 mg/kg, i.p). 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, thermostatized 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$_2$PO$_4$, 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 an optimal passive tension of 2 g and allowed to equilibrate for 1h. After this period, isometric tension was induced by addition of 1 μM norepinephrine (Nor). When tension reached a plateau, aliquots of 6-AAMetNO$_2$ and AANO$_2$ (1-30 μM) were added. The mechanisms involved in the effects of 6-AAMetNO$_2$ were evaluated by incubation of the aortic rings the nitric oxide synthase (NOS) inhibitor L-NAME (30 μM) [36], added 30 min before

6-AAMetNO$_2$ or the ·NO scavenger OxyHb (10 µM), added 10 min before 6-AAMetNO$_2$; alternatively, the soluble guanylate cyclase (sGC) inhibitor ODQ (30 µM) in DMSO (5mg/mL, <0,1%) [2, 3] or the cyclooxygenase inhibitor indomethacin (10 µM) in MiliQ water, [3, 30] were added to the bath 15 min before 6-AAMetNO$_2$. In all cases, following preincubations aortic rings were contracted with Nor. For experiments with endothelium-denuded arteries, aorta preparations were rubbed in their internal surface with a rough glass capillary; this process was confirmed by the lack of vasorelaxation in response to 1 µM ACh. Control experiments were performed with AAMet and drug vehicle methanol (MeOH) at comparable concentrations.

Nitric oxide releasing activity of 6-AAMetNO$_2$- The capacity of 6-AAMetNO$_2$ to release ·NO was evaluated by electron paramagnetic resonance (EPR), using the specific ·NO scavenger carboxy-PTIO ($k= 10^4$ M^{-1} s^{-1}) as before [2] and DAF as ·NO probe. For EPR, the Bruker EMX spectrometer was tuned as follows: frequency, 9.76 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; receiver gain, 2.83 x 10^3; time constant, 1.28 ms; sweep time, 5.24 s; center field, 3479 G; sweep width, 80.64 G; power, 19.97 mW; scan parameter, 16 scans. Experiments using DAF were performed by incubating confluent bovine aortic endothelial cells (BAECs) with 6-AAMetNO$_2$, AAMet (both at 10, 20 and 50 µM), NOC-18 (5, 10 and 20 µM) or vehicle (MeOH <0,1%, control) in M199 medium for 15 min. In parallel, incubations were performed in the presence of L-NAME (5 mM) or carboxy-PTIO (600 µM) to evaluate at which extent DAF fluorescence response was mediated by ·NO. Next, DAF was added to cell culture at 10 µM and fluorescence (λ_{exc} 485nm, λ_{em} 520nm) was monitored immediately (time 0) and after 90, 150, 210 and 270 min. For comparison, incubations of 6-AAMetNO$_2$ with DAF were carried out in the absence of endothelial cells.

Cyclic GMP assay- Aortic rings with and without endothelium were prepared as described above. Rings were placed in 10 mL oxygenated Krebs-Henseleit solution and incubated during 30 min with 100 μM IBMX. The induction of cGMP in aortic rings by 6-AAMetNO$_2$ or AANO$_2$ was also analyzed using their corresponding fatty acid precursors. A positive control of sGC activation was performed using SNP or NOC-18. Incubations were carried out for 10 min, the time needed by 6-AAMetNO$_2$ to mediate aortic ring vasorelaxation. In addition, spontaneous cGMP generation was evaluated by incubating aortic rings with buffer under same experimental conditions. For sGC inhibition experiments, rings were preincubated during 15 min in the presence of ODQ (30 μM) before adding the stimuli (6-AAMetNO$_2$ or controls). For cGMP extraction, after incubation rings were frozen in liquid N$_2$, macerated, homogenized in ice with 5% trichloroacetic acid, centrifuged at 15000 x g for 10 min and then supernatants were collected. Trichloroacetic acid was removed by extracting 5 times with 5 volumes of H$_2$O-saturated ether and heating at 70ºC for 5 min. Then samples were bubbled with N$_2$ for 1 hour to remove solvent. Extracts were reconstituted with 50 mM sodium acetate, pH 6.2 and cGMP levels were quantified by ELISA using an inmunoassay kit (Cayman Chemical). cGMP concentrations were normalized to protein content, determined by Bradfors' method using serum albumin as standard.

Data and Statistical Analysis- The relaxation response was quantified by the decrease in vascular tension and expressed as percentage of Nor-induced vascular tension. The cGMP level in vascular smooth muscle was expressed (mean±SD) relative to the value obtained for the positive control SNP, which was considered 100%. Statistical analyses were performed using Newman-Keuls test for platelet aggregation, Mann-Withney test

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for vasorelaxation and multiple comparisons test for cGMP data. p values below 0.05 were considered as statistically significant.

Results

Nitration of AAMet was performed by its reaction with $NaNO_2$ under acidic conditions (see Materials and Methods), the reaction mixture was fractioned and the main nitrated product was characterized. From the chromatographic column four fractions were isolated: the fraction having higher R_f (F1) identified as *trans* AAMet followed by the main product of nitration (5%, F2), and two secondary nitrated products (F3 and F4) exhibiting lower R_f, both near 1% yield (see in supporting information). The positive ion ESI-MS analysis of the main nitrated product (F2) showed the ion with *m/z*364, characteristic of $[M+H]^+$ ion for $AAMetNO_2$. Fragmentation of the *m/z* 364 ion yielded a *m/z* 317 daughter ion, corresponding to the neutral loss of the nitro ($-NO_2$) group ($[M+H-HNO_2]^+$ Fig. 1a). In addition, the IR analysis of this product confirmed the presence of a $-NO_2$ group attached to the carbon chain of the fatty acid. In accordance to previous reports by us and others [2, 5, 11], the IR spectrum of the product obtained under our experimental conditions showed characteristic absorption bands at 1524 and 1378 cm^{-1}, which corresponds to the nitro $N=O$ bonds (data not shown). Further structural information was obtained from NMR ^1H, COSY, HMQC and HMBC experiments (exemplified in Fig. 1b, see also supporting information). New protons and carbons, respect to AA spectra, appear in the region near to 7 ppm and 130 and 150 ppm, respectively, corresponding to the new system $CH=CNO_2$ (7.10 for proton, and 135.0 and 150.0 for carbons). Besides, changes in the olefinic protons integrations were observed. On the other hand, with respect to allylic protons, changes in the chemical shifts and multiplicities were found. COSY, HMQC and HMBC-correlations with carbons at the 130 and 150 ppm regions unequivocal protons and carbons assignments were done. Besides, for head's and tail's protons, COSY experiment allow us to assign the rest of protons and carbons signals. The complete spectroscopic analysis confirmed

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that nitration occurred at carbon C6 being the product obtained the 6-AAMetNO$_2$ isomer. For fractions F3 and F4 we were unable to determine the exact chemical structures with the same spectroscopic tools. However, according to ESI-MS spectrometry, IR spectroscopy and NMR experiments (see in supporting information) we could claim that nitro-olefination processes took place. In accordance with the chemical characterization of AAMet nitration products, we choose fraction F2 for biological studies and referred to it as 6-AAMetNO$_2$.

In order to compare the stability of 6-AAMetNO$_2$ and AANO$_2$ in aqueous conditions, both nitroalkenes at 10 μM were incubated in 50 mM phosphate buffer, pH 7.4, 100 μM dtpa at 37ºC (see supporting information, Fig. 1S). The decay of both products was analyzed spectrophotometrically for 30 min following the appearance of a peak at 320 nm, in accordance to [23]. 6-AAMetNO$_2$ exhibited greater stability than AANO$_2$ since decayed significantly slowly in aqueous solution.

Next, the effects of 6-AAMetNO$_2$ on human platelet aggregation were evaluated. Addition of 1 and 5 μM 6-AAMetNO$_2$ to washed platelets lead to 50 and 80% inhibition of thrombin- induced aggregation, respectively (Fig.2). Control experiments were performed with AAMet, AANO$_2$ or 6-AAMetNO$_2$ (tested up to 50 μM) which were unable to induce platelet aggregation in these experimental conditions (not shown). Of note, the observed inhibition of platelet aggregation was greater in the presence of 6-AAMetNO$_2$ compared with AANO$_2$ reaching statistically significance at 5 μM (Fig. 2).

The vasorelaxating properties of 6-AAMetNO$_2$ were also evaluated. Similarly to AANO$_2$ [2], at micromolar concentrations 6-AAMetNO$_2$ showed a dose-dependent vasodilator effect on precontracted aortic rings (Fig 3a). The magnitude of vasorelaxation exerted by 10 μM 6-AAMetNO$_2$ was due to the presence of the nitroalkene, since neither the precursor (AAMet, 10 μM) nor the vehicle (MeOH, final

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concentration <0.1%) had any effect on the active tension of contracted aortic rings (40±13.8%, 0.8±1.1% and 1.1±1.5%, respectively; Fig. 3b). Furthermore, the vasorelaxating properties of 6-AAMetNO$_2$ were found to be similar to those exerted by AANO$_2$ (Fig 3c). However, a detailed kinetic analysis showed that 6-AAMetNO$_2$ (10 μM) caused a greater decrease on active tension of precontracted aortic rings than AANO$_2$ (10 μM) at the time of addition. Anyway, the final level of tension at end points was similar in both cases (Fig. 3S). Next, the involvement of endothelium was analyzed: 6-AAMetNO$_2$ induced-vasorelaxation was endothelium independent, since similar extents of vasorelaxation were obtained using denuded aortic rings (Fig. 4). Moreover, the observed effects were independent of endothelial nitric oxide synthase (NOS1) and cyclooxygenase (COX) activities since preincubation of aortic rings with L-NAME or indomethacin did not significantly reduced the vasorelaxing capacity of 6-AAMetNO$_2$ (Fig. 4). On the other hand, the involvement of sGC in our system was determined due to the significant decrease on 6-AAMetNO$_2$ induced-vasorelaxation observed on aortic rings preincubated with the sGC inhibitor ODQ (Fig. 4).

Since sGC activation is triggered by $^{\cdot}$NO, and nitroalkenes -including AANO$_2$- have shown to be capable of releasing $^{\cdot}$NO in aqueous media, we evaluated the potential ability of 6-AAMetNO$_2$ to release $^{\cdot}$NO. In contrast to AANO$_2$, we were unable to detect $^{\cdot}$NO by EPR during 6-AAMetNO$_2$ incubation in aqueous solution (at 300 μM each one, Fig. 5a). Similarly, when 6-AAMetNO$_2$ was incubated in cell-free conditions, DAF fluorescence was not detected (data not shown). Nevertheless, in the presence of endothelial cells, 6-AAMetNO$_2$ -but not its precursor AAMet- leads to a dose-dependent fluorescence response (Fig. 5b). This dose-response was similar in magnitude when using AANO$_2$ or 6-AAMetNO$_2$ (data not shown). 6-AAMetNO$_2$-induced fluorescence was not inhibited by L-NAME (not shown) but was partially inhibited by carboxy-PTIO

(Fig. 5b). Importantly, this inhibition (20-30% at 20 μM) could only be associated with $^{\cdot}NO$ capture when nitroalkenes were assayed at concentrations ≥ 20μM as the presence of carboxy-PTIO caused fluorescence quenching (10-14%, evaluated in cuadruplicates in two independent experiments). As positive control, NOC-18 promoted similar DAF fluorescence response than 6-AAMetNO$_2$ at comparable low μM concentrations; as a difference, this was completely inhibited by carboxy-PTIO (data not shown). Taken together, these suggest that 6-AAMetNO$_2$ was capable of generating modest amounts of $^{\cdot}NO/^{\cdot}NO$ like species in a cellular environment. This is also in accordance with the fact that the vasorelaxation capacity of 6-AAMetNO$_2$ was partially reduced (from 31% to 23%, n=2) when aortic rings were preincubated with 10 μM oxyHb (data not shown). Furthermore, to confirm the involvement of sGC activation on 6-AAMetNO$_2$-mediated vasorelaxation, we determined the generation of cGMP during AANO$_2$ or 6-AAMetNO$_2$ (both at 10 μM) incubation with aortic rings. Results showed that both nitroalkenes were capable of inducing cGMP formation (Fig. 6a). The extents of cGMP were similar for AANO$_2$, 6-AAMetNO$_2$ and SNP and significantly greater than those caused by AA or AAMet. This response was independent of the presence of endothelium, since similar results were observed using endothelium-free aortic rings (Fig. 6b). The increase on cGMP levels generated by 6-AAMetNO$_2$ or SNP was abolished by preincubation of rings with ODQ (Figs. 6a and 6b), further supporting the involvement of sGC in nitroalkene induced-cGMP generation.

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Discussion

Herein, we described the synthesis of a novel nitroalkene from an esterified fatty acid, AAMet, which exerted relevant vascular protective actions. Furthermore, since we have previously demonstrated the formation of mononitrated-alkenes from AA nitration [2], we compared the products obtained from AAMet and AA nitration. Similarly to the results achieved from AA nitration, the *cis*-alkene isomerization of AAMet was the main reaction (90%) occurring during the incubation with $NaNO_2$, and the nitrated products were obtained in lower yields (between 1 to 5%). The presence of a $-NO_2$ group in the carbon chain of AAMet was confirmed in terms of IR data, with absorbance at 1524 and 1378 cm^{-1} corresponding to N=O moiety [2, 5, 11]. ^{1}H-NMR spectra confirmed this structure, due to new signals assigned to both new olefinic-proton and new olefinic-carbon, i.e. 7.10 ppm and 150.0 ppm corresponding to C$\underline{H}$=C-NO_2 and C=$\underline{C}$-NO_2, respectively (Fig. 1b). However, using AAMet as the fatty acid precursor the main product obtained is the result of the substitution of the alkenic proton 6 by the $-NO_2$ moiety instead of alkenic protons 9-, 12-, 14- or 15-substitutions as occurred with AA [2]. This observation is based on IR and NMR studies, since HMQC, HMBC and COSY experiments offered additional information with respect to allylic protons, changes in the chemical shifts and olefinic protons, and correlations with carbons in the 130-150 ppm regions. The observed differential reactivities between AA and the corresponding methyl ester can be explained by a) the difference in the 5-alkene-electronic density when the ester is present, and b) how the ester derivative of AA it is folded in the milieu and, consequently, how the 5-alkene is exposed to reactive species.

6-AAMetNO$_2$ was able to inhibit thrombin-induced platelet aggregation in a dose-dependent manner, exerting more potent effects than AANO$_2$ (Fig. 2). On the other

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hand, AANO$_2$ and 6-AAMetNO$_2$ promoted comparable extents of vasorelaxation on precontracted aortic rings that were inhibited by the presence of ODQ, suggesting sGC activation (Fig. 4). This was confirmed by the fact that both 6-AAMetNO$_2$ and AANO$_2$ showed similar ability to induce cGMP formation in vascular smooth muscle cells (SMCs) (Figs. 6a and 6b), as detected in intact and denuded aortic rings. Moreover, vasorelaxation and cGMP formation induced by 6-AAMetNO$_2$ were endothelium-independent, suggesting that its interaction with the endothelium did not contribute to generate ·NO/·NO-like species responsible for sGC activation (Figs. 4 and 6b). This is consistent with the fact that NOS1 was not involved in 6-AAMetNO$_2$-mediated vasorelaxation since the percentage of vasorelaxation was similar to that obtained in the presence of L-NAME (Fig. 4). To explore the involvement of ·NO on 6-AAMetNO$_2$ -mediated sGC activation [37], we evaluated ·NO release in aqueous solution (cell-free conditions) as well as during 6-AAMetNO$_2$ incubation with cells. In contrast with AANO$_2$, 6-AAMetNO$_2$ was not able to release ·NO in cell-free conditions (Fig. 5a) but, in a cell environment, 6-AAMetNO$_2$ led to generation of ·NO /NO-like species. This is based on the fact that 6-AAMetNO$_2$, but not AAMet, induced a DAF fluorescence response during incubation with endothelial cells which was partially inhibited by cPTIO, but not by L-NAME (data not shown). So far, there have been reports on several and controversial mechanisms by which nitroalkenes release ·NO spontaneously [2, 3, 5, 19, 23, 38]. Differences between the ability of AANO$_2$ and 6-AAMetNO$_2$ to release ·NO in an aqueous milieu could be explained by the lower solubility of the ester compared with the free fatty acid. Alternatively, the position of the -NO$_2$ group (closer to the ester moiety) could decrease the alkene-capability to act as Michael acceptor preventing the formation of an intermediary nitronate for ·NO release (Nef mechanism, ref. [39]). It is known that, under neutral aqueous conditions, nitroalkene equilibrium

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with vicinal nitrohydroxy derivatives will facilitate formation of the nitronate anion and, following this event, the resulting nitroso intermediate provides a pathway that yields ˙NO *via* a reaction facilitated by reductants. This nitroso intermediate has a weak C-N bond that yields ˙NO and a carbon-centered radical product stabilized by conjugation with both the alkene and the OH group [21]. Nitric oxide detection during 6-AAMetNO$_2$ incubation with endothelial cells (Fig. 5b) suggests that 6-AAMetNO$_2$ could be converted to AANO$_2$ upon hydrolytic release by cell lipases and esterases followed by ˙NO release. If this is true, 6-AAMetNO$_2$ may be relevant as an esterified hydrolyzable reserve of AANO$_2$ in hydrophobic compartments that could contribute to modulate vascular tone through sGC activation.

The involvement of ˙NO in the vasorelaxating properties of 6-AAMetNO$_2$ is in agreement with the fact that ˙NO scavengenging by oxyHb led to a reduction of its vasorelaxating activity. However, since this reduction was partial, activation of sGC by other mechanisms cannot be ruled out. Arachidonic metabolites have been described as modulators of vasorelaxation; in particular prostacyclin (PGI2) [40, 41] together with hyperpolarizing K$^+$ fluxes triggered by epoxyeicosatrienoic acids can trigger relaxation of vascular SMCs [42]. Thus, we analyzed whether the generation of COX-derived metabolites of 6-AAMetNO$_2$ could contribute to its ability to promote vasorelaxation. However, this was not the case since vascular relaxation induced by 6-AAMetNO$_2$ was not affected by indomethacin (Fig. 4). This is in agreement with results showing that 6-AAMetNO$_2$ is not a good substrate for prostaglandin endoperoxide H synthase and did not interfere with the normal AA metabolic pathway evaluated in terms of PGI2 production, adenylate cyclase activation and cAMP levels [43, 44] (unpublished data). Alternatively, other cGMP-dependent signaling actions of 6-AAMetNO$_2$ on SMCs may be involved in the modulation of vascular tone [3, 14, 16]. Endothelium was not critical

for mediating the vasoactive action of 6-AAMetNO$_2$, since inhibition of NOS1 as well as denudation of endothelium revealed similar extents of vessel relaxation (Fig. 4). Overall, our results suggest that 6-AAMetNO$_2$-mediated vasorelaxation involves sGC activation, leading to an increase in cGMP in SMCs. As reported for AA(OH)NO$_2$ [3], 6-AAMetNO$_2$ metabolization by vascular SMCs is likely needed, at least for the $^{\cdot}$NO-mediated effects. Similarly, metabolism of 6-AAMetNO$_2$ by platelets may regulate the bioavailability of intracellular Ca^{2+} and signaling cascades involving AMPc or GMPc mechanisms.

In conclusion, 6-AAMetNO$_2$ synthesized and characterized for a first time here could be considered as a potential novel vascular protective agent, where the presence of the methyl group serves to improve the bio-disposal of the parent molecule (AANO$_2$). While most of the signaling activities described for nitroalkenes require low concentrations, our data suggest an important pharmacological potential for 6-AAMetNO$_2$, since local concentrations in the nanomolar range would be enough to activate sGC and enhance cGMP-mediated vasorelaxation. Current studies are focusing on the administration of AANO$_2$ and 6-AAMetNO$_2$ in animal models of inflammatory diseases to effectively demonstrate if the presence of this methyl group increases its pharmacological potential *in vivo*.

Acknowledgments

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List of Abbreviations

$^.$NO, Nitric oxide

AAMet, methyl-arachidonic acid

AAMetNO$_2$, methylnitroarachidonate

6-AAMetNO$_2$, 6-methyl-nitroarachidonate

AA, arachidonic acid

m/z, mass to charge ratio

MS, mass spectrometry

IR, infrared radiation

NMR, nuclear magnetic resonance

cGMP, cyclic guanosine monophosphate

NOS2, inducible nitric oxide synthase

PPARγ, peroxisome proliferator-activated receptor-γ

$^.$NO$_2$, nitrogen dioxide

AA(OH)NO$_2$, nitrohydroxyarachidonate

AANO$_2$, nitroarachidonate

COX, cyclooxygenase

TLC, thin-layer chromatography

HPLC, high pressure liquid chromatography

ODQ, 1*H*-[1,2,4]oxadiazole[4,3-*a*]quinoxalin-1-one

L-NAME, N^G-nitro-L-arginine methyl ester

SNP, sodium nitropruside

Nor, norepinephrine

Ach, acetylcholine

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IBMX, 3-isobutyl-1-methylxanthine

HPLC-MS, HPLC tandem mass spectromerty

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

SMCs, smooth muscle cells

$NaNO_2$, sodium nitrate

ESI-MS/MS, Electrospray ionization tandem mass spectrometry

NOS1, endothelial nitric oxide synthase

PGI2, prostacycline

cAMP, cyclic adenosine monophosphate

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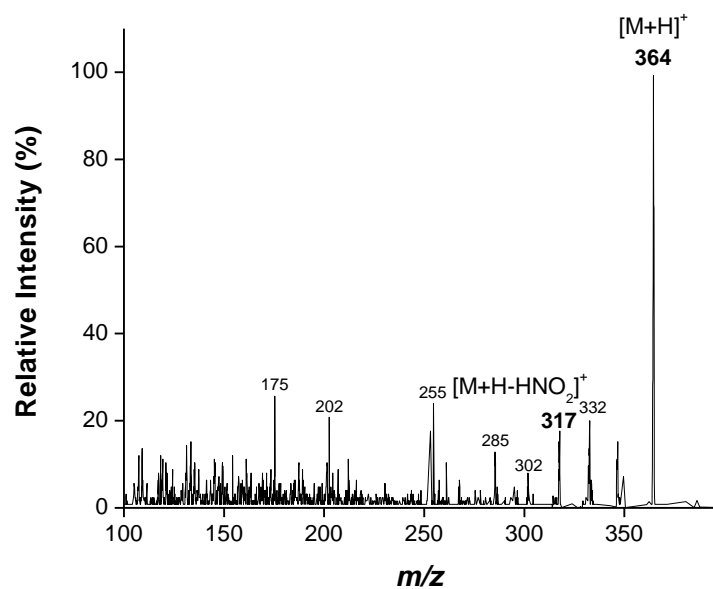
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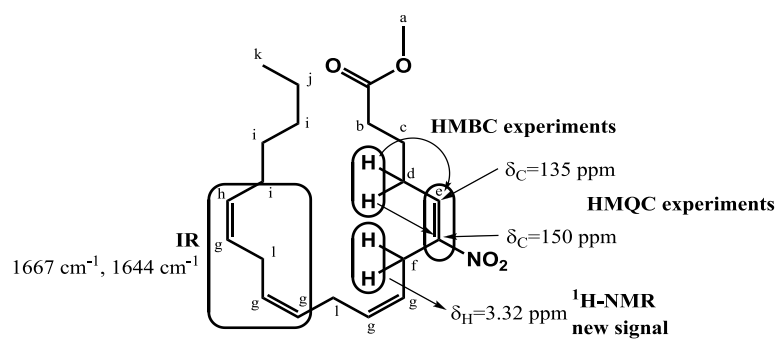
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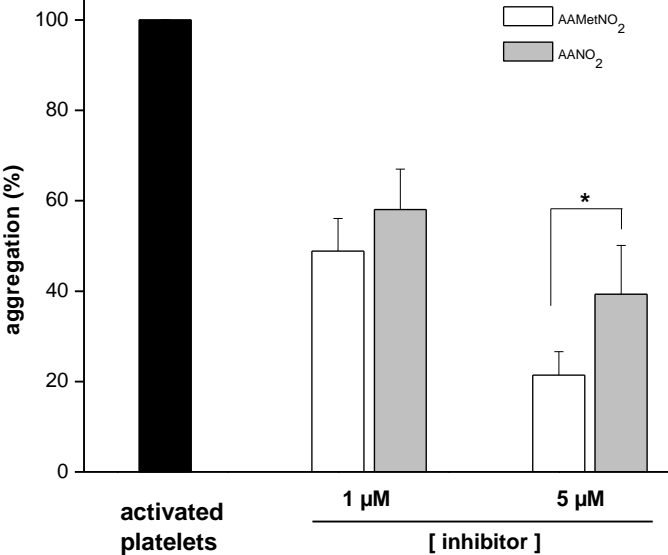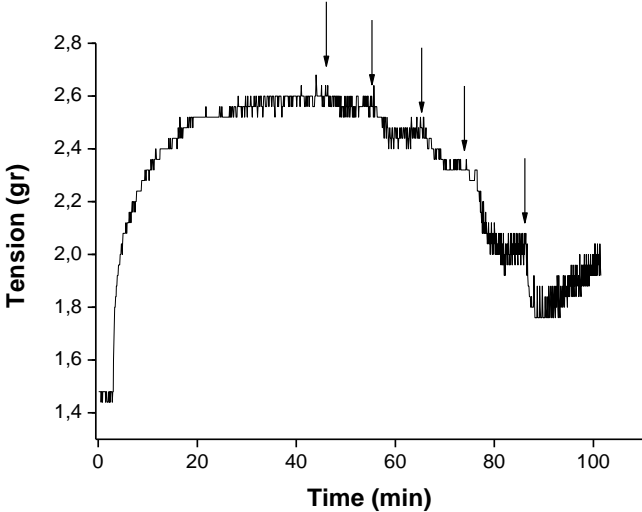
Figure 1-

a)

b)

^{1}H-RMN (CDCl$_3$): δ(ppm)= 3.65(Ha), 2.30(Hb), 1.70(Hc), 2.20(Hd), 7.10(He), 2.95-2.35(Hf), 5.42(Hg), 5.54(Hh), 2.02(Hi), 1.32(Hj), 0.93 (Hk), 3.30-3.40(Hl).
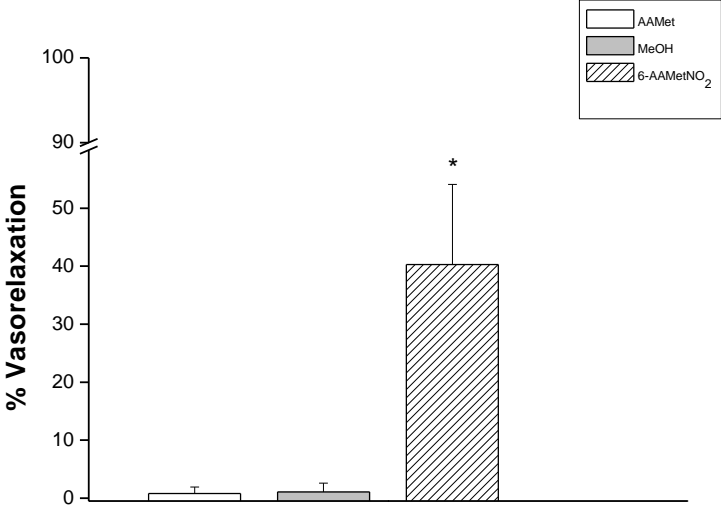
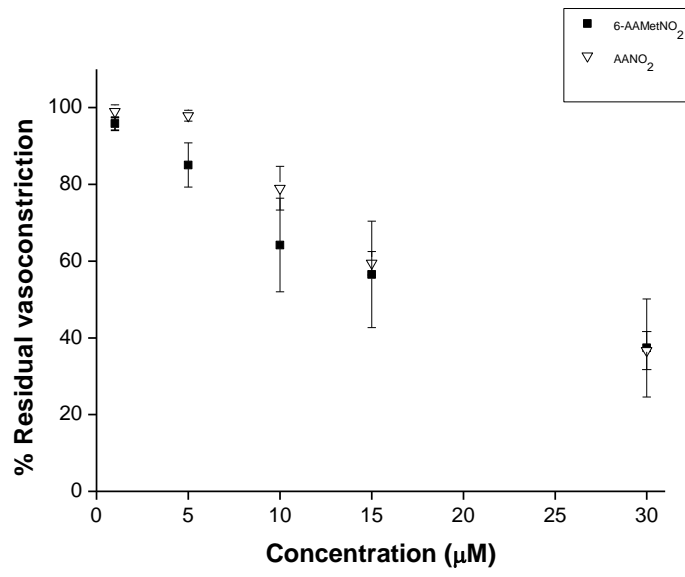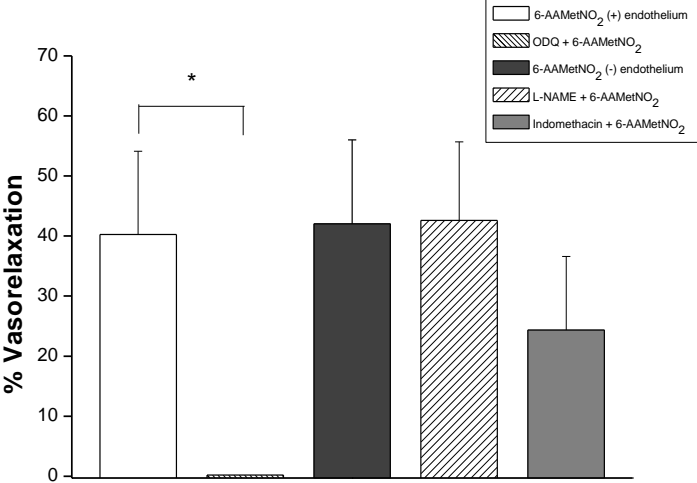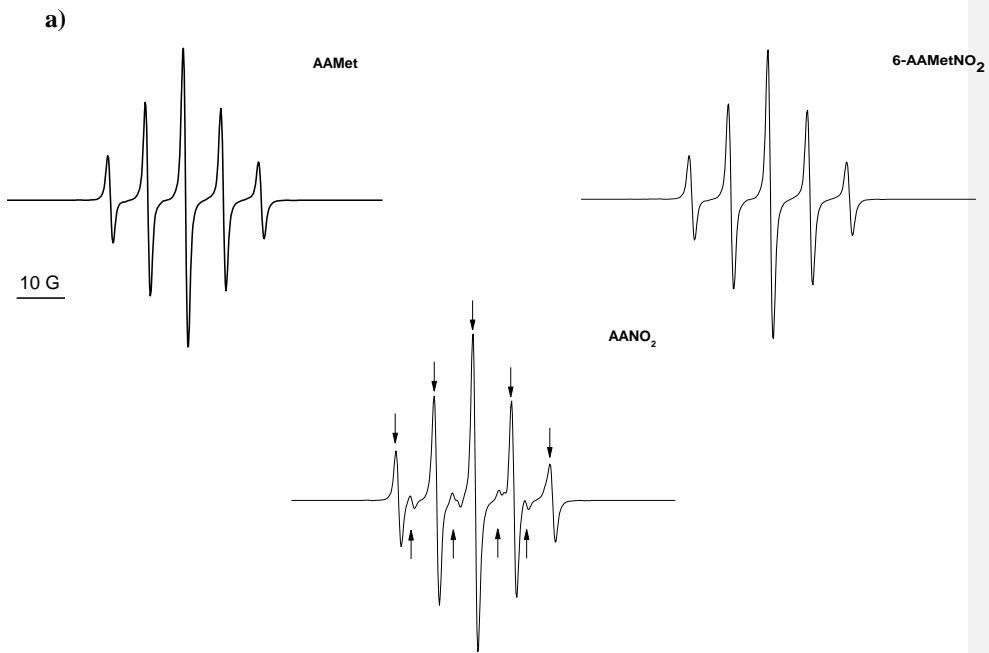26

Figure 2-

Figure 3-
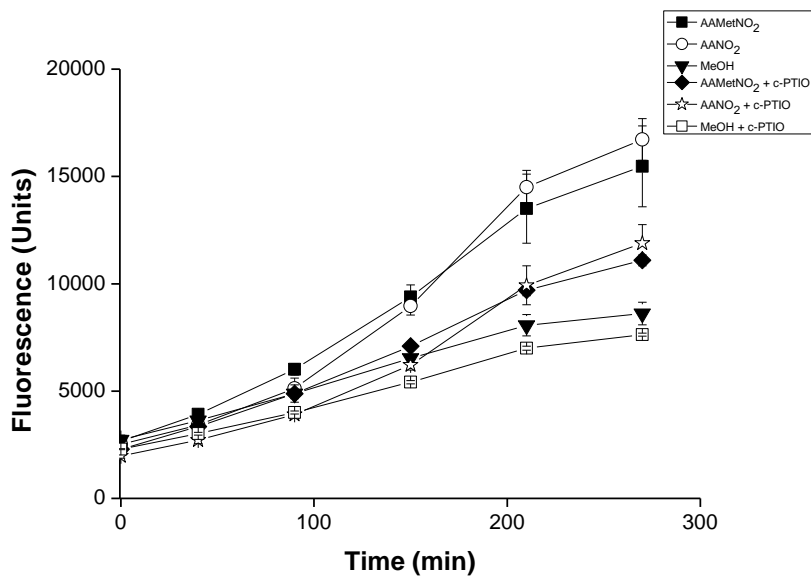
a)

b)

c)

Figure 4-

Figure 5-
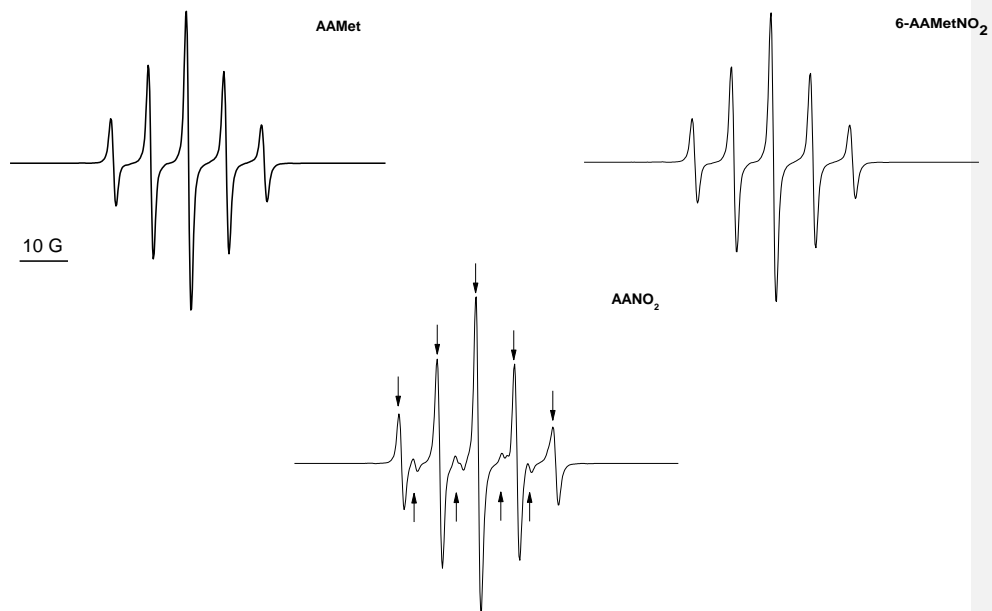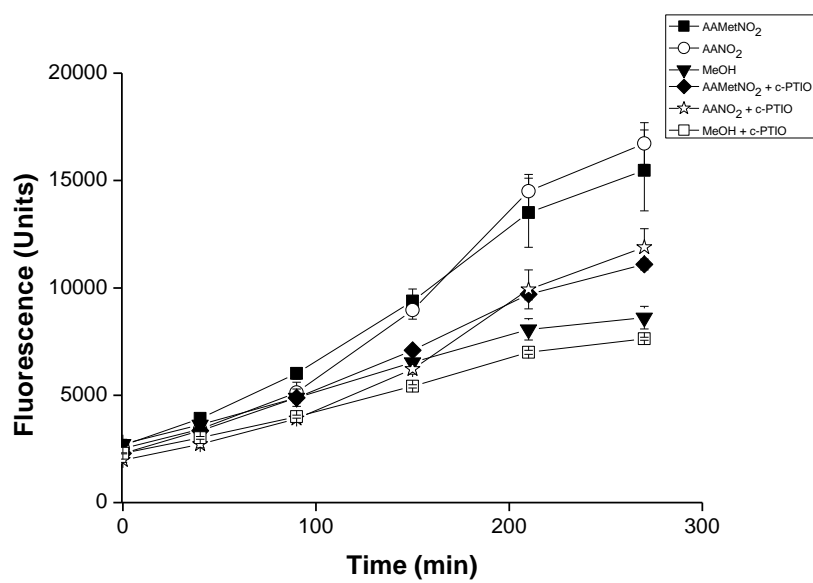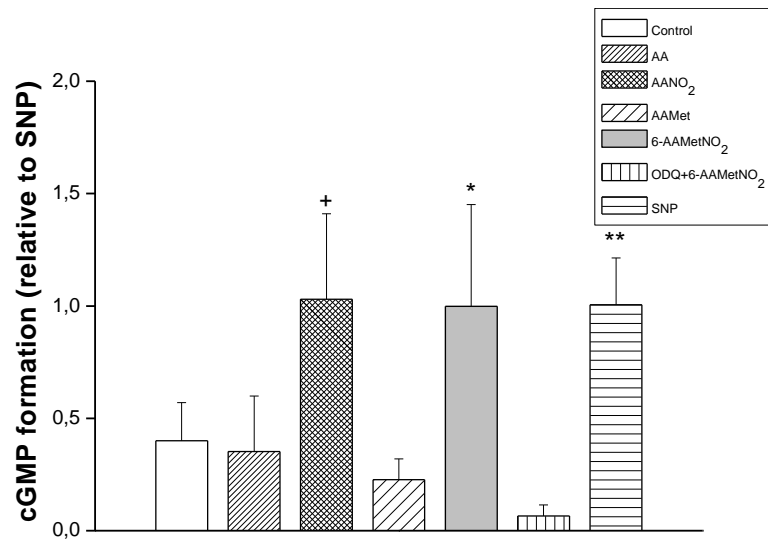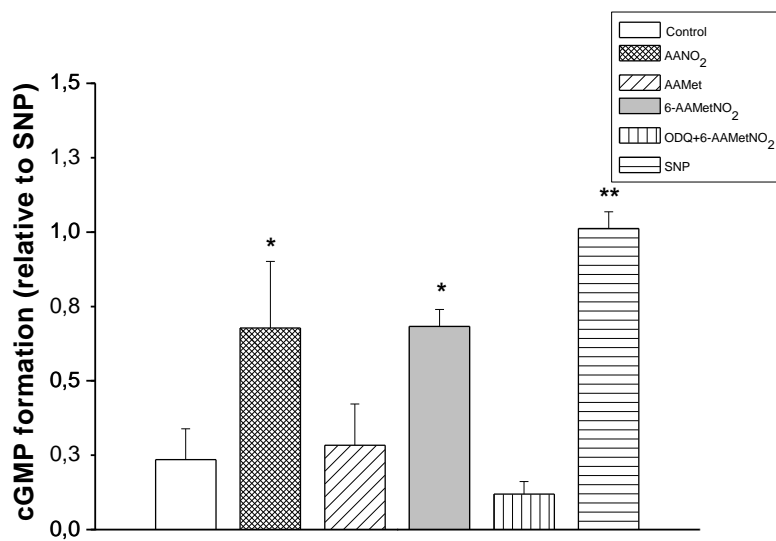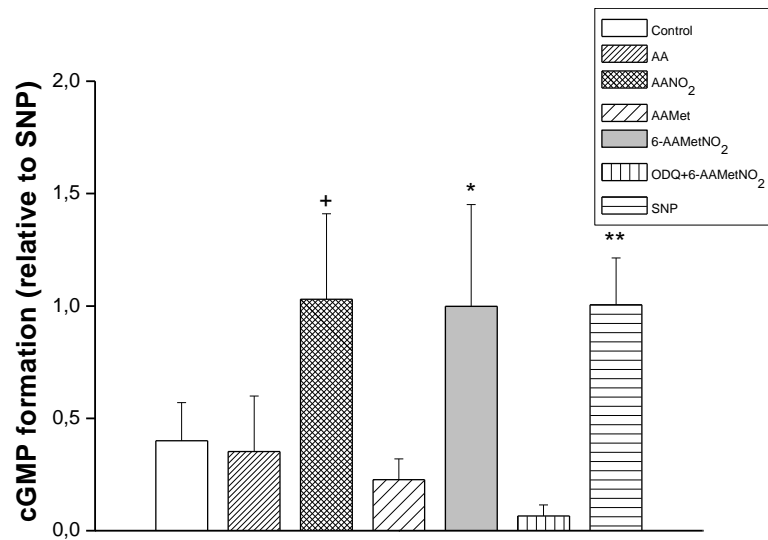
a)

AAMet

6-AAMetNO$_2$

10 G

AANO$_2$

b)

31

Figure 6-

a)

b)

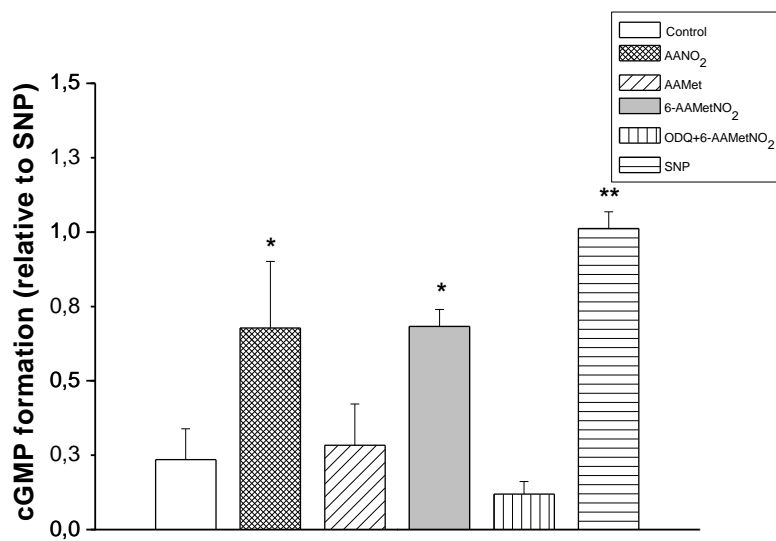**Figure legends**

Figure 1. Chemical characterization of 6-AAMetNO$_2$.

a) ESI-MS analysis of AAMetNO$_2$. Product analysis was performed by MS in the positive ion mode as explained in Material and Methods, following transitions consistent with the neutral loss of the nitro group (*m/z* 364/317). **b)** Summary of NMR and IR analysis of 6-AAMetNO$_2$. ^{1}H NMR spectroscopy in addition to 2D-COSY, HBMC and HMQC analysis were performed to confirm the structure of 6-AAMetNO$_2$.

Figure 2. 6-AAMetNO$_2$ is a powerful inhibitor of platelet aggregation.

Washed platelets (0.5×10^8 cells/mL) were preincubated for 2 min at 37°C in Tyrodes buffer with Ca^{+2} (1 mM) in absence (black bar) or presence of either AAMetNO$_2$ (white bar) or AANO$_2$ (gray bar). In all cases, aggregation was initiated by thrombin addition (0.05 U/mL). Light transmission was recorded at 700 nm for 10 min. Data correspond to mean±SEM, n=4, * p< 0.05. AAMet up to 10 μM did not inhibit platelet aggregation (data not shown).

Figure 3. 6-AAMetNO$_2$ induces a doses-dependent vasorelaxation.

a) Temporary course of tension by intact aortic rings contracted with 1 μM Nor. Arrows indicates 6-AAMetNO$_2$ addition to reach final concentrations of 1, 5, 10, 15 and 30 μM. **b)** Percentages of vasorelaxation induced by 10 μM AAMet, MeOH (<0.1%) and 10 μM 6-AAMetNO2 in contracted intact aortic rings added to bath after reaching the contraction plateau generated by 1 μM Nor. Data are represented as mean±SD, n=5, * p<0.05 compared to control conditions. **c)** Percentages of residual vasoconstriction induced by 1, 5, 10, 15 and 30 μM of 6-AAMetNO$_2$ and AANO$_2$ in contracted intact aortic rings. Data are represented as mean±SD, n=3.

Figure 4. 6-AAMetNO$_2$ induces endothelium-independent vasorelaxation.

Vessels with or without endothelium were contracted with 1 μM Nor and then exposed to 10 μM 6-AAMetNO$_2$. In parallel, experiments were done with 6-AAMetNO$_2$ in the presence of 30 μM L-NAME or 10 μM indomethacin. Results are expressed as the mean±SD, n=5. In addition, aortic rings were preincubated with 30 μM ODQ prior to 6-AAMetNO$_2$ addition. Significant differences between the ODQ+6-AAMetNO$_2$ and 6-AAMetNO$_2$ conditions were observed. Results are expressed as the mean±SD, n=5, * p<0.05.

Figure 5. 6-AAMetNO$_2$ releases ·NO or ·NO like species in the presence of cells.

a) Nitric oxide release by AAMetNO$_2$ or AANO$_2$ (300 μM each) was determined by EPR as explained in "Materials and Methods"; control was performed using AAMet (300 μM). 6-AAMetNO2 was unable to release ·NO in aqueous solution. **b)** Confluent bovine aortic endothelial cells were incubated during 15 min with AAMetNO$_2$ or AAMet (both at 20 μM) before adding 10 μM DAF, and fluorescence (λ_{exc} 485nm, λ_{em} 520nm) was recorded along time (0, 40, 90, 150, 210 and 270 minutes). Experiments were carried out in medium M199 (full square for 6-AAMetNO$_2$, open circle for AANO$_2$ and full triangle for MeOH) or in the presence of 600 μM cPTIO (full diamond for 6-AAMetNO$_2$, open star for AANO$_2$ and open square for MeOH). Controls with AAMet and AA were done and the results were similar to obtained with MeOH. Results are expressed as mean±SD of two independent assays.

Figure 6. 6-AAMetNO$_2$ induces cGMP formation.

Aortic rings with (**a**) and without (**b**) endothelium were exposed 10 min to 10 μM of the following compounds: AA, AANO$_2$, AAMet, 6-AAMetNO$_2$ or SNP. Under similar conditions, rings were incubated with ODQ (30 μM, 15 min) before adding 6-

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AAMetNO$_2$. Then cGMP levels were determined using an immunoELISA kit as described in Materials and Methods. Data is expressed as cGMP levels (pmol/mg protein) relative to the maximum response obtained by incubation with SNP, and expressed as mean±SD, with at least n=3. *p<0.05; **p<0.01compared to control, AAMet and ODQ+AAMetNO$_2$ conditions.

Supplementary Material