Electrophilic characteristics and aqueous behavior of fatty acid nitroalkenes

Valentina Grippo a, Milos Miovic b, Aleksandra Pavicevic b, Martin Kabelac c, Frantisek Hubatka d, Jaroslav Turanek d, Martina Zatloukalova a, Bruce A. Freeman e, Jan Vacek a,b,*

a Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University, Hnevetinska 3, Olomouc, 775 15, Czech Republic
b Faculty of Physical Chemistry, University of Belgrade, Studentski Trg 12-16, Belgrade, Serbia
c Department of Chemistry, Faculty of Science, University of South Bohemia, Bramsisova 31, Ceske Budejovice, 370 05, Czech Republic
d Department of Pharmacology and Immunotherapy, Veterinary Research Institute, v.v.i., Hadcova 70, 621 00, Brno, Czech Republic
e Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15261, USA
f The Czech Academy of Sciences, Institute of Biophysics, Kralovopolska 135, Brno, 612 65, Czech Republic

ARTICLE INFO

Keywords:
Nitro-fatty acid
Electrophile
Nitric oxide
Free radical
Micelle

ABSTRACT

Fatty acid nitroalkenes (NO2-FA) are endogenously-generated products of the reaction of metabolic and inflammatory-derived nitrogen dioxide (NO2) with unsaturated fatty acids. These species mediate signaling actions and induce adaptive responses in preclinical models of inflammatory and metabolic diseases. The nitroalkene substituent possesses an electrophilic nature, resulting in rapid and reversible reactions with biological nucleophiles such as cysteine, thus supporting post-translational modifications (PTM) of proteins having susceptible nucleophilic centers. These reactions contribute to enzyme regulation, modulation of inflammation and cell proliferation and the regulation of gene expression responses. Herein, focus is placed on the reduction-oxidation (redox) characteristics and stability of specific NO2-FA regioisomers having biological and clinical relevance; nitro-oleic acid (NO2-OA), bis-allylic nitro-linoleic acid (NO2-LA) and the conjugated diene-containing nitro-conjugated linoleic acid (NO2-cLA). Cyclic and alternating-current voltammetry and chronopotentiometry were used to study the reduction potentials of these NO2-FA. R-NO2 reduction was observed around −0.8 V (vs. Ag/AgCl/3 M KCl) and is related to relative NO2-FA electrophilicity. This reduction process could be utilized for the evaluation of NO2-FA stability in aqueous milieu, shown herein to be pH dependent. In addition, electron paramagnetic resonance (EPR) spectroscopy was used to define the stability of the nitroalkene moiety under aqueous conditions, specifically under conditions where nitric oxide (NO) release could be detected. The experimental data were supported by density functional theory calculations using 6-311++G (d,p) basis set and B3LYP functional. Based on experimental and computational approaches, the relative electrophilicities of these NO2-FA are NO2-cLA > NO2-LA > NO2-OA. Micellarization and vesiculation largely define these biophysical characteristics in aqueous, nucleophile-free conditions. At concentrations below the critical micellar concentration (CMC), monomeric NO2-FA predominate, while at greater concentrations a micellar phase consisting of self-assembled lipid structures predominates. The CMC, determined by dynamic light scattering in 0.1 M phosphate buffer (pH 7.4) at 25 °C, was 6.9 (NO2-LA) 10.6 (NO2-OA) and 42.3 µM (NO2-cLA), respectively. In aggregate, this study provides new insight into the biophysical properties of NO2-FA that are important for better understanding the cell signaling and pharmacological potential of this class of mediators.

1. Introduction

Elevated levels of nitrated amino acids, nucleotides and lipids have been detected in tissues and fluids of patients undergoing inflammatory conditions [1,2]. These derivatives are generated in vivo and in vitro upon exposure to nitric oxide (NO)- and nitrite (NO2)-derived RS that yield nitrogen dioxide (NO2). For example, NO and NO2 are precursors for the generation of peroxynitrous acid (ONOOH), nitroso-peroxocarbonate (ONOOCO2) and dinitrogen trioxide (N2O3). Also, NO2 can be oxidized by heme peroxidases to yield NO2 [3-5]. When NO2 reacts with the double bond of an unsaturated acyl chain, forming

* Corresponding author. Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University, Hnevetinska 3, Olomouc, 775 15, Czech Republic.
E-mail address: jan.vacek@upol.cz (J. Vacek).

https://doi.org/10.1016/j.redox.2020.101756
Received 20 July 2020; Received in revised form 28 September 2020; Accepted 8 October 2020
Available online 12 October 2020
2213-2317/© 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license
a nitroalkene substituent, the products have generically been termed nitro-fatty acids (NO$_2$-FA). NO$_2$-FA found in living organisms are a result of the nitration of oleic (C18:1) [6], linoleic (C18:2) [7], linolenic (C18:3) and arachidonic (C20:4) [8] acids. Current data supports that NO$_2$-FA display anti-inflammatory and adaptive signaling actions. The administration of synthetic natural and non-natural nitroalkenols also can mitigate pathogenic responses in preclinical models of cardiovascular, ischemic, metabolic, inflammatory and fibrotic diseases [9–12].

NO$_2$-FA undergo reversible Michael addition [13] with cellular nucleophiles, predominantly Cys, that frequently reside in functionally-significant cell signaling, metabolic and transcriptional regulatory proteins. The nitro group on the alkene moiety has a strong electron-withdrawing behavior that confers electron deficiency to the β-carbon and responsiveness to nucleophilic attack by thiolates. In nitro-conjugated linoleic acid, both the β- and δ-carbons with respect to the nitro group are susceptible to Michael addition [14]. The β-adduct is the kinetic product while the δ-adduct is the thermodynamic product. This Michael addition is reversible and kinetically rapid, which differentiates the NO$_2$-FA from other endogenous signaling electrophiles (such as aldehydic fatty acid oxidation products and cyclopentenone prostaglandins) that react slowly and irreversibly with nucleophiles [15, 16].

NO$_2$-FA show NO donor activities in vitro [17–19]. The mechanism(s) underlying aqueous NO release by nitroalkenols has not been definitively established, but may include a modified Nef reaction. A possible mechanism is that under neutral aqueous conditions, the nitroalkene facilitates the formation of the nitronate anion; then followed by protonation and deprotonation steps resulting in a nitroso intermediate. This nitroso intermediate has a weak C–N bond that yields NO [20]. The presence of the nitrite radical was supported by chemiluminescence measurements and EPR-based methodologies [21]. It is noted that in these aqueous-based NO donor studies and buffer-perfused vascular rings that claim a vasodilatory action of NO$_2$-FA, experimental results might have been complicated by a) contaminating nitrite in NO$_2$-FA preparations or b) unique reactions that occur in pure aqueous milieu as opposed to those operative in more complex biological fluids and tissues. For example, extensive preclinical pharmacokinetics and toxicology studies, as well as human Phase 1 and Phase 2 trials do not show an acute effect of intravenous or oral NO$_2$-FA on blood pressure or pulse rate, suggesting that NO and cGMP-dependent signaling mechanisms have not been engaged (unpublished). In any event, these previous studies motivated the present more detailed analysis of the kinetics of NO generation by fatty acid nitroalkenoles and mitigating factors.

The endogenous formation of NO$_2$-FA [3–5] will be strongly influenced by diet, which may contain pre-formed NO$_2$-FA or a combination of precursor fatty acids and sources of the organic nitrates required for fatty acid nitration (i.e., NO$_2$ coming from animal and plant-derived products or NO$_2$-based preservatives) [22]. Clinical studies have revealed that dietary supplementation of organic nitrates, with and without co-administration of conjugated linoleic acid (cLA), resulted in a sustained level of NO$_2$-cLA only when cLA was co-administered [23]. This indicates that cLA is rate limiting and its simultaneous presence in the gastric compartment together with NO$_2$ and its precursor nitrate (NO$_3$) is needed for the formation of the NO$_2$-cLA. Related to this, the cardiovascular health-promoting properties attributed to the Mediterranean diet may be due in part to these dietary constituents that could react and upregulate NO$_2$-FA generation [24–26]. Thus, interest in characterizing the biophysical and biochemical properties of NO$_2$-FA has grown [27].

The detailed formation, metabolism, distribution and excretion of NO$_2$-FA, as well as the mechanisms accounting for net signaling responses to NO$_2$-FA are still not fully resolved [7,20,28]. From a methodological point of view, NO$_2$-FA are complex analytes because of aqueous insolubility, an amphiphilic nature, a net negative charge and different chemical reactivities in aqueous and lipophilic biological microenvironments. MS-based approaches [29] as well as spectrophotometry and crystallographic analysis [21] have been used for structural characterization and quantification of unesterified NO$_2$-FA, with mass spectrometry using electrospray ionization (ESI) coupled with liquid chromatography (LC-MS) being the most suitable method [20,30].

Herein, we expand on a previous study [31] and compare the biophysical properties of NO$_2$-OA, NO$_2$-LA and NO$_2$-cLA (Scheme 1) using multiple analytical and computational approaches. Native FA without NO$_2$ substitution (OA, LA and cLA) served as negative controls in all experiments. These analyses define the reactivity, stability, NO release characteristics, conformational properties and micellization of key NO$_2$-FA.

## 2. Experimental

### 2.1. Chemicals

Chemicals were purchased from Merck KGaA (Darmstadt, Germany) or BioRad Laboratories (Hercules, CA, USA). 10-nitrolinoleate (NO$_2$-LA) as pure positional isomer was from Cayman Chemical (Ann Arbor, MI, USA). NO$_2$-OA as an equimolar mixture of the 9- and 12-nitrooctadec- (9)-enoic acid and NO$_2$-cLA as an equimolar mixture of the 9- and 12-nitrooctadec-a(9,11)-dienoic acid were synthesized as previously [32]. The purity of NO$_2$-FA was higher than 98% for all samples. The stock solutions were prepared using methanol. With exception of NO$_2$-LA, NO$_2$-OA and NO$_2$-cLA were used throughout the study as an appropriate mixture of both positional isomers (Scheme 1). The unmodified fatty acids (LA, cLA and OA) were purchased from Merck KGaA (Darmstadt, Germany). All solutions were prepared using Milli-Q water (18.2 MΩ cm$^{-1}$). Millipore, Bedford, MA, USA. All other used chemicals were of analytical grade.

### 2.2. CMC determination by DLS

The micelles were prepared by dissolving individual fatty acids and
their nitro derivatives in 1 mM NaOH solution [33]. Each solution was diluted to the final concentration of 100 μM fatty acid (as sodium salt) with 0.1 M phosphate buffer (pH 7.4). Samples were diluted to several lower concentrations before performing the experiments. Dynamic light scattering (DLS) measurements were performed using Zetasizer Nano ZSP (Malvern Panalytical Ltd, Malvern, UK) at 25 °C. Finally, light scattering intensities (Derived Count Rate, Kcps) were plotted against solute concentration. Critical micellar concentration (CMC) was determined as the intersection between the straight line coming from the light scattering intensity values below CMC with the straight line derived from the light scattering intensities above CMC (region of rapid intensity increase) [34].

2.3. Electrochemistry

All electrochemical measurements were performed at room temperature with a μAutolab III analyzer (EcoChemie, Utrecht, Netherlands) in a three-electrodes cell with an Ag/AgCl/3 M KCl electrode as the reference and platinum wire as the auxiliary electrode. Two types of working electrodes were used: HMDE (hanging mercury drop electrode; area 0.4 mm$^2$) for constant-current chronopotentiometric stripping analysis (CPSA) and alternating-current voltammetry (ACV), and PGE, a basal-plane pyrolytic graphite electrode (electrode area 9 mm$^2$, Momentive Performance Materials, USA) for cyclic voltammetry (CV). Individual settings for electrochemical experiments, as well as concentrations of the compounds, are given in the Fig. legends. The electrochemical analyses were performed using two supporting electrolytes: 0.1 M phosphate and Britton-Robinson buffers [35]. For the voltammetry of NO$_2$-FA, all electrolytes were purged with argon. Degassing was not employed with CPSA because the dioxygen does not interfere with the technique. The pH measurements were performed with a HI 2211 pH/ORP Meter (HANNA instruments, IT).

2.4. Stability of NO$_2$-FA

The stability of 8 μM NO$_2$-FA solutions was investigated following the decrease in intensity of the NO reduction peak for more than 24 h by using CPSA. The compounds investigated were dissolved in Britton-Robinson buffer as supporting electrolyte at pH 5, 7.4 and 9 in the presence of atmospheric oxygen. Incubation and CPSA were performed at 25 °C (controlled by thermostat) and room temperature, respectively.

2.5. Detection of NO release from NO$_2$-FA

In order to study whether NO$_2$-FA are able to release NO, the Fe(II) (DTCS)$_2$, 10-nitro-octadec-9-enoic acid, DTCS (Chemos Cz, Prague, Czech Republic) was used as an NO trapping agent. For all three of NO$_2$-FA, 150 mM ethanol stock solutions were prepared. DTCS was dissolved in water and bubbled with N$_2$ for at least 30 min to remove O$_2$. For the purpose of EPR measurements, water solutions of FeSO$_4$ $\times$ 7H$_2$O (final concentration 4 mM) and DTCS (final concentration 5.5 mM) were added to Britton-Robinson buffer (pH 7.4), followed by adding small volume of NO$_2$-cLA, NO$_2$-LA or NO$_2$-OA ethanol stock solutions (final concentration 25 mM). Final samples were drawn into gas-permeable Teflon tubes (Zeus Industrial Products, Orangeburg, SC, USA), and placed into the SHQE EPR resonator cavity. Between the measurements, all of the samples were kept at 25 °C. Since NO production from NO$_2$-OA has already been inspected [31], previous work was used as a reference.

2.6. EPR data acquisition and processing

EPR spectra were recorded using a Bruker Elexsys II E540 EPR X-band spectrometer (Germany) at 9.85 GHz and 10 mW microwave power. For all measurements, the modulation amplitude was 2 G, modulation frequency 100 kHz, conversion time 14.65 ms and sweep width was 77 G.

2.7. Theoretical calculations

Since the finding of global energy minimum for OA by systematical torsion search was found to be too time consuming and impractical in our previous work [31], a limited set of twenty energetically favorable structures was generated by program FROG2 [36]. This program allows the generation of conformational ensembles of small molecules using a two-stage Monte Carlo approach in the dihedral space from initial structure. The conformational search was limited to desired (E, Z)-isomers of anionic form of studied FA. The retrieved conformers were further reoptimized at DFT level of theory employing 6–311++G (d, p) basis set and B3LYP functional. Analysis of frontier molecular orbitals

Scheme 1. Structures and identification of NO$_2$-FA used in this study.
and molecular electrostatic potentials were performed using the program Avogadro [37]. The role of solvent (water, methanol and n-octanol) was described in implicit way using PCM model [38]. All quantum mechanical calculations were treated in Gaussian 16 program [39].

3. Results and discussion

3.1. CMC determination by DLS

Fatty acids are amphiphilic molecules with an aliphatic tail which can be either saturated or unsaturated and have a polar headgroup that can be protonated (COOH) or deprotonated (COONa) was described in implicit way using PCM model [38]. All quantum gram Avogadro [37]. The role of solvent (water, methanol and octanol) was described in implicit way using PCM model [38]. All quantum mechanical calculations were treated in Gaussian 16 program [39].

3.2. Redox behavior of NO2-FA

In next experimental work we focused on the redox behavior of NO2-CLA and NO2-LA; the redox transformations of NO2-OA were described recently [31], thus the redox behavior of NO2-cLA and NO2-LA was evaluated and compared with NO2-OA. The redox behavior of NO2-cLA, an equimolar mixture of the 9- and 12-NO2 regioisomers was evaluated by cyclic voltammetry (CV) with a basal-plane pyrolytic graphite electrode (PGE) (Fig. 1). Native conjugated-linoleic acid (cLA) was used as a control. The reduction peak (peak NO) of the modified fatty acid is

![Table 1](image-url)

Schematic representation of the self-assembly of fatty acids with respect to critical micellar concentration (CMC). CMC values for free FA and NO2-FA were determined using density light scattering.

![Fig. 1](image-url)

Cyclic voltammetry (CV) of NO2-cLA at pyrolytic graphite electrode. (a) CV of 20 μM NO2-cLA and cLA in 0.1 M phosphate buffer at pH 7.4. CV parameters: initial potential 0 V, first vertex potential +1 V, second vertex potential +1 V. (b) CV of 20 μM NO2-cLA and cLA in 0.1 M phosphate buffer at pH 7.4. CV parameters: initial potential 0 V, first vertex potentials +1 V, second vertex potential +1 V. (c) The pH effect on the reduction NO peak coming from 5 μM NO2-cLA was recorded in Britton-Robinson buffer at pH 3–12. CV parameters: initial potential 0 V, first vertex potential +1 V, second vertex potential +1 V. Inset: dependence of the peak NO potential on pH. Prior to the experiments, the electrolytes were deaerated for 10 min with a stream of argon. Time of accumulation 30 s (Ea = 0 V), step potential 5 mV and scan rate 1 V/s were applied in all cases. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

visible at ca. −0.8 V in 0.1 M phosphate buffer at pH 7.4 while for the native fatty acid no peak appears (Fig. 1a).

This reduction process is irreversible and of an adsorptive nature [31]. The final reduction product for the nitro moiety has not yet been established but it has been proposed to proceed via a hydroxylamine to an amine derivative via a complex reduction cascade [20]. For example,
the reduction of cis-nitrostilbene in acetonitrile occurs by initial formation of the radical anion followed by irreversible dimerization [45]. When the trans isomer was studied, a different mechanism was operative, where the initial radical anion rapidly isomerizes to the cis form followed by a partial oxidation to cis-nitrostilbene. This process was then followed by the second-order dimerization [45]. The oxidation of individual reduction products of NO2-cLA can be demonstrated by switching the first vertex potential in CV experiments (Fig. 1b). After reduction of the NO2 moiety, the anodic peak appears at ca. +0.3 V, most probably corresponding to the oxidation of the first reduction product in the cascade. If the potential of ≈0.5 V (or a less negative one) is applied, the anodic peak was not observed in reverse branch of cyclic voltammograms, (black line, Fig. 1b). The NO reduction peak for the NO2-cLA acid is also pH dependent, as reported for NO2-OA (Fig. 1c). The peak NO potential is shifted toward negative values with increasing of pH. In the neutral pH range the maximum of peak current intensity for the reduction process was obtained.

The electrochemical behavior of NO2-cLA was also studied by constant-current chronopotentiometric stripping analysis (CPSA) with a hanging mercury drop electrode (HMDE). These measurements showed the presence of a second peak (Ads) at negative potential ca. −1.3 V (Fig. 2a). Alternating current voltammetry (ACV) at HMDE was used for a more detailed view of the interfacial behavior of NO2-cLA [46]. Comparison of the out-of-phase (sensitive to adsorption properties) and in-phase (sensible to both diffusion control and adsorption processes) AC voltammograms suggests that peak Ads is most probably due to adsorption/desorption or reorientation processes [31,47]. The out-of-phase ACV shows only a tensametric Ads peak (or double peak) for NO2-cLA (or cLA). In contrast to in-phase, out-of-phase ACV measurement is not as sensitive to peak NO in agreement with previous findings [31] (Fig. 2b and c). In the same way, the reduction of NO2-LA was tested and quite similar redox behavior as for NO2-cLA was found (Fig. 3).

NO2-cLA redox properties were compared with the corresponding methylene-interupted diene species (i.e. NO2-LA) and to the monoenoic nitroalkene NO2-OA by using CPSA. The NO2-cLA reduction peak NO appears at a less negative potential compared with NO2-LA and NO2-OA (Fig. 4a, inset). The same behavior was observed for PGE using the CV method (Fig. 4b). CPS and CV measurements indicated that both conjugated and methylene-interupted diene containing nitro-linoleic acids have stronger electrophilic character compared with NO2-OA. This is consistent with the concept that the electrophilic (electron acceptor) properties of NO2-FA increase with the shift of the NO peak towards less negative potentials.

3.3. Evaluation of NO2-FA stability

When NO2-FA electroreduction was inspected over the time, it was apparent that depending on pH, that under aqueous buffer conditions NO2-FA can undergo decomposition reactions, with a range of pH values (5, 7.4 and 9) showing that the greatest degradation rate was in acidic conditions (Fig. 5). The degradation rate was greater for NO2-cLA than for NO2-OA and NO2-LA, a response consistent with the greater electron acceptor capacity of NO2-cLA (Fig. 4).

EPR spectroscopy, using Fe(II) (DTCS)2 as the spin-trapping agent, revealed rates and extents of NO2-cLA and NO2-LA aqueous decay and generation of -NO, and allowed comparison with NO2-OA (Fig. 6). By comparing the EPR signal intensities of spin-adducts, NO2-cLA generated a significantly greater yield of -NO than NO2-LA and NO2-OA. The amount of -NO produced by NO2-LA and NO2-OA was almost equivalent. The rate constant of -NO release over 24 h could be calculated for all three NO2-FA by applying a consecutive reaction fitting model to the kinetic data (Fig. 7, Table 2). This is a simplified model, since multiple simultaneous processes are contributing to the overall kinetics. Namely, this simplified model takes into account the -NO release from the NO2-FA, the rapid reaction of -NO with Fe(II) (DTCS)2, and the decay of the

![Fig. 2. Chronopotentiometric stripping analysis (CPSA) and alternating current (AC) voltammetry of NO2-cLA at hanging mercury drop electrode. All measurements were in 0.1 M phosphate buffer at pH 7.4. (a) CPS record of 10 μM NO2-cLA (blue line) and cLA (red line); Istr = −35 μA, time of accumulation tstr = 30 s (Estr = 0 V). (b) In-phase and (c) out-of-phase AC voltammograms of 30 μM NO2-cLA and cLA; initial (0 V) and end (−1.95 V) potentials, frequency: 66.2 Hz, amplitude: 5 mV, phase angle: 0◦ (in-phase) and 90◦ (out-of-phase). For AC voltammetry the electrolyte was degassed for 10 min with argon. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)](image-url)
EPR-active complex, resulting in a loss of the EPR signal. One of the causes for the EPR signal decay of the NO–Fe(II) (DTCS)₂ complex may be the reaction between this complex and another ⋅NO molecule, yielding EPR-silent dinitrosyl species, thus affecting the ⋅NO trapping efficiency [48]. Also, in spite of all the solutions used for spin trapping procedure were N₂-equilibrated, it is possible that a small fraction of iron from Fe(II) (DTCS)₂ was quickly oxidized, giving the Fe(III) (DTCS)₃, which is also able to complex with ⋅NO. Notably, NO–Fe(III) (DTCS)₃ can also be converted to NO–Fe(II) (DTCS)₂ [49, 50]. Accordingly, the extent of Fe²⁺ oxidation to Fe³⁺ and the rate of subsequent steps resulting in the formation of NO–Fe(II) (DTCS)₂ complex could also determine the reduction rate of the EPR signal. Finally, dithiocarbamate (DTC)-Fe complexes can also be employed for indirect detection of NO₂/N₂O₄ [51] via the following reactions:

Fe(DTC)₃ + N₂O₄ → Fe(NO₃) (DTC)₂ + NO + DTC

Fe(DTC)₃ + NO → NO–Fe(DTC)₂ + DTC

3.4. Conformation of NO₂-FA

The MM and QM calculations performed on fatty acids showed that the conformation of aliphatic chain [52-56] is different for saturated versus unsaturated compounds. In saturated chains the anti orientation of the C–C–C–C torsion angles (180°) represents the global minimum, whereas its gauche orientation (60°) is a local minimum, being less stable than the global one by approx. 2 kJ/mol. The skew orientation (120°) belongs to the transition state with a barrier height around 14 kJ/mol.

When a double bond is present in the system, the global minimum is represented by a skew, skew conformation of two C–C–C=C dihedrals (i.e. like in 3-hexene), also being the case for dienes. In 2,5-heptadiene, a common model for the flexibility of aliphatic chains of FA [53, 55], the +skew, +skew (or -skew, -skew) orientation is a global minimum. A positive value of the torsion angle corresponds to counterclockwise rotation of the furthest substituent with respect to the central bond. In this conformation, double-bond directions are aligned in parallel, which leads to the extended aliphatic chain. Almost the same stability occurs...
The two most stable energy conformers of NO\textsubscript{2}-FA and their physical-chemical properties (relative stability, electrostatic potential, HOMO and LUMO) are depicted in Figs. 8–10 and Table 3. From this, it is evident that for all NO\textsubscript{2}-FA the shape of HOMO and LUMO orbitals is similar, the HOMO orbital is spread around the carboxylic group, whereas the LUMO is composed from contributions in the π* orbitals of the nitro group and of C–C bond(s). The energies of HOMO and LUMO in any given FA are insensitive to the position of the nitro group and its molecular geometry. The increase of polarity of the solvent enlarges the HOMO–LUMO energy gap (by ca. 0.35 eV comparing the n-octanol and water solvents). The lowest HOMO-LUMO energy gap resulting to the highest reactivity (by 0.2 eV in comparison with other NO\textsubscript{2}-FA) was found for NO\textsubscript{2}-cLA what can be caused by the presence of two conjugated C=C bonds in this compound.

3.5. Biological relevance of reported data

Herein three nitro-FA with biological signaling capabilities were biophysically characterized under aqueous conditions. The C-10 isomer of NO\textsubscript{2}-OA (CXA-10) is being evaluated in Phase 2 clinical trials as a potential new drug candidate [11]. In addition, we have evaluated NO\textsubscript{2}-La and its conjugated diene conformer, the latter of significant biological relevance because of its endogenous levels [57]. Comparative redox and EPR analysis indicate NO radical release is a spontaneous decay mechanism for NO\textsubscript{2}-FA, yet likely does not activate soluble guanylate cyclase to exert physiological responses because of low yields and a kinetically-preferential reaction with nucleophilic amino acid residues [58]. In particular, NO\textsubscript{2}-FA form adducts with Cys residues of signaling proteins, redox sensors, proteins involved in stress responses and redox-sensitive transcription factors. The important driving factor for
these low molecular weight nucleophile (i.e., GSH) or protein alkylation reactions will be steric hindrance, specifically the availability and reactivity of particular Cys residues with NO$_2$-FA. The structural data from this study can facilitate the study and prediction of downstream NO$_2$-FA reactions. For example, the pKa of Cys targets is crucial factor [58], since thiolate anions are so readily nitroalkylated. The involvement of vicinal basic residues that promote Cys deprotonation is also critical and under investigation. Another key parameter that defines downstream signaling responses will be the stability of NO$_2$-FA in plasma and at cellular and tissue levels. At the same time, excessive protein target accumulation of NO$_2$-FA can become

Table 2
Rate constants for NO release from three different fatty acid nitroalkenes maintained under aqueous conditions.

| NO donor | $k_1$ (arbitrary EPR units/h) |
|----------|-----------------------------|
| NO$_2$-cLA | $1.3 \times 10^6$ |
| NO$_2$-LA | $5.5 \times 10^5$ |
| NO$_2$-OA | $5.1 \times 10^5$ |

The structural data from this study can facilitate the study and prediction of downstream NO$_2$-FA reactions. For example, the pKa of Cys targets is crucial factor [58], since thiolate anions are so readily nitroalkylated. The involvement of vicinal basic residues that promote Cys deprotonation is also critical and under investigation. Another key parameter that defines downstream signaling responses will be the stability of NO$_2$-FA in plasma and at cellular and tissue levels. At the same time, excessive protein target accumulation of NO$_2$-FA can become
toxic, compromising their action as signaling molecules. In this regard, enzymatic reduction of the nitroalkene, mitochondrial $\beta$-oxidation, NO$\_2$-FA incorporation into complex lipids such as triglycerides and the concerted GSH alkylation and export by multi-drug resistant proteins can limit toxic accumulation of this class of lipid electrophiles [59–63].

Depending on buffer, media or in vivo conditions, NO$\_2$-FA are stable for several minutes to several hours [11, 64]. This time perspective corresponds very well to the pharmacokinetics that would support activation of the expression of adaptive signaling-related genes and the modulation of both acute and chronic inflammatory responses [65]. The relationship between the degradation of NO$\_2$-FA and the aqueous -NO release phenomenon is documented by the comparison of 24 h CPSA-degradation profiles in Fig. 5, which fits the EPR-degradation kinetics shown in Fig. 7. It is viewed that the generation of NO by all nitroalkenes will be a consequence of aqueous decay via a modified Nef reaction, a phenomenon reported for in nitroalkene derivatives of bis-allylic linoleic acid [20]. Herein, we suggest that conjugated diene-containing FA nitroalkenes are also capable of undergoing the same protonation-deprotonation cycle followed by reaction with the hydroxide anion always present in aqueous milieu. Because of the adjacent conjugated double bonds in NO$\_2$-cLA, it is viewed that the nitroso intermediate would have a weaker C–N bond and thus be more susceptible to scission to yield NO radical. Prior mass spectrometric

Fig. 8b. Physico-chemical properties of 10-NO$\_2$-OA presented for two most stable conformers (I, II). From top to bottom: molecular electrostatic potential (ESP); the highest occupied molecular orbital (HOMO); the lowest unoccupied molecular orbital (LUMO).

![Figure 8b](image_url)

| Water | Methanol | n-Octanol |
|-------|----------|-----------|
| 12-NO$\_2$cLA | | |
| 0.00 | −8.278 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 2.15 | −8.289 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 0.01 | −8.302 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 10-NO$\_2$cLA | | |
| 7.48 | −8.297 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 9-NO$\_2$OA | | |
| 0.00 | −8.286 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 0.02 | −8.285 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 1.02 | −8.286 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 10-NO$\_2$OA | | |
| 0.00 | −8.287 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 1.02 | −8.286 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 9-NO$\_2$OA | | |
| 0.00 | −8.287 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 0.02 | −8.285 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |
| 1.02 | −8.286 | 0.00 | 0.00 | −7.813 | −0.851 | 7.187 |

[1] relative energy of the conformers (in kJ/mol).
[2] HOMO, LUMO energy and their difference (all in eV).

Fig. 9. Physico-chemical properties of 10-NO$\_2$-LA presented for two most stable conformers (I, II). From top to bottom: molecular electrostatic potential (ESP); the highest occupied molecular orbital (HOMO); the lowest unoccupied molecular orbital (LUMO).
detection of the expected oxidized fatty acid products and the present
direct detection of NO formation support this pathway of nitroalkene
decay. This acid/base chemistry may potentially be employed by en-
zymes that might also catalyze NO release from the multiple lipid
nitroalkene derivatives that have been detected biologically. It is noted
in the latter context however that the main physiological reaction
pathway for NO$_2$-cLA is more likely to be a) stabilization by esterifica-
tion into complex lipids, b) incorporation into hydrophobic compart-
ments such as hydrophobic pockets of proteins, micelles, chylomicrons,
lipid droplets or membranes that will in turn limit
protonation-deprotonation of the nitroalkene and subsequent NO for-
mation and c) the alkylation of proteins that in turn also inhibits the
chemistries associated with the Nef reaction.

NO$_2$-FA are generated by the reactions of unsaturated FA with NO,$\cdot$ and
NO$_2$-derived nitrating species (Scheme 2). The life-time of these
species are all very short, e.g. for ONOO$^{-}$, a $t_{1/2}$ of 0.15 s was reported
[66]. The generation of these species can be upregulated by inflamma-
tion, metabolism and other physiological and pathological manifesta-
tions [67]. One common denominator is that these reactive species act
vicinal to the location where they are generated, with further distribution
limited by a low $t_{1/2}$ and high reactivity. In contrast, the nitration of
unsaturated FA to a more stable product results in broader distribution
and more systemic signaling potential. Thus, the transformation of
various nitrogen oxides to NO$\cdot$ and the formation of NO$_2$-FA transduces
the effects of often toxic mediators of inflammation into a species that
induces adaptive metabolic and gene expression responses. The rela-
tionship between the stability and biological reactivity of NO$_2$-FA in
the context of their origin, distribution and storage is depicted in Scheme 2.

4. Conclusions

This study affirms that NO$_2$-FA biological actions are pleiotropic and
that understanding the physicochemical properties of NO$_2$-FA is crucial
for clearer insight into the mechanisms of action of these electrophilic
products of inflammation and metabolism. Despite a number of phar-
caceutical and clinical studies [68], only limited studies describe the
structure, stability and reactivity of NO$_2$-FA. NO$_2$-FA are electrochemically reducible around $0.8$ V, which cor-
responding to reduction of R$\cdot$NO$_2$ group. Based on the potential of
reduction peak NO we can evaluate electrophilic capacity or reactivity
of NO$_2$-FA, which decreases, with NO$_2$-cLA $\gg$ NO$_2$-LA $>$ NO$_2$-OA. This
electrochemical data is in good agreement with DFT calculations. In this
respect, the correlation of LUMO energy and the peak NO potential is an
important parameter. The LUMO energy of the tested derivatives ranges
from $-0.7$ to $-0.95$ eV and, the electron deficient region is located at the
R$\cdot$NO$_2$ group for all compounds studied. Furthermore, the mechanism of
NO release [69] was investigated by EPR spectroscopy. The formation of
NO$\cdot$ or NO$_2$ radicals is due to the spontaneous degradation of NO$_2$-FA.
A highly efficient donor of NO radical(s) is NO$_2$-cLA, for which a rate
constant for radical(s) formation of $1.3 \times 10^{10}$ was observed. This value
differs from the rate constants for NO$_2$-LA and NO$_2$-OA by one order of
magnitude. From theoretical studies [70] as well as from pharmaco-
logical studies of adipose tissue [71], it is evident that NO$_2$-FA exhibit
different behavior when they are an integral part of organized lipid
structures such as membranes, micelles, chylomicrons and lipid droplets

Fig. 10a. Physico-chemical properties of 9-NO$_2$-cLA presented for two most
stable conformers (I, II). From top to bottom: molecular electrostatic potential
(ESP); the highest occupied molecular orbital (HOMO); the lowest unoccupied
molecular orbital (LUMO).

Fig. 10b. Physico-chemical properties of 12-NO$_2$-cLA presented for two most
stable conformers (I, II). From top to bottom: molecular electrostatic potential
(ESP); the highest occupied molecular orbital (HOMO); the lowest unoccupied
molecular orbital (LUMO).
or if they are freely available in solution (Scheme 2). This has to be taken into account with respect to the stability of the NO$_2$-FA in various model systems.

When working in an aqueous environment, the concentration of NO$_2$-FA also plays an important role, because of self-assembly processes [43]. If the concentration of NO$_2$-FA is lower than the CMC, monomeric fractions are present in the solution. Conversely, when the CMC is exceeded, the NO$_2$-FA monomers are transformed into self-assembled structures, most often liposome-like micelles. In the native (non-pathological) cellular environment, free NO$_2$-FA can be found at nM, or sub-nM, concentrations [64]. Thus, when conducting an in vitro experiment with μM NO$_2$-FA concentrations, it is important to take into account micellization, vesicle formation, and aggregation phenomena.

Defining the endogenous or pharmacological actions of the heterogeneous and pleiotropically-acting NO$_2$-FA class requires a comprehensive and multi-disciplinary approach. In particular, conformational polymorphism is an inseparable property of NO$_2$-FA where, in addition to the positional isomers, we have to consider cis/trans isomerism and the formation of “open” and “closed” conformer populations [54]. These will achieve a dynamically changing equilibrium in the aqueous solution depending on the experimental conditions. In general, the presence of the nitro group has a rather negligible effect on the conformation of NO$_2$-FA as compared to unsubstituted FA, but a profound effect on chemical reactivity and downstream effects.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of competing interest

B.A.F. acknowledges financial interest in Complexa, Inc. and Ceregh Pharmaceuticals.

Acknowledgments

This work was supported by the Czech Science Foundation, grants 19-21237Y (M.Z.) and 19-09212S (J.V.). The authors would like to acknowledge Ministry of Education, Youth and Sports (CZ.02.2.69/0.0/ 0.0/16_027/0008482, V.G.) and (CZ.02.1.01/0.0/0.0/15_003/ 0000495, J.T.) and NIH R01-HL64937, R01-HL132550 and P01- HL103455 (B.A.F.) for financial support. Computational resources were supplied by the project "e-Infrastruktura CZ" (e-INFRA LM2018140) provided within the program Projects of Large Research, Development and Innovations Infrastructures. A partial financial support from project LTUSA17160 (Ministry of Education, Youth and Sports) is also acknowledged.

References

[1] S. Kalayanaraman, Nitrated lipids: a class of cell-signaling molecules, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 11527-11529.
[2] M. Delmastro-Greenwood, B.A. Freeman, S.G. Wendell, Redox-dependent anti-inflammatory signaling actions of unsaturated fatty acids, Annu. Rev. Physiol. 76 (2014) 79-105.
[3] S. Baranaghi, R. Radi, Fundamentals on the biochemistry of peroxynitrite and protein tyrosine nitration, Redox Biol 14 (2018) 618-625.
[4] C. Bregere, I. Rebrin, R.S. Sohal, Detection and characterization of in vivo nitration and oxidation of tryptophan residues in proteins, Methods Enzymol. 441 (2008) 339-349.
[5] J. Khan, D.M. Brennan, N. Bradley, B. Gao, R. Bruckdorfer, M. Jacobs, 3-Nitrotyrosine in the proteins of human plasma determined by an ELISA method, Biochem. J. 330 (1996) 795-801.
[6] D. Tsikas, A.A. Zeiwer, J. Jordan, Oxidized and nitrated oleic acid in biological systems: analysis by GC-MS/MS and LC-MS/MS, and biological significance, Biochim. Biophys. Acta 1811 (2011) 694-705.
[7] P.R.S. Baker, F.J. Schofer, S. Sweeney, B.A. Freeman, Red cell membrane and plasma linoleic acid nitration products: synthesis, clinical identification, and quantitation, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 11577-11582.
[8] M. Balazy, C.D. Poff, Biological nitration of arachidonic acid, Curr. Vasc. Pharmacol. 2 (2004) 81-93.
[9] A.J. Deen, V. Silvola, J. Harkonen, S. Ahdinoffi, A.L. Levonen, Regulation of stress signaling pathways by nitro-fatty acids, Nitric Oxide : biology and chemistry 78 (2018) 170-175.
[10] T. Melo, J.F. Montero-Bullon, P. Domingues, M.R. Domingues, Discovery of bioactive nitrated lipids and nitro-lipid-protein adducts using mass spectrometry-based approaches, Redox Biol 23 (2019) 101106.
[11] F.J. Schofer, D.A. Vitturi, D.K. Jorkasky, B.A. Freeman, Nitro-fatty acids: new drug candidates for chronic inflammatory and fibrotic diseases, Nitric Oxide : biology and chemistry 79 (2018) 31-37.
[12] B.A. Freeman, M. Pekarova, H. Rubbo, A. Trotschansky, Electrophilic nitro-fatty acids: nitric oxide and nitrite-derived metabolic and inflammatory signaling mediators, in: L.J. Ignarro, B.A. Freeman (Eds.), Nitric Oxide: Biology and Pathobiology, third ed., Elsevier, London, England, 2017, pp. 213–229.
[13] F.A. Carey, R.J. Sundberg, Advanced Organic Chemistry: Part B: Reaction and Synthesis, fifth ed., Springer US, 2007.
[14] L. Turell, D.A. Vitturi, E.L. Costino, L. Lebrato, M.N. Moller, C. Sagasti, S. Ignarro, Nitric Oxide: Biology and Pathobiology, third ed., Elsevier, London, England, 2017, pp. 213–229.
[15] P.B.S. Baker, F.J. Schofer, V.B. O'Donnell, B.A. Freeman, Convergence of nitric oxide and lipid signaling: anti-inflammatory nitro-fatty acids, Free Radic. Biol. Med. 46 (2009) 989-1003.
[16] F.J. Schofer, C. Gippilina, B.A. Freeman, Formation and signaling actions of electrophilic lipids, Chem. Rev. 111 (2012) 5997-6021.
[17] C. Bathuyan, F.J. Schofer, P.R.S. Baker, R. Duran, L.M.S. Baker, Y. Huang, C. Cervenansky, B.P. Branchaud, B.A. Freeman, Reversible post-translational modification of proteins by nitrated fatty acids in vivo, J. Biol. Chem. 281 (2006) 20450-20463.
[18] M.J. Gorczynski, J. Huang, H. Lee, S.B. King, Evaluation of nitroalkenes as nitric oxide donors, Bioorg. Med. Chem. Lett. 17 (2007) 2013-2017.
[19] T.G. Lin, S. Sweeney, A. Bloodsworth, C.R. White, P.H. Chumley, N.R. Krishna, F. Schofer, V.B. O'Donnell, J.P. Eiseirich, B.A. Freeman, Nitrolineolate, a nitric oxide-derived mediator of cell function: synthesis, characterization, and vasomotor activity, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 15941–15946.
