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Nitro-linolenic acid is a nitric oxide donor

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ABSTRACT

Nitro-fatty acids (NO_2-FAs), which are the result of the interaction between reactive nitrogen species (RNS) and non-saturated fatty acids, constitute a new research area in plant systems, and their study has significantly increased. Very recently, the endogenous presence of nitro-linolenic acid (NO_2-Ln) has been reported in the model plant *Arabidopsis thaliana*. In this regard, the signaling role of this molecule has been shown to be key in setting up a defense mechanism by inducing the chaperone network in plants. Here, we report on the ability of NO_2-Ln to release nitric oxide (NO) in an aqueous medium with several approaches, such as by a spectrofluorometric probe with DAF-2, the oxyhemoglobin oxidation method, ozone chemiluminescence, and also by confocal laser scanning microscopy in Arabidopsis cell cultures. Jointly, this ability gives NO_2-Ln the potential to act as a signaling molecule by the direct release of NO, due to its capacity to induce different changes mediated by NO or NO-related molecules such as nitration and *S*-nitrosylation or by the electrophilic capacity of these molecules through a nitroalkylation mechanism.

1. Introduction

Nitric oxide (NO) is a relatively stable and highly diffusible free radical which is endogenously generated in plant cells. This molecule has a key role in a wide range of plant processes ranging from seed germination or senescence to several situations of biotic and abiotic stress such as plant-microbial interactions or salinity [8,10,19,54]. Furthermore, NO is also an important signaling molecule affecting the function of different proteins by post-translational modifications (NO-PTMs) such as protein tyrosine nitration and *S*-nitrosylation which are mediated by NO-derived molecules as for example peroxynitrite ($ONOO^-$) or *S*-nitrosoglutathione (GSNO) [58,9].

In mammals, NO is enzymatically produced through an oxidative mechanism utilizing a NO synthase (NOS) which catalyzes the NADP-dependent oxidation of L-arginine to NO and citrulline. Nevertheless, the enzymatic source of NO remains unidentified in plant systems. Several possible pathways for NO generation have been proposed in plants, which are widely classified depending on either reductive or oxidative chemistry. Among oxidative pathways of NO synthesis, NO and citrulline production from L-arginine in plant extracts from higher plants has been reported together with a significant decrease in this activity by typical NOS inhibitors [7,12,15]. Furthermore, rises in the levels of polyamines (spermine and spermidine) have been shown to induce NO release [53,3] and also NO formation via hydroxylamine-mediated synthesis [45]. On the other hand, reductive pathways of NO synthesis include nitrate reductase (NR) catalyzing the reduction of nitrate to nitrite and also the reduction of nitrite to NO [40]. However, the efficiency of this reaction is low and requires small oxygen tensions, light, and high

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nitrite concentrations [40,38]. Moreover, a membrane-bound nitrite-NO reductase (NiNOR) generates extracellular NO, and a role in sensing nitrate availability has been suggested [35]. Finally, NO can also be generated in the mitochondrial inner membrane via cytochrome c oxidase and/or reductase, and a xanthine oxidoreductase (XOR) enzyme can also reduce nitrite to NO [23,38]. In conclusion, the ability of land plants to assimilate and reduce nitrate together with hypoxic conditions encountered by plants under different environmental conditions in terrestrial habitats have led these organisms to develop an efficient NO production from NR as their main survival strategy [29].

In animal systems, cGMP-dependent signaling of NO has been widely described. Nonetheless, how plants sense NO by guanylate cyclases (GC) and subsequent cGMP production is an emerging area regarding its implications for the defense response and the response to hormone-related processes [37,27]. In fact, a candidate GC has been characterized *in vitro* in Arabidopsis [36]. In this respect, it has also been shown that the NO production from an unknown NOS-like enzyme activity reduces cold-responsive pollen germination, inhibits tube growth, and reduces proline accumulation partly via the cGMP signaling pathway in *Camellia sinensis* [56]. Moreover, NO can act indirectly through GC to activate cGMP-dependent cellular responses, e.g. through PTMs such as nitration and *S*-nitrosylation and through the activation of phosphatases and protein kinases including MAPKs. All these events may lead to changes in tertiary structure and therefore in the functioning of ion channels [21,25,30,55,60].

NO-derived molecules interacting with unsaturated fatty acids have been identified as signaling mediators in animal systems. This reaction yields a variety of oxidized and nitrated products [42]; highlighting a new family of important signaling molecules termed nitro-fatty acids (NO_2-FAs) which are chemically stable and have a longer half-life [22]. These NO_2-FAs are present endogenously as free, esterified, and nucleophilic-adducted species [41], and their main properties include the notable capacity to mediate several anti-inflammatory effects [11,44,57].

Due to the chemical structure of NO_2-FAs, they present different biological properties such as the NO release in aqueous solutions [32,49,26]. Basically, two mechanisms have been proposed for NO release in aqueous mediums, including a modified Nef reaction, which involves mainly the formation of a hydroxy-nitroso intermediate with the subsequent release of either HNO or NO. The other proposed mechanism is through a nitroalkene-rearrangement to a nitrite ester followed by N–C bond hemolysis to form NO and the corresponding enol group [31,32,26]. Although this NO generation has been considered to be of minor significance *in vivo* and less than 1% *in vitro*, we cannot rule out that this NO release can have important consequences in key cellular targets. In this sense, it has been shown that NO released from nitro-linoleic acid (NO_2-LA) mediates the *S*-nitrosylation of the proinflammatory member CD40 with subsequent inactivation, thereby triggering an anti-inflammatory response [17]. Otherwise, NO_2-FAs display more functional capacities, acting mainly via post-translational modification. The β-carbon adjacent to the nitro group ($-NO_2$) has strong electrophilicity and represents an attractive target for nucleophilic addition. This reaction is termed Michael addition, involving a nucleophile which donates a pair of electrons to the electrophile to form a covalent bond [20,48]. In the case of NO_2-FAs, this addition is specifically called nitroalkylation and consists of a reversible reaction which is important in terms of signaling [22]. In this respect, the ability of different NO_2-FAs to modulate key cell targets by this electrophilic capacity has been widely reported. For example, both nitro-oleic (NO_2-OA) and nitro-linoleic (NO_2-LA) acids covalently bind to the cysteine 285 present in the structure of peroxisome proliferator-activated receptor (PPAR), which is consequently activated [5,13]. Moreover, nitroalkenes inhibit nuclear factor-kappa B (NF-κB) through a Michael addition with Cys 38 of the p65 subunit [13]. Thus, nitro-fatty acids tightly regulate different signaling pathways, thereby promoting anti-inflammatory responses.

On the other hand, research on NO_2-FAs in plant systems has been scarcely explored [46,18]. However, very recently Mata-Pérez et al. [34] have described the endogenous occurrence of nitro-linolenic acid (NO_2-Ln) in the model plant *Arabidopsis thaliana*. In this regard, it has been shown that this molecule is able to prompt the transcriptional expression of a large set of heat-shock proteins (HSPs) and induce an antioxidant defense response against abiotic stress processes such as those involving ascorbate peroxidase (APX) or methionine sulfoxide reductase (MSR). Nevertheless, the mechanisms by which NO_2-Ln is able to launch this defense remain unknown. In this sense, the present study evaluates the ability of NO_2-Ln to release NO in aqueous solutions by different experimental approaches including a fluorometric assay using DAF-2 (4,5-diaminofluorescein) as NO fluorescent probe, oxyhemoglobin oxidation method and chemiluminescence ozone analyzer. Additionally, this NO release by NO_2-Ln was also observed in Arabidopsis cells by confocal laser scanning microscopy (CLSM) using the DAF-FM DA (4-aminomethyl-2′,7′-difluorofluorescein diacetate) as a cell-permeable fluorescent NO probe. Jointly, these results confirm the capacity of the nitro-linoleic acid (NO_2-Ln) to release NO and therefore the potential of this acid to modulate specific cellular targets by processes such as nitration or *S*-nitrosylation and by the electrophilic ability of NO_2-Ln.

2. Material and methods

2.1. Plant material

9-day-old Arabidopsis cell-suspension cultures (ACSCs) were grown as previously described [33].

2.2. Nitro-fatty acids synthesis and plant treatments

NO_2-LA was synthesized by a nitroselenation procedure as established elsewhere [5,2] and NO_2-Ln was also produced by a similar methodology described previously [34]. For spectrophotometric characterization of NO_2-Ln, NO_2-Ln stock solutions were used to determine dilution concentrations for subsequent biochemical analysis. An absorbance spectrum of NO_2-Ln from 200 to 400 nm was generated using 100 μM NO_2-Ln in phosphate buffer (100 mM, pH 7.4) containing 100 μM DTPA. The extinction coefficient (ε) for NO_2-Ln was measured (λ_{264}) using a UV–visible spectrophotometer Cary 50 Bio (Varian). Absorbance values for increasing concentrations of NO_2-Ln were plotted against the concentration to calculate ε (Fig. S1).

For CLSM analysis, ACSCs were pre-incubated with distilled water, methanol or 100 μM linolenic acid (Ln) used as negative controls and 100 μM NO_2-Ln for 1 h.

2.3. Mass spectrometric analysis of NO_2-Ln decay

For HPLC-MS/MS analysis of NO_2-Ln decay in aqueous solutions, 10 μM NO_2-Ln was incubated in phosphate buffer (100 mM, pH 7.4) containing 100 μM DTPA and the reaction was followed for 1 h. Measurements made at different times were plotted vs. the relative area of chromatographic peaks of NO_2-Ln (Fig. S2). In this sense, 100% corresponds to NO_2-Ln area at zero time.