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In situ generation, metabolism and immunomodulatory signaling actions of nitro-conjugated linoleic acid in a murine model of inflammation

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ABSTRACT

Conjugated linoleic acid (CLA) is a prime substrate for intra-gastric nitration giving rise to the formation of nitro-conjugated linoleic acid (NO_2-CLA). Herein, NO_2-CLA generation is demonstrated within the context of acute inflammatory responses both *in vitro* and *in vivo*. Macrophage activation resulted in dose- and time-dependent CLA nitration and also in the production of secondary electrophilic and non-electrophilic derivatives. Both exogenous NO_2-CLA as well as that generated *in situ*, attenuated NF-κB-dependent gene expression, decreased pro-inflammatory cytokine production and up-regulated Nrf2-regulated proteins. Importantly, both CLA nitration and the corresponding downstream anti-inflammatory actions of NO_2-CLA were recapitulated in a mouse peritonitis model where NO_2-CLA administration decreased pro-inflammatory cytokines and inhibited leukocyte recruitment. Taken together, our results demonstrate that the formation of NO_2-CLA has the potential to function as an adaptive response capable of not only modulating inflammation amplitude but also protecting neighboring tissues via the expression of Nrf2-dependent genes.

1. Introduction

Inflammatory responses are central to survival in the face of sterile and infectious insults. However, equally important as being able to mount an effective response is the ability to terminate this process when the threat has been removed [1]. Indeed, chronic inflammation is a driving force behind metabolic syndrome development [2], atherogenesis [3], occurrence of acute cardiovascular events [4,5] and progression to heart failure [6]. As a result, the immune system must be endowed with mechanisms that allow it to sensitively respond to a changing inflammatory environment and achieve self-limitation [1]. Inflammation is initiated by a complex series of events, that include the activation of toll-like receptors (TLR) in resident macrophages, dendritic cells and non-immune cells by pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively). This activation leads in turn to microvasculature changes, which mediate the recruitment of neutrophils and monocytes into the inflamed tissues [7]. At these sites, leukocyte activation results in increased nitric oxide ($^{\bullet}NO$) and superoxide generation by iNOS and NADPH-oxidases respectively, leading to peroxynitrite and ultimately $^{\bullet}NO_2$ formation [8]. In addition, myeloperoxidase (MPO) released by neutrophil degranulation in the presence of hydrogen peroxide and nitrite also contributes to $^{\bullet}NO_2$ formation [9]. The generation of reactive nitrogen oxides is an essential component of the early response to invading pathogens [8,10]. Herein

Abbreviations: MRM, multiple reaction monitoring; iNOS, inducible Nitric Oxide Synthase (NOS2); LOQ, limit of quantification; BME, β-mercaptoethanol; NO_2-FA, nitrated fatty acids; CLA, octadeca-(*9Z,11E*)-dienoic acid; NO_2-CLA, (mixture of 9-NO_2-CLA [9-nitro-octadeca-9,11-dienoic acid] and 12-NO_2-CLA [12-nitro-octadeca-9,11-dienoic acid]); dinor-NO_2-CLA, (mixture of 7-NO_2-CLA [7-nitro-hexadeca-7,9-dienoic acid] and 10-NO_2-CLA [7-nitro-hexadeca-7,9-dienoic acid]; tetranor-NO_2-CLA, (mixture of 5-NO_2-CLA [5-nitro-hexadeca-5,7-dienoic acid] and 8-NO_2-CLA [8-nitro-hexadeca-5,7-dienoic acid]. The prefix "dihydro" refers to non-electrophilic nitroalkane derivatives of NO_2-FA. The designations "9-NO_2-" and "12-NO_2-"CLA are used to describe position of the nitro group in conjugated dienes and do not refer to IUPAC nomenclature

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we show that in addition to their well-characterized microbicidal actions, reactive nitrogen oxides fine-tune the inflammatory response via the formation and signaling actions of NO_2-CLA.

Nitrated fatty acids (NO_2-FA) are generated upon $^{\bullet}NO_2$ addition to double bonds in unsaturated fatty acids [11]. The resulting nitroalkene moiety confers NO_2-FAs with electrophilic reactivity thus allowing them to reversibly interact with nucleophilic cysteines both in proteins and in low molecular weight compounds [12,13]. This covalent reactivity is essential for their numerous biological actions, including Nrf2 activation, partial PPARγ agonism, heat shock response induction and TLR4/NF-κB/STAT1 inhibition [14–16]. Administration of the prototypical NO_2-FA, nitro-oleic acid (NO_2-OA) is associated with improved outcomes in a wide range of animal models of disease, which has propelled the pharmacological development of NO_2-FA as drug candidates for clinical use (http://www.complexarx.com/).

CLA has recently been identified as a prominent substrate for NO_2-CLA formation *in vivo* [17]. CLA is present in dairy and meat-derived products and becomes nitrated upon reaction with nitrite derived from saliva and diets rich in leafy vegetables in the acidic conditions of the stomach, [18–21]. As a result, NO_2-CLA is detected in varying amounts in the plasma (1–3 nM) and urine (0.5–43 pmol/mg creatinine) of healthy human subjects, and its levels can be further modulated by oral supplementation with CLA in combination with either nitrite or nitrate [19,22]. Interestingly, although consumption of CLA alone has been sporadically associated with anti-inflammatory effects both in animal models and human studies, the clinical significance of these findings remains poorly defined [21,23–25].

NO_2-CLA levels under healthy conditions are most likely determined by the diet [19]. However, the generation of $^{\bullet}NO_2$ during inflammatory responses has the potential to be a prominent pathway for *in situ* NO_2-CLA formation. Using high resolution mass spectrometry and stable isotope dilution quantitation techniques, we demonstrate that inflammatory macrophages mediate CLA nitration in a dose- and time-dependent manner. This reaction is dependent on iNOS activity and leads to the formation of two positional NO_2-CLA isomers which are further metabolized by macrophages. Both isomers activated Nrf2 signaling and inhibited the transcription of NF-κB-dependent genes, resulting in the suppression of pro-inflammatory cytokine production and in the expression of cytoprotective phase 2 proteins. Notably, the anti-inflammatory effects of NO_2-CLA were corroborated *in vivo* using a mouse model of zymosan-A induced peritonitis that promotes *in situ* CLA nitration. Taken together, our results support that NO_2-CLA formation is an adaptive response capable of down-regulating inflammation and protecting neighboring tissues against the deleterious effects of reactive oxygen and nitrogen species.

2. Results

2.1. LPS/IFN-γ activated macrophages mediate CLA nitration and subsequent NO_2-CLA metabolism

RAW264.7 macrophages were activated in the presence of CLA (50 μM) and the formation of nitrated derivatives was assessed in the media 24 h later. Fig. 1 shows the formation of both NO_2-CLA (Fig. 1A) and its two-electron reduction product dihydro-NO_2-CLA (Fig. 1B). A set of two closely-eluting isobaric peaks were detected for both NO_2-CLA and dihydro-NO_2-CLA. In the case of NO_2-CLA, MS^2 fragmentation analysis and accurate mass determinations identified these species as the positional 12- and 9-NO_2-CLA isomers (peaks 1 and 2 respectively, Fig. S1A). Unlike nitroalkene-containing fatty acids, collision induced dissociation of nitroalkane dihydro-derivatives does not result in structurally-informative fragment ions beyond the typical neutral losses of H_2O, CO_2 and HNO_2 [26]. Therefore, the identity of the two dihydro-NO_2-CLA peaks was established by a combination of high resolution accurate mass determinations (Fig. S1B) and co-elution with a synthetic dihydro-9-$^{15}NO_2$-CLA standard (Fig. 1B). As expected, the formation of NO_2-CLA and dihydro-NO_2-CLA was dependent on CLA concentration and time after activation for both RAW264.7 and bone marrow-derived macrophages (BMDM) (Fig. 1C-E). Interestingly, CLA nitration was only observed in the presence of the classic M1 inducers LPS/IFN-γ, with no NO_2-CLA formation obtained by either non-activated or M2-polarized macrophages (Fig. 1E). These results suggested an important role for the NF-κB-regulated gene iNOS, as this protein in the main source of $^{\bullet}NO$ production under inflammatory conditions. Consistent with this hypothesis, NO_2-CLA formation was completely abrogated by both the iNOS-specific inhibitor 1400 W or the use of $iNOS^{-/-}$ derived BMDM (Fig. 1F).

In addition to nitroalkene reduction to the corresponding dihydro-derivative, NO_2-FA also undergo β-oxidation giving rise to dinor, tetranor and hexanor metabolites [27]. Incubation of RAW264.7 cells with synthetic NO_2-CLA resulted in the generation of two series of metabolites separated by 28 amu corresponding to successive losses of C_2H_4 from both the parent compound and the dihydro-NO_2-CLA derivative (Fig. 2A, top). This pattern was fully recapitulated when activated RAW264.7 cells are treated with CLA (Fig. 2A, middle), indicating that macrophages can modulate NO_2-CLA levels by mediating both its formation and catabolism. Previous work indicates that only nitroalkene-containing NO_2-FA are electrophilic [22,28]. To test this, macrophage media containing endogenously generated NO_2-CLA was incubated with excess β-mercaptoethanol (BME) nucleophile before lipid extraction. As expected, BME treatment resulted in selective consumption of nitroalkene-containing NO_2-CLA metabolites whilst having no effect on the levels of non-electrophilic dihydro-NO_2-CLA derivatives (Fig. 2A, bottom). In line with the results obtained with NO_2-CLA formation, the levels of both nitroalkene-containing and dihydro β-oxidation metabolites increased as a function of incubation time and CLA concentration (Fig. 2B-E).

2.2. Positional isomers of NO_2-CLA have different catabolic rates

Nitrogen dioxide adds to carbons C9 and C12 in the diene moiety of 9,11-CLA forming 9-NO_2-CLA and 12-NO_2-CLA with similar efficiency [17,29]. However, isomer-specific analysis of NO_2-CLA formation by activated RAW264.7 cells revealed preferential accumulation of the 9-NO_2-CLA derivative (Fig. 3A). This coincided with a more predominant formation of the reduced metabolite dihydro-12-NO_2-CLA, suggesting that 12-NO_2-CLA is metabolized more readily than the 9-NO_2-CLA isomer (Fig. 3B). To test this concept, RAW264.7 cells were independently treated with synthetic 9- and 12-NO_2-CLA and catabolism was monitored. Consistent with the hypothesis, 12-NO_2-CLA was consumed at a significantly higher rate and resulted in a more prominent formation of the corresponding dihydro-12-NO_2-CLA derivative than 9-NO_2-CLA (Fig. 3C-D).

2.3. NO_2-CLA inhibits pro-inflammatory signaling and promotes expression of Nrf2-dependent genes

To test the role of NO_2-CLA in modulating pro-inflammatory signaling, RAW264.7 cells were activated with LPS/IFN-γ in the presence of increasing does of either 9-NO_2-CLA or 12-NO_2-CLA. The more efficient reduction of 12-NO_2-CLA versus 9-NO_2-CLA (see Fig. 3) suggested that the latter might be a more potent signaling mediator. However, Fig. 4A demonstrates that both isomers inhibited the expression of the NF-κB target protein iNOS with comparable potency. Consistent with this observation, NO_2-CLA co-treatment also dose-dependently inhibited the secretion of the pro-inflammatory cytokines IL-6 and MCP-1 by RAW264.7 cells (Fig. 4B-C) and directly antagonized the expression of NF-κB-dependent genes as demonstrated using a luciferase-based reporter construct (Fig. S2A). Finally, these results were recapitulated in BMDM, where NO_2-CLA potently inhibited LPS/IFN-γ induced iNOS and IL-6 expression (Fig. 4D-E). Interestingly, the observation that macrophage activation with LPS/IFN-γ leads to iNOS-dependent CLA