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Nitroarachidonic acid (NO_2AA) inhibits protein disulfide isomerase (PDI) through reversible covalent adduct formation with critical cysteines

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ABSTRACT

Background: Nitroarachidonic acid (NO_2AA) exhibits pleiotropic anti-inflammatory actions in a variety of cell types. We have recently shown that NO_2AA inhibits phagocytic NADPH oxidase 2 (NOX2) by preventing the formation of the active complex. Recent work indicates the participation of protein disulfide isomerase (PDI) activity in NOX2 activation. Cysteine (Cys) residues at PDI active sites could be targets for NO_2AA- nitroalkylation regulating PDI activity which could explain our previous observation.
Methods: PDI reductase and chaperone activities were assessed using the insulin and GFP renaturation methods in the presence or absence of NO_2AA. To determine the covalent reaction with PDI as well as the site of reaction, the PEG-switch assay and LC–MS/MS studies were performed.
Results and conclusions: We determined that both activities of PDI were inhibited by NO_2AA in a dose- and time-dependent manner and independent from release of nitric oxide. Since nitroalkenes are potent electrophiles and PDI has critical Cys residues for its activity, then formation of a covalent adduct between NO_2AA and PDI is feasible. To this end we demonstrated the reversible covalent modification of PDI by NO_2AA. Trypsinization of modified PDI confirmed that the Cys residues present in the active site a' of PDI were key targets accounting for nitroalkene modification.
General significance: PDI may contribute to NOX2 activation. As such, inhibition of PDI by NO_2AA might be involved in preventing NOX2 activation. Future work will be directed to determine if the covalent modifications observed play a role in the reported NO_2AA inhibition of NOX2 activity.

1. Introduction

Nitrated fatty acids, in particular nitroalkenes, are pleiotropic anti-inflammatory compounds found in several biological systems [1–7]. It is well established that lipid nitration occurs under both physiological and pathophysiological conditions with end products capable of modulating cell responses [2–3,5,8–14]. Nitroalkenes are detected *in vivo* in the nanomolar to low micromolar range concentrations [8,15–19], sufficient for triggering peroxisome proliferator-activated receptor γ (PPARγ) signaling pathways [3,8,13], releasing •NO in aqueous milieu [6,20–22] and promoting endothelium-independent vessel dilation [10].

We have demonstrated several important biological actions for nitroarachidonic acid (NO_2AA), the nitrated derivative of AA (20:4) [4,22–24]. For example, NO_2AA alters superoxide ($O_2^{\bullet-}$) production from the phagocytic NADPH oxidase (NOX2) in activated macrophages [25]. This inhibitory action of NO_2AA involved the prevention of active complex formation at the membrane in the absence of altering the phosphorylation state of the cytosolic subunit [25]. Yet, the exact molecular mechanisms underpinning this process remain unknown.

Recently published work indicates that protein disulfide isomerase (PDI) exerts a supportive effect on NOX2 activity [26–27]. PDI is a ubiquitous dithiol-disulfide oxidoreductase located primarily in the endoplasmic reticulum (ER). PDI is a highly conserved redox chaperone enzyme from the thioredoxin superfamily which supports redox protein-folding through oxidation and multiple intramolecular thiol-disulfide exchange reactions [28–29]. The primary activity of PDI is the introduction of disulfide bonds in unfolded proteins at the ER, the best demonstrated function for the enzyme [28]. However, PDI is also

Abbreviations: AA, arachidonic acid; NO_2AA, nitroarachidonic acid; NOX2, phagocytic NADPH oxidase; NOS2, inducible nitric oxide synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species; PDI, Protein disulfide isomerase; DTT, dithiothreitol; GFP, green fluorescence protein; NEM, N-ethylmaleimide; PEG-maleimide, Methoxypolyethylene glycol maleimide.

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reported to be present in the cytosol, the surface of platelets, smooth muscle cells, the endothelium and in neutrophils [29]. Alternative to its chaperone activity, PDI participates at the integrin αIIbβ3 activation on the surface of platelets: a process requisite for platelet aggregation [30]. Interestingly, PDI signaling also contributes to redox-dependent events such as smooth muscle cell migration induced by PDGF and TNFα-dependent angiogenesis.

Previous studies have shown that intracellular PDI regulates the expression and activity of the NADPH oxidase family of proteins (NOX), which are enzymes dedicated to ROS generation. For example, NOX2 is an enzymatic complex consisting of a membrane-imbedded 91 kDa flavoprotein (Nox2) and $p22^{phox}$. The complex is activated when the cytosolic subunits (e.g. $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) are phosphorylated, activated and subsequently translocated with Rac1/2-GTP to the plasma membrane to interact with Nox2. It has been reported that PDI redox dependently participates in the NOX-family enzyme complex activity [26–27,31–34]. In macrophages, PDI overexpression increases *Leishmania chagasi* phagocytosis and NOX activation, whereas PDI silencing decreases both processes [27]. In neutrophils, PDI functions as a redox-dependent enzyme complex organizer thus, plays an important role in the control of $p47^{phox}$ migration to the membrane and perhaps modulates proper assembly and subsequent activation of NOX2 [26]. There is evidence for $p67^{phox}$ playing a key role in the induction of a conformational remodeling of NOX2, resulting in the induction of the electron flow from NADPH to oxygen [35–37]. Therefore, NOX2 inhibition by NO_2AA could be due to interaction with different cytosolic or membrane protein targets. A potential mechanism may be interaction of NO_2AA with PDI without affecting NOX2 components. Since PDI has been reported to affect signaling pathways other than Nox2 activation such as platelet activation and aggregation and thrombosis prevention, then modulation of PDI by NO_2AA may affect other biological activities [38–40].

Due to their electrophilic nature, nitroalkenes are capable of forming reversible covalent adducts with nucleophilic residues, e.g. Cys or His residues through Michael addition reactions [11,41–43]. Our hypothesis proposes a covalent modification of critical residues in PDI by NO_2AA which could affect enzyme activity.

2. Material and methods

2.1. Materials and chemicals

Nitroarachidonic acid was synthesized and quantitated as previously described [22–23]. Insulin, EDTA, dithiothreitol (DTT), N-acetyl maleimide, methoxypolyethylene glycol maleimide (PEG-maleimide), β-mercaptoethanol and Sodium pyrophosphate were from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) was from Mallinckrodt. Seq. grade modified trypsin porcine was from Promega and NOC-7 was from Dojindo Molecular Technologies Inc., Japan. All solvents were of HPLC grade from Pharmco (Brookfield, CT).

2.2. Preparation of recombinant human PDI

Recombinant human PDI protein was cloned in pET28a (Novagen) and expressed in *E. coli* [26]. PDI containing a His-tag was purified by affinity chromatography using a nickel-containing column. Protein purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomasie Blue staining as well as western blot with a monoclonal anti-PDI antibody (RL90, Abcam). Protein concentration determined by Bradford using BSA as standard.

2.3. PDI reductase activity

Enzyme reductase activity was measured by using the insulin turbidity assay [44–45]. Recombinant PDI (1 μM) was incubated in 100 mM phosphate buffer pH 7.2, 2 mM EDTA and the reaction initiated by adding 1 μM DTT and 1 mg/mL insulin. Insulin precipitation was measured by changes in absorbance at 600 nm in a microplate reader coupled to a plate spectrophotometer (Varioskan). PDI reductase activity was obtained from the initial slopes of the primary plot. When the effect of NO_2AA was analyzed, the enzyme was incubated with different concentrations (0–30 μM) or pre-incubated at different times (0–60 min) with the nitroalkene or its non-nitrated precursor (AA). Then the reaction was initiated with DTT and insulin. To determine if ·NO was involved, PDI reductase activity was performed with a constant flux of ·NO. We performed the experiments by using the ·NO-donor NOC-7 (1 μM), which releases ·NO at an equivalent flux to 10 μM NO_2AA, in both the absence and presence of NO_2AA.

2.4. PDI chaperone activity

PDI chaperone activity was measured by using the Green Fluorescence Protein (GFP) - renaturation assay in accordance to [46]. The roGFP plasmid was kindly given by Dr. Marcelo Comini from Pasteur Institute, Montevideo. Briefly, recombinant roGFP was acid denatured and the chaperone activity was determined by following the fluorescence ($\lambda exc = 485$ nm, $\lambda em = 538$ nm) due to the renaturation of roGFP. A 5 μM acid-denatured roGFP solution (pH 1.5) was prepared by mixing a 10 μM recombinant roGFP solution in denaturing buffer (0.3 mM EDTA, 1 mM DTT, 50 mM Tris-HCl, pH 7.5) with an equal volume of 125 mM HCl and incubated at room temperature for 1 min. Low fluorescence confirmed protein denaturation. Then PDI (2 μM) was added to 5 μM acid-denatured roGFP in renaturing buffer (25 mM $MgCl_2$, 100 mM KCl, 50 mM Tris-HCl, pH 7.5) and the chaperone activity followed by fluorescence at room temperature. Fluorescence was detected using a microplate fluorometer (Varioskan). The chaperone activity was obtained from the initial slopes of the primary plots. To evaluate the effect of NO_2AA, 2 μM PDI was incubated with different nitroalkene concentrations (0–30 μM) for 15 min. Then the reaction was initiated adding the acid-denatured GFP solution. As a control, non-nitrated AA was also studied.

2.5. PEG-switch assay

To determine the formation of a reversible covalent adduct between NO_2AA and PDI, we used a modified PEG-switch assay [47]. PDI was incubated with DTT for 30 min and the excess of DTT was removed using Amicon Ultra-0.5 mL Centrifugal Filters MWCO 10 kDa (Merck KGaA, Germany). In all cases, reduced thiols were measured with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to confirm total reduction of the enzyme [48]. Reduced PDI was treated with NO_2AA (1:5 or 1:10 molar ratio) for 30 min at 37 °C and then alkylated with N-acetyl maleimide to block the remaining free thiols. Then, PDI was treated with β-mercaptoethanol to release the adducted nitroalkene and the thiols labelled with Polyethylene glycol monomethyl ether 5,000. The untreated and treated proteins were separated by SDS-PAGE. The gel was stained with Coomassie blue. Changes in protein migration due to PEG-alkylation were correlated with molecular weights standards.

2.6. Mass spectrometry studies

Liquid chromatography- electrospray ionization tandem mass spectrometry (LC–MS/MS) was performed to analyze the formation of an adduct PDI-NO_2AA. Reduced or oxidized PDI treated with NO_2AA as explained previously was analyzed in a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP4500, ABSciex, Framingham, MA). Reduced PDI was prepared by incubating the enzyme with DTT in a 10:1 ratio for 30 min at room temperature. Oxidized PDI was prepared by oxidizing PDI with 10 mM hydrogen peroxide (1:500 ratio), 1 h at 4 °C. The samples were incubated with NO_2AA (1:10) for 30 min at 37 °C. In all cases, after passing the reaction mixture through Amicon Ultra-0.5 mL Centrifugal Filters MWCO 10 kDa (Merck KGaA, Germany), the protein