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Atypical PKCζ transduces electrophilic fatty acid signaling in pulmonary epithelial cells ☆,,☆☆

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Abstract

Nitric oxide and secondary oxides of nitrogen react with unsaturated fatty acids such as linoleic acid to yield oxidized and nitrated products. Fatty acid nitroalkene derivatives, (e.g. nitrolinoleate [LNO_2]) are produced by oxidative inflammatory reactions, detected clinically, display potent electrophilic reactivity and induce post-translational protein modifications that mediate adaptive inflammatory signaling responses. LNO_2 signaling was examined in lung epithelial cells because the alveolar compartment is a rich site for the transduction of redox and inflammatory reactions. LNO_2 did not directly induce Ca^{2+} influx in cultured lung epithelial cells, but inhibited bradykinin-induced Ca^{2+} influx in a cGMP-independent manner. In contrast, LNO_2 activated MAP kinase (Erk1/2) by a mechanism independent of bradykinin. It was hypothesized that these unique responses were transduced by activation of different protein kinase C isotypes, supported by the observation that LNO_2-mediated inhibition of Ca^{2+} influx was blocked by the non-selective PKC inhibitors chelerythine chloride and calphostin C, but not by the calcium dependent "classic" PKC inhibitor Gö6976. Western blot analysis showed that atypical PKCf was activated by LNO_2 stimulation, with PKCf and Erk activation also demonstrated in primary culture of human lung type II cells. Addition of pseudotypical PKCζ substrate peptide reversed LNO_2-mediated induction of Ca^{2+} influx and MAP kinase activation. Finally, the electrophilic nature of LNO_2 resulted in a novel mode of PKCζ activation, covalent adduction of the enzyme. In summary, LNO_2 mediated signaling in lung type II epithelial cells occurs via a unique pathway involving PKCζ.

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Keywords

Signal transduction; Protein kinase C; Pulmonary epithelial cell; Calcium mobilization; Nitrated lipids

Introduction

Nitric oxide (NO) is a diffusible, reactive gaseous second messenger that regulates biologic functions including blood pressure, neurotransmission, and platelet aggregation. The pluripotent signaling actions of NO are exemplified in the lung, where NO displays a wide range of both physiologic and pathophysiologic effects, including the control of airway and vessel tone, regulation of fluid homeostasis and microvascular permeability, and the mediation of inflammatory processes such as septic responses, reperfusion injury and asthma [1,2].

The principal signaling mechanism by which NO operates is the activation of guanylate cyclase [3], however this alone cannot explain the functional diversity of NO. What separates NO from many other second messenger molecules is its complex biological chemistry. Not only is NO a free radical, but it is also a redox active molecule and thus capable of reacting with multiple biological targets [4]. In particular, the reactivity of NO with oxygen-derived species yields a range of secondary oxides of nitrogen (NO_x) that expand its molecular targets via oxidation, nitrosation and nitration reactions.

The reactions of NO and its products in hydrophobic tissue compartments (e.g., lipoproteins, membranes) are particularly facile and may be important aspects of the transduction of NO signaling. Due to its small molecular radius and lipophilicity, NO concentrates in lipid environments where it can more readily participate in a rich spectrum of reactions with oxygen, thiols, free radicals and lipids [5]. Depending on the underlying redox milieu, these reactions will either inhibit or transduce NO signaling. Previously NO has been shown to react with lipids to inhibit chain peroxidation events [6], while higher oxides of nitrogen can directly react with unsaturated fatty acids [7] to yield nitrated fatty acids that display potent signaling functions, particularly within the vascular compartment [8–10].

Fatty acid nitration in the pulmonary compartment was initially indicated in studies aimed at modeling acute and chronic of exposures of pulmonary surfactant lipids to NO_2 in polluted urban atmosphere providing evidence that at low ppm levels, NO_2 reacts with unsaturated fatty acids, in part yielding nitroalkene derivatives such as nitrolinoleic acid (9, 10, 12 or 13-nitro-octadecadienoic acid, LNO_2). The synthesis and physical characterization of LNO_2 derivatives have provided a basis to investigate the reactivities and biological signaling actions of nitrated lipids [11]. This species and other nitrated fatty acid derivatives have now been detected in plasma lipoproteins and red blood cell membranes [8], with nitrated fatty acid identified as potent endogenous ligands for peroxisome proliferator-activated receptors (PPAR) that activate receptor dependent gene expression [12]. Moreover, nitrofatty acids display biologic activities such as induction of endothelium independent vasorelaxation, inhibition of platelet aggregation and inhibition of neutrophil superoxide generation, degranulation and integrin expression [13]. These data indicate that LNO_2 is a pluripotent-signaling molecule particularly within the inflammatory system.

Pulmonary inflammation induces both nitrosative and oxidative reactions, thus provides ideal conditions for biomolecule nitration. Unlike the vascular compartment where the signaling actions of LNO_2 have been studied, the cell signaling consequences of nitrated fatty acids within the pulmonary system are not well understood. Herein, we investigated the

effects of LNO_2 on lung type II epithelial cell function and reveal that LNO_2 covalently reacts with the atypical PKC isotype-PKCξ, resulting in its activation and a consequent down-regulation of cell responsiveness to adrenergic stimulation of Ca^{2+} influx. These observations indicate that fatty acid nitroalkene derivatives display potent signaling actions on the pulmonary epithelium with significant consequences predicted for modulating pulmonary cell responses during inflammatory processes.

Materials and methods

Reagents

LNO_2 and LA were prepared as previously [8]. Bradykinin was purchased from Sigma (St. Louis, MO). Antibody to the activated phosphorylated form of p42/p44 ERKs was purchased from Cell Signaling Technology (Danvers, MA). Antibodies to α, β1, β2, δ and ζ PKC isoforms were from Santa Cruz Biotechnology (Santa Cruz, CA). PKC inhibitors, chelerythine chloride, calphostin C and Gö6976 were from Calbiochem (San Diego, CA). Fura-2 AM and pluronic F-127 were from Molecular Probes (Eugene, OR). PKCζ-pseudosubstrate inhibitor (PKCζ-PI) was from ECL Reagents and horseradish peroxidase-conjugated anti-rabbit IgG was from Amersham (Little Chalfont, Bucks, UK). All other reagents were of the purest grade available.

NO preparation

NO saturated saline was prepared freshly and sterilely for the experiments under a fume hood. Fifty milliliters of saline were bubbled with pure nitrogen for 15 min in a rubber caped flask. This deoxygenated saline was then bubbled with NO gas that had been scrubbed with 1 M NaOH. During the gas bubbling the room temperature was maintained at 22–23 °C. The actual concentration of the NO in the saturated saline solution was measured by NO electrode. In our system, the NO content was about 1.4 mM.

Cell culture

A549 cells were obtained from the ATCC (CCL-185) and maintained in MEM medium containing 5% fetal calf serum (FCS) with 100 IU/ml penicillin and 0.1 mg/ml streptomycin in an incubator at 37 °C with 5% CO_2. For most experiments, cells were seeded into six-well plates (well diameter: 3.5 cm) in 2 ml of medium. Human fetal lung samples, 14–22 weeks gestation, were obtained from Advanced Bioscience Resources (Alameda, CA) and the Birth Defects Laboratory at the University of Washington (Seattle, WA) under protocol (#2008-7-6105) approved by Children's Hospital of Philadelphia. Undifferentiated fetal alveolar epithelial cells were isolated from these lungs via collagenase-trypsin digestion, differential adhesion to remove fibroblasts and plating on coverslips coated with extracellular matrix produced by Madin-Darby canine kidney cells. Final cultures contained fewer than 10% fibroblasts. Cells were cultured in Waymouth's medium in 35-mm dishes. The following day, a combination of stimuli termed DCI [dexamethasone (Dex, 10 nM), cAMP (0.1 mM), and isobutylmethylxanthine (IBMX, 0.1 mM)] was added to the media for the remainder of the culture period. Media was changed daily, and cells were studied at day 4 of culture. Treatment of human fetal lung cells with DCI results in the differentiation of precursor cells into type II-like epithelial cells that contain lamellar bodies, express surfactant protein-A, -B, and -C mRNAs, process SP-B and -C proproteins to mature forms, and display regulated exocytosis of phospholipids [14].

Cell viability assay

The effect of LA or LNO_2 on A549 cell viability was carried out using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium]

(Promega, Madison, WI) assay. Briefly, exponentially growing A549 (3×10^5 cells/well) were seeded in 24-well plates and incubated for 24 h in complete medium. Then A549 cells were incubated in the presence of indicated doses of LA or LNO_2 for 24 and 48 h. To each well, MTS (final concentration of 333 μg/ml)/PMS (25 μM) were added, and the color was developed at 37 °C for 2 h. The absorbance was measured at 490 nm with an enzyme-linked immunosorbent assay reader.

Western blotting

After treatments, cells were washed twice in ice-cold PBS and extracted in SDS/PAGE sample buffer [2% (w/v) SDS, 63.5 mM Tris–HCl, pH 6.8, 10% glycerol, 200 μM Na_3VO_4, 5 μg/ml leupeptin, and 0.1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF)]. Cell lysates were clarified by centrifugation (14,000g, 20 min at 4 °C), heated to 95 °C for 10 min, run on SDS–PAGE gels and transferred onto PVDF membranes (Millipore). The membranes were blocked with 5% non-fat milk in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and incubated with primary antibody diluted in the blocking solution. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL Plus, Amersham, Little Chalfont, Bucks, UK).

PKC activity assay and PKC translocation

PKC activity assay was determined by commercial PKC activity assay kit (Upstate Biotechnology, Lake Placid, NY) followed the manufacturer's procedure. After adding 10 μl of substrate cocktail, 10 μl of inhibitor cocktail, 10 μl of assay dilution buffer, 10 μl of lipid activator, 50 ng of recombinant PKCζ (Upstate Biotechnology) that was preincubated with or without LA (10 μM) or LNO_2 (10 μM) for 2 min in kinase buffer (50 mM HEPES, 100 mM NaCl, 100 mM $MgCl_2$, 50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 0.1% Tween 20, pH 7.4) and 10 μl of diluted [γ-^{32}P]-ATP (1 μCi) were added to the reaction. After incubation at 30 °C for 10 min, 10 μl of the reaction was dotted on Waterman 81 paper. The paper was air-died for 10 min and washed 3 times with 0.75% H_2PO_4. The paper was washed once with acetone and air-dried. The paper dots of the reaction were cut and put into 3 ml scintillation tubes and read on β-counter for 1 min. The data of PKC activity were expressed as count per minute (CPM). Each condition was done at least triplicate. Total PKC activity was measured in cell extracts (Calbiochem, San Diego, CA). After treatment with various mediators in serum-free medium at 37 °C for the indicated times, cells were collected in ice-cold homogenization buffer [20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.3% (v/v) β-mercaptoethanol, 0.1 mM AEBSF, 10 mM benzamidine] and sonicated on ice (3 × 5 s at 20 Hz). Cell lysates were centrifuged at 10,000g for 10 min at 4 °C. The supernatant was collected and used as the cytosolic fraction. The pellet was resuspended in homogenization buffer containing 1% Triton X-100, sonicated for 10 s on ice and centrifuged at 100,000*g* for 60 min. The supernatant of this fraction was saved and used as the particulate fraction. Both fractions were run on Nupage 4–12% Bis-Tris SDS–PAGE gels and then immunoblotted.

ERK assay

Cell extracts were prepared in SDS–PAGE sample buffer, collected by scraping, heated to 95 °C for 10 min, run on 4–12% Bis-Tris SDS/PAGE gels and immunoblotted with an antibody that specifically recognizes p42 and p44 MAP kinases (ERK-1 and ERK-2) activated by phosphorylation at Tyr-204.

Ca^{2+} mobilization analysis

A549 cells (3×10^5 cells) or human type II cells (5×10^5 cells) were plated on glass coverslips (Fisher Scientific, Pittsburgh, PA) sized to fit a homeothermic perfusion chamber platform of an inverted Nikon microscope. Cells were loaded with 5 μM fura-2 acetoxymethylester (Molecular Probes, Eugene, OR) and 0.2 mg/ml pluronic F-127 (Molecular Probes, Eugene, OR) in 2 ml HBSS supplemented with 1% FBS and 1.25 mM $CaCl_2$ was added for 30 min at 37 °C. Cells were stimulated with HBSS supplemented with 1% FBS and 1.25 mM $CaCl_2$ at 37 °C containing LA or LNO_2 at indicated concentrations and excitation induced at 334 and 380 nm with two narrow bandpass filters. Emitted fluorescence was filtered (520 nm), captured with a Hamamatsu CCD video camera (512 × 480-pixel resolution), digitized (256 gray levels) and analyzed with SimplePCI software (Version 3.7.9, Cranberry, PA). Intracellular Ca^{2+} concentration was calculated by comparing the ratio of fluorescence at each pixel to an *in vitro* 2-point calibration curve. The Ca^{2+} concentrations presented herein were obtained by averaging the values of all pixels over a cell body. All data points were collected at intervals of 5 s.

Statistical analysis

Statistical analysis of the differences between means obtained from multiple experiments was performed using one-way ANOVA. $P < 0.05$ was considered to be significant.

Results

LNO_2 inhibits bradykinin-induced Ca^{2+} influx in type II epithelial cells

Bradykinin is a potent adrenergic stimulator of type II cells that induces a significant increase in intracellular calcium [15]. Using A549 cells as a model of pulmonary epithelial type II cells, the effect of LNO_2 on bradykinin-mediated Ca^{2+} influx was examined (Fig. 1A). LNO_2 inhibited Ca^{2+} influx in a dose-dependent manner. LNO_2 is membrane soluble and lipoprotein stabilized, but in aqueous environments can undergo slow aqueous decay via the Nef reaction to release NO [16]. Thus, the effect of LNO_2 on bradykinin-mediated Ca^{2+} influx was evaluated in the context of chemical sources of NO in the presence and absence of the guanylate cyclase inhibitor ODQ. Both NO and the nitrosonium donor SNAP mimicked the effect of LNO_2 upon calcium influx (Fig. 1B), with the effect of both of these compounds entirely reversible by ODQ, indicating a component of NO-mediated, cGMP-dependent actions. The impact of LNO_2 on Ca^{2+} homeostasis was not affected by ODQ. However, indicating a separate component of NO-independent action. We further determined whether the inhibition of cytosolic calcium by LNO_2 was due to its cytotoxicity on pulmonary epithelial cells. Incubation with LA or LNO_2 from the range of (0.1–100 μM) did not induce cell cytotoxicity in A549 cells up to 48 h (Fig. 1C).

LNO_2-induced Erk phosphorylation

One of the downstream targets of adrenergic stimulation in type II cells is the kinase Erk, which becomes activated via phosphorylation upon bradykinin administration. Fig. 2A shows that both bradykinin and LNO_2 stimulate phosphorylation of Erk while similar concentrations of native linoleic acid had no effect. In addition, the actions of LNO_2 were separate from those of bradykinin, as the combined actions of the two treatments were at least cumulative. LNO_2 stimulated Erk phosphorylation dose-dependently (Fig. 2B).

LNO_2-induced changes in intracellular Ca^{2+} are PKC-dependent

Protein kinase C (PKC) enzymes phosphorylate Erk, thus the effect of PKC inhibitors on LNO_2-mediated changes in Ca^{2+} homeostasis were examined. Non-specific inhibitors of PKC reversed the inhibitory actions of LNO_2 towards bradykinin-induced Ca^{2+} influx (Fig.

3). However, the cPKC inhibitor Gö6976 had no effect on LNO_2-mediated inhibition of Ca^{2+} influx, suggesting that LNO_2 activates PKC-dependent signaling via atypical isoforms.

Since PKC activation is associated with membrane association, the effect of LNO_2 on the cellular distribution of PKCs was examined (Fig. 4A). The basal level of association of the typical PKCs α, β_1, and β_2 with A549 cell membranes was not affected by LNO_2 and PKCδ was not membrane-associated in the presence or absence of LNO_2. There was an increase in membrane-associated PKCζ upon LNO_2 treatment. When this property was studied in human type II epithelial cells, LNO_2-mediated PKCζ-membrane association was also demonstrated (Fig. 4B). A549 cell PKCζ activity was inhibited by the peptide substrate analog PS-ζ, attenuating LNO_2-dependent induction of Erk phosphorylation (Fig. 4C). PKCζ-mediated, LNO_2-dependent Erk phosphorylation and downstream effects on inhibition of A549 cell Ca^{2+} influx was indicated by PS-ζ peptide abrogation of LNO_2 inhibition of bradykinin-induced Ca^{2+} influx.

LNO_2 activates PKCζ upon covalent binding

The electrophilic reactivity of the β-carbon of fatty acid nitroalkene derivatives supports Michael addition reaction with nucleophilic residues in proteins such as cysteine and histidine [17]. In order to investigate whether this property contributed to LNO_2 activation of PKCζ, purified PKCζ was treated with a biotinylated derivative of LNO_2, which similarly induced direct PKCζ activation while linoleic acid had no effect (Fig. 5A). Purified PKCζ was treated with biotinylated LNO_2 or biotinylated linoleic acid and analyzed by electrophoresis and Western blotting for covalent LNO_2 reaction with PKCζ (Fig. 5B). LNO_2 treatment resulted in a dose dependent biotinylation of PKCζ, while there was no effect of linoleic acid. Specificity of this reaction was further indicated by the observation that excess unmodified LNO_2 added to biotinylated LNO_2-PKCζ reaction systems competed for LNO_2 reaction with PKCζ (Fig. 5C). These results show that LNO_2 covalently modifies atypical PKCζ to induce activation, with this addition of the hydrophobic nitro-fatty acid also inducing membrane association of PKCζ (Fig. 3).

Discussion

The nitration of unsaturated fatty acids to electrophilic nitroalkene derivatives has been detected in both healthy and inflammatory conditions. Multiple mechanisms can account for fatty acid nitration by NO-derived species such as $^{\cdot}NO_2$, $ONOO^-$, HNO_2 and NO_2^+ [18–20]. During inflammation, lipid oxidation occurs at an accelerated rate, yielding radical intermediates that can support fatty acid nitration. Also, $ONOO^-$ diffuses through membranes and lipoproteins and can induce the nitration of unsaturated fatty acids [21,22]. Fatty acid nitration can also be mediated by nitronium ion (NO_2^+) and $^{\cdot}NO_2$ reaction with unsaturated fatty acids. Nitrating species such as $^{\cdot}NO_2$ yield oxidized and nitrated products by either ionic addition or H-atom abstraction reactions. In acidic tissue compartments, NO_2^- can be protonated to HNO_2 ($pK_a = 3.4$), which readily nitrates unsaturated fatty acids and lipid hydroperoxides. The mechanisms of biological fatty acid nitration, the structural isomer distribution of nitrated fatty acids and both the metabolism and signaling actions of specific nitrated fatty acid regioisomers remain incompletely characterized [23–26].

In this study, we investigated the effects of synthetic LNO_2 on human lung epithelial cell signaling in order to understand the potential role these compounds may play in inflammatory lung disease. Of significance, LNO_2 inhibited type II cell Ca^{2+} influx in response to bradykinin (Fig. 1). This inhibition was independent of potential NO release during the Nef decay reaction of the nitroalkene and occurred via a mechanism dependent upon PKCξ activation as indicated by blockade with the specific inhibitor peptide PS-ξ. The

corresponding native fatty acid (linoleic acid, LA) did not alter type II cell signaling capacity, indicating the dependence of signaling upon the nitroalkene moiety.

These results also reveal the importance of the atypical PKCξ as a mediator of LNO_2-dependent regulation of type II cell function and represents a novel mechanism of signaling for nitroalkene derivatives. Previously reported nitroalkene signaling pathways have relied on NO release, thiol modification and activation of peroxisome proliferator activated receptors and cAMP-dependent protein kinases [9,27]. Functionally nitrated fatty acids have been shown to activate receptor-dependent gene expression at physiological concentrations, initiate anti-inflammatory signaling pathways in neutrophils, and induce vessel relaxation in an endothelium-independent manner [11,13,28]. These studies all support that fatty acid nitroalkene derivatives are pluripotent-signaling mediators that act via both receptor-dependent and receptor-independent pathways, with PKCs now also identified as mediators of LNO_2-dependent signaling.

Previous research has established the capability of nitroalkenes to attenuate induced Ca^{2+} signaling within both neutrophils and platelets [13]. Bradykinin induces Ca^{2+}-influx within epithelial cells via the B2 G-protein coupled receptor and activation of phospholipases. In this study, we observed that LNO_2 was a potent inhibitor of BK-induced Ca^{2+}-influx (Fig. 1). Through its activation of the phospholipase-signaling pathway, BK has downstream effects on MAP kinase activity. Despite its inhibition of Ca^{2+}-influx, it was found that LNO_2 itself directly stimulated MAP kinase activity (Fig. 2). These two observations suggested that LNO_2 may act via PKC signaling, was confirmed when the nonselective PKC inhibitors, chelerythine chloride and calphostin C, which blocked the inhibitory effects of LNO_2. The superfamily of PKC serine-threonine kinase enzymes is divided into three groups of isoenzymes, according to their mode of activation. The conventional PKCs (cPKCs; α, β, and γ) are activated by diacylglycerol (DAG), calcium or phosphatidylserine; while the novel (nPKCs; δ, ε, η, and θ) are activated by only DAG or phosphatidylserine; in contrast, the atypical (aPKCs; ζ and λ) are independent of both calcium and DAG in their activation. The ability of LNO_2 to inhibit Ca^{2+} mobilization in lung epithelial cells provided the first clue that cPKCs were not involved in LNO_2 signaling (Fig. 3), affirmed by the observation that Gö6976, a specific cPKC inhibitor, had no effect on LNO_2-mediated inhibition of Ca^{2+} influx. In order to directly investigate the activation of PKC isotypes, a membrane translocation assay was conducted. Western blotting analysis clearly showed that that atypical PKCζ was activated by LNO_2 both in the A549 cell line as well as primary cultured human lung type II cells (Fig. 4).

PKCζ acts as a regulator of intracellular signaling induced by a variety of extracellular stimuli. PKCζ activation occurs primarily via phosphorylation by 3′-PI-dependent protein kinase 1 in a PIP_3-mediated manner. Downstream actions of PKCζ include activation of the MAP kinase cascade and the transcription factor NFκB, ribosomal S6-protein kinase signaling and the control of cellular polarity. PKCζ is particularly abundant within the lung, where it mediates NFκB activation and nuclear translocation via IKK. In the present study, LNO_2 activation of PKCζ also results in downstream MAP kinase stimulation, notably Erk (Fig. 2). PKCζ activation has been viewed to date to be primarily via phosphorylation, which results in translocation of the kinase to the membrane. Herein we report that LNO_2 activated type II epithelial cell PKCf in a manner that induced translocation to the membrane without phosphorylation. Previous work has shown that, via kinetically rapid Michael addition reaction, nitroalkenes directly alkylate thiols [27]. This addition of an aliphatic group to the protein increases membrane association and can also impact activity [17]. In this regard, it is important to note that LNO_2 directly activates recombinant PKCζ *in vitro* (Fig. 5) under conditions that result in direct covalent linkage between the nitroalkene and PKCζ, presumably via Michael addition (Fig. 5). We thus propose that membrane-

associated binding and modification by fatty acid nitroalkene derivatives initiates signaling within pulmonary epithelial cells.

LNO_2-mediated functions are likely to be highly cell type-dependent. For example, LNO_2 mediates smooth muscle relaxation through exclusively cGMP-dependent mechanisms [11], whereas in platelets and neutrophils, there is no role for NO, with signaling actions in part being cAMP dependent [29]. Indeed, it is expected that most nitro-fatty acids will be esterified to complex lipids, suggesting that during inflammatory responses, esterases and A_2-type phospholipases can also hydrolyze and mobilize membrane-stabilized LNO_2 for mediating cell-signaling actions. This regulated disposition of LNO_2 in lipophilic *versus* aqueous environments thus represents a “hydrophobic switch” that can control the nature of LNO_2 signaling activity. LNO_2 mediated regulation of lung type II cells thus occurs via a unique pathway, which is independent of NO release and guanylate cyclase activation, as it is resistant to inhibition by ODQ. It is apparent that LNO_2 mediated functions in different cell systems are based on the mechanisms that favor LNO_2 decay to release NO, react with thiols or both.

LNO_2 directly induces the activation of PKCζ through modification of this protein kinase leading to translocation, which may have relevance to pulmonary pathophysiology. For instance, it has been shown that disruption of PKCf in the lung attenuates ovalbumin-induced airway inflammation [30]. PKCζ was elevated in patients with COPD indicating the importance of this enzyme in chronic lung diseases [31]. Recently, it was shown that PKCζ mediates cigarette smoke/aldehyde and LPS-induced lung inflammation and histone modification [32]. Nitrating agents, such as nitrogen dioxide, have been found in the urban atmosphere and to be capable of reacting with lipids in the lung lining fluid [33]. Further, LNO_2 has been detected in the lung lining fluid of individuals with a variety of pulmonary inflammatory diseases including respiratory distress syndrome. Our observations raise the possibility that nitroalkenes, formed either by disease or by pollutant exposure, modulate epithelial cell function and as such may play a significant role in pulmonary pathophysiology. In summary, our studies have identified atypical PKCξ as a key participant in nitro-fatty acid regulation of pulmonary epithelial cell function and provide additional insight into molecular mechanisms of nitroalkene signaling during inflammation. The observation that LNO_2 specifically activates PKCξ reveals a novel mechanism for understanding the actions of this transducer of NO signaling.

Abbreviations

LNO_2	nitrolinoleate, 9, 10, 12 or 13-nitro-octadecadienoic acid
PKC	protein kinase C
BK	bradykinin
NO	nitric oxide

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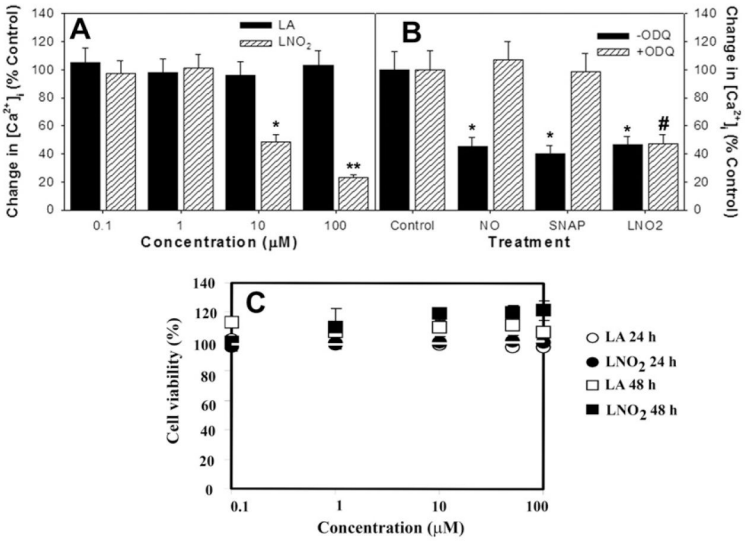

Fig. 1.

LNO_2 inhibits bradykinin-mediated calcium influx in A549 cells. (A) A549 cells were preloaded with fura-2 AM and incubated at 37 °C with different concentrations of LNO_2 or linoleic acid (LA) for 2 min. prior to Bk addition and monitoring of fluorescence ratios (340 and 380 nm, emission at 510 nm). Data represent the mean ± SEM ($n = 6$). *$P < 0.05$ *versus* control or LA treatment. **$P < 0.001$ versus control or LA treatment. (B) A549 cells grown on cover slips were loaded with fura-2 AM. When added, 4 μM ODQ was given 20 min prior to assay, and NO (100 μM), SNAP (1 mM) or LNO_2 (33 nmol/10^6 cells or 10 μM) was given 2 min before Bk addition. Data represent the mean ± SEM ($n = 3$). **$P < 0.05$ versus control, #N.S. *versus* LNO_2 plus ODQ. (C) Cell viability in LA and LNO_2 treated cells. Cells were treated with LA or LNO_2 at concentration indicated for 24 and 48 h, followed by performance of MTS assay as described under 'Materials and methods'. The data shown are two experiments.

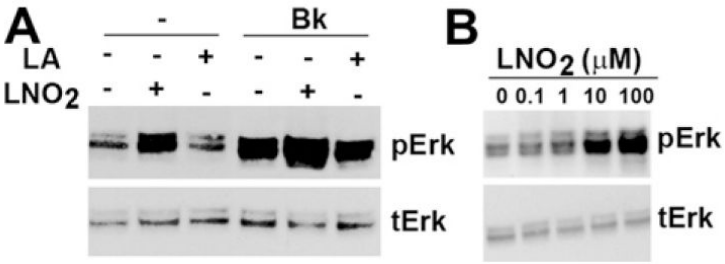

Fig. 2.

LNO_2 induces phosphorylation of Erk. (A) A549cells were incubated with or without (33 nmol/10^6 cells or 10 μM) LNO_2, (10 μM) LA and/or (10^{-7} M) Bk for 5 min. LNO_2 or LA was preincubated for 2 min prior to addition of Bk. Whole cell lysates were analyzed by Western blotting for phosphorylated Erk (p-Erk) or total Erk content. (B) Primary cultures of human type II cells were incubated with different concentrations of LNO_2 for 5 min. Cell lysates were analyzed by Western blotting for phosphorylated Erk (P-Erk) and total Erk content.

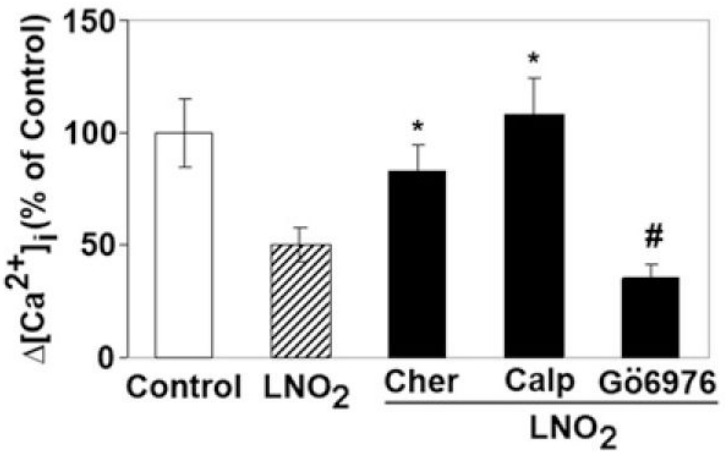

Fig. 3.

LNO_2 signaling is transduced via an atypical PKC. A549 cell were pretreated with the nonselective PKC inhibitors chelerythine chloride (1 μM) (Cher) and (1 μM) calphostin C (Calp) or (1 μM) Gö6976 for 1 h. The cells were loaded with fura-2 AM and incubated with (33 nmol/10^6 cells or 10 μM) LNO_2 for 2 min and stimulated by BK (10^{-7} M). LNO_2 mediated inhibition of Ca^{2+} flux was monitored. Data represent the mean ± SEM ($n = 3$). *$P < 0.01$ *versus* LNO_2 treatment, #N.S. *versus* LNO_2 treatment.

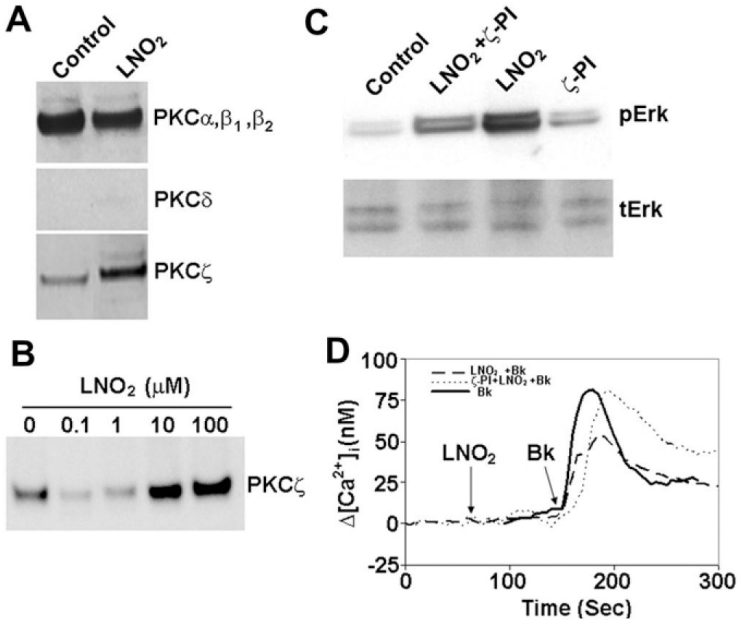

Fig. 4.

LNO_2 signaling actions are mediated by atypical PKCζ. (A) A549 cells were treated with (33 nmol/10^6 cells or 10 μM) LNO_2 for 5 min followed by ice-cold PBS washing and cell harvesting. The distribution of PKCα, β1, β2, δ and ζ on the membrane was analyzed following differential centrifugation by SDS–PAGE and Western blotting. (B) Primary cultures of human type II cells were treated with LNO_2 for 5 min followed by washing with ice-cold PBS and cell harvesting. The distribution of PKCξ on the membrane was analyzed following differential centrifugation by SDS–PAGE and Western blot. (C) A549 cells were stimulated with (33 nmol/10^6 cells or 10 μM) LNO_2 for 5 min after pretreatment with PS-ξ peptide (10 μM, 60 min) and analyzed for p-Erk by Western blot. (D) A549 cells grown on coverslips were incubated in the presence and absence of PKCξ -PI (10 μM, 60 min). Following loading with fura-2 AM and incubation with LNO_2 (33 nmol/10^6 cells or 10 μM), cells were stimulated with Bk (10 nM) and fluorescence ratios were monitored (e.g. 340 and 380 nm, emission at 510 nm). Tracings are an average from recording of 50–70 cells.

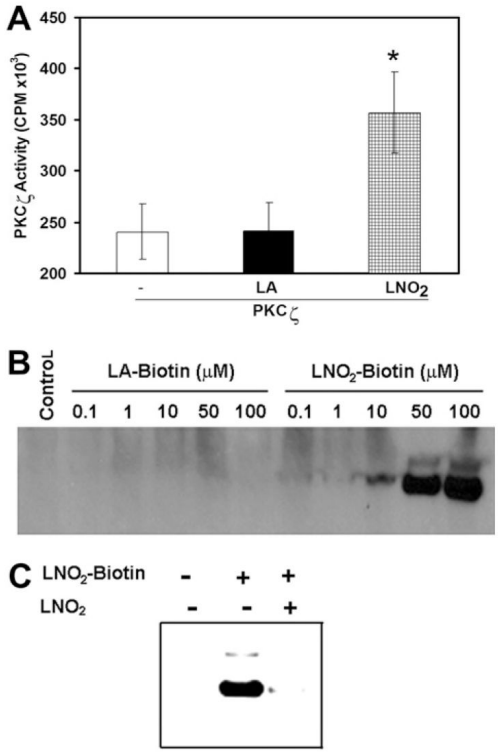

Fig. 5.

LNO_2 covalently modifies PKCζ to induce PKCζ activation. (A) Recombinant PKCζ (50 ng) was used in an *in vitro* reconstitution activity assay. The recombinant PKCζ was incubated with LA (10 μM) or LNO_2 (10 μM) and the activity of PKC was based on phosphorylation of a specific substrate peptide via transfer of [λ-^{32}P] ATP by PKC kinase activity. The quantitation of PKCζ activity is shown. Data represent the mean ± SEM (n = 3). *$P < 0.01$ *versus* control and LA treatment. (B) Biotinylated LA or LNO_2 at indicated concentrations was incubated with PKCζ in kinase buffer. Labeled proteins were separated by SDS–PAGE and analyzed by Western blot using an anti-biotin primary antibody. A biotin positive band was detected at 66 kDa indicating PKCζ-LNO_2 formation. The data shown are representative Western blot of two separate experiments. (C) These experiments were repeated using non-biotinylated (1 mM) LNO_2 as a competitive reactant to ensure specificity. The data shown are representative of two separate experiments.