Nitrolinoleic acid: An endogenous peroxisome proliferator-activated receptor γ ligand

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Nitroalkene derivatives of linoleic acid (nitrolinoleic acid, LNO₂) are formed via nitric oxide-dependent oxidative inflammatory reactions and are found at concentrations of $\approx$ 500 nM in the blood of healthy individuals. We report that LNO₂ is a potent endogenous ligand for peroxisome proliferator-activated receptor γ (PPAR γ ; K_i $\approx$ 133 nM) that acts within physiological concentration ranges. This nuclear hormone receptor (PPAR γ) regulates glucose homeostasis, lipid metabolism, and inflammation. PPAR γ ligand activity is specific for LNO₂ and not mediated by LNO₂ decay products, NO donors, linoleic acid (LA), or oxidized LA. LNO₂ is a significantly more robust PPAR γ ligand than other reported endogenous PPAR γ ligands, including lysophosphatidic acid (16:0 and 18:1), 15-deoxy- $\Delta^{12,14}$ -PGJ₂, conjugated LA and azelaoyl-phosphocholine. LNO₂ activation of PPAR γ via CV-1 cell luciferase reporter gene expression analysis revealed a ligand activity that rivals or exceeds synthetic PPAR γ agonists such as rosiglitazone and ciglitazone, is coactivated by 9 *cis*-retinoic acid and is inhibited by the PPAR γ antagonist GW9662. LNO₂ induces PPAR γ -dependent macrophage CD-36 expression, adipocyte differentiation, and glucose uptake also at a potency rivaling thiazolidinediones. These observations reveal that NO-mediated cell signaling reactions can be transduced by fatty acid nitration products and PPAR-dependent gene expression.

fatty acid | nitric oxide | free radical | adipocyte differentiation | redox

The reaction of nitric oxide with tissue-free radical and oxidative intermediates yields secondary oxides of nitrogen that mediate oxidation, nitration, and nitrosation reactions (1, 2). Of present relevance, the reaction of NO and NO-derived species with oxidizing unsaturated fatty acids is kinetically rapid and exerts a multifaceted impact on cell redox and signaling reactions. NO readily outcompetes lipophilic antioxidants for the scavenging of lipid radicals, resulting in the inhibition of peroxyl radical-mediated chain propagation reactions (3). Both the catalytic activity and gene expression of eicosanoid biosynthetic enzymes are also regulated by NO, affirming a strong linkage between NO and fatty acid oxygenation product synthesis and signaling (4, 5). Consistent with this latter precept, fatty acid nitration products generated by NO-derived species inhibit multiple aspects of inflammatory cell function, indicating that nitrated fatty acids are both by-products and mediators of redox-signaling reactions (6–8).

Recently, the structural characterization and quantitation of nitrolinoleic acid (LNO₂) in human red cells and plasma revealed this unsaturated fatty acid derivative to be the most abundant bioactive oxide of nitrogen in the vasculature. Net blood levels of $\approx$ 80 and 550 nM free and esterified LNO₂, respectively, were measured in healthy humans (9). The observation that NO-dependent oxidative inflammatory reactions yield nitroalkene derivatives of unsaturated fatty acids displaying cGMP-independent cell signaling properties (5) motivated identifying a receptor that might transduce LNO₂ signaling. Affymetrix oligonucleotide microarray analysis of cRNA prepared

from methanol (vehicle), linoleic acid (LA)-, and LNO₂-treated human aortic smooth muscle cells indicated that LNO₂ specifically and potently regulated the expression of key inflammatory, cell proliferation and cell differentiation-related proteins (data not shown). Multiple peroxisome proliferator-activated receptor γ (PPAR γ) target genes were significantly regulated, suggesting that LNO₂ serves as an endogenous PPAR γ ligand.

PPAR γ is a nuclear hormone receptor that binds lipophilic ligands. Downstream effects of PPAR γ activation include modulation of metabolic and cellular differentiation genes and regulation of inflammatory responses (e.g., integrin expression and lipid transport by monocytes), adipogenesis, and glucose homeostasis (10, 11). In the vasculature, PPAR γ is expressed in monocytes, macrophages, smooth muscle cells, and endothelium (12) and plays a central role in regulating the expression of genes related to lipid trafficking, cell proliferation, and inflammatory signaling (13). Although synthetic thiazolidinediones such as rosiglitazone and ciglitazone are appreciated to be the most potent PPAR γ ligands yet described, considerable interest and debate remains focused on the identity of endogenous PPAR γ ligands because of therapeutic potential and their intrinsic value in understanding cell signaling. At present, tissue and plasma levels of putative PPAR ligands are frequently not precisely defined and when so, are found in concentrations sometimes orders of magnitude lower than those required to activate specific α , γ , or δ PPAR subtypes (14–16). Herein we report that nitroalkene derivatives of fatty acids are robust endogenous PPAR γ ligands that act within physiological concentration ranges to modulate key PPAR γ -regulated signaling events including adipogenesis, adipocyte glucose homeostasis, and CD36 expression in macrophages.

Materials and Methods

Materials. LNO₂ and [¹³C]LNO₂ were synthesized and purified by using LA (NuCheckPrep, Elysian, MN) and [¹³C]LA (Spectra Stables Isotopes, Columbia, MD) subjected to nitroselenylation as described (9). LNO₂ concentrations were quantified spectroscopically and by chemiluminescent nitrogen analysis (Antek Instruments, Houston) by using caffeine as a standard (9). Anti-CD36 Ab was kindly provided by F. Debeer (University of Kentucky Medical Center, Lexington, KY); anti-PPAR γ and anti- β -actin Abs were from Santa Cruz Biotechnology; and anti- α P2 Ab was from Chemicon. Horseradish peroxidase-linked

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Abbreviations: LNO₂, nitrolinoleic acid; PPAR, peroxisome proliferator-activated receptor; PPPE, PPAR response elements; LA, linoleic acid; RXR, retinoic X receptor; azPC, 1-O-hexadecyl-2-azelaoyl-sn-glycero-3-phosphocholine; 9-oxoODE, 9-oxo-10(E),12(Z)-octadecadienoic acid; 13-oxoODE, 13-oxo-9(E),11(Z)-octadecadienoic acid; LPA, lysophosphatidic acid.

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goat anti-rabbit IgG and Coomassie blue were from Pierce. [^3H]rosiglitazone was from American Radiolabeled Chemicals (St. Louis). 2-deoxy-D-[^3H]glucose was from Sigma. ScintiSafe Plus 50% was from Fisher Scientific. Rosiglitazone, ciglitazone, 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$, conjugated LA (CLA1 and CLA2), and GW9662 were from Cayman Chemical (Ann Arbor, MI). The 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate [16:0 lysophosphatidic acid (LPA)], 1-O-9-(Z)-octadecenyl-2-hydroxy-*sn*-glycero-3-phosphate (18:1 LPA), 1-O-hexadecyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (azPC), and 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (azPC ester) were from Avanti Polar Lipids.

Cell Transient Transfection Assay. CV-1 cells from American Type Culture Collection were grown to $\approx 85\%$ confluence in DMEM/F12 supplemented with 10% FBS and 1% penicillin-streptomycin. Then, cells were transiently cotransfected with a plasmid containing the luciferase gene under regulation by four Gal4 DNA-binding elements (UAS $_G \times 4$ TK-luciferase, a gift from Ronald M. Evans, The Salk Institute, La Jolla, CA), in concert with plasmids containing the ligand-binding domain for the different nuclear receptors fused to the Gal4 DNA-binding domain. For assessing full-length PPAR receptors, CV-1 cells were transiently cotransfected with a plasmid containing the luciferase gene under the control of three tandem PPAR response elements (PPRE) (PPRE $\times 3$ TK-luciferase) and hPPAR γ , hPPAR α , or hPPAR δ expression plasmids, respectively. In all cases, GFP expression plasmid was cotransfected as the control for the transfection efficiency. After the transfection (24 h), cells were cultured for another 24 h in OPTIMEM (Invitrogen). Then, cells were treated with different compounds as indicated in the figure legends for 24 h in OPTIMEM. Reporter luciferase assay kits from Promega were used to measure the luciferase activity, according to the manufacturer's instructions, with a luminometer (Victor II, Perkin-Elmer). Luciferase activity was normalized by GFP units. Each condition was performed with $n \geq 3$ for each experiment. All experiments were repeated at least three times.

PPAR γ Competition-Binding Assay. Human PPAR γ 1 cDNA was inserted into pGEX (Amersham Pharmacia Biosciences) containing the gene encoding GST. GST-PPAR γ protein induction and receptor binding was assessed as described in ref. 17.

The 3T3-L1 Differentiation and Oil Red O Staining. The 3T3-L1 preadipocytes were propagated and maintained in DMEM containing 10% FBS. To induce differentiation, 2-d postconfluent preadipocytes (designated day 0) were cultured in DMEM containing 10% FBS plus 3 μM LNO $_2$ for 14 d. The medium was changed every 2 d. Rosiglitazone (3 μM) and LA (10 μM) were used as the positive and negative controls, respectively. The differentiated adipocytes were stained by Oil red O as described in ref. 18.

The 2-Deoxy-D-[^3H]glucose Uptake Assay in Differentiated 3T3-L1 Adipocytes. The 2-Deoxy-D-[^3H]glucose uptake assay was performed as described in ref. 19. 3T3-L1 preadipocytes were grown in 24-well tissue culture plates; 2-d postconfluent preadipocytes were treated by 10 $\mu\text{g}/\text{ml}$ insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (all from Sigma) in DMEM containing 10% FBS for 2 d, and then cells were kept in 10 $\mu\text{g}/\text{ml}$ insulin also in DMEM containing 10% FBS for 6 d (changed medium every 3 d). After induction of adipogenesis (8 d), test compounds in DMEM containing 10% FBS were added for an additional 2 d (changed medium every day). The PPAR γ -specific antagonist GW9662 was included 1 h before other additions. After two rinses with serum-free DMEM, cells were incubated for 3 h in serum-free DMEM and rinsed at room

temperature three times with freshly prepared KRPB buffer (5 mM phosphate buffer/20 mM Hepes/1 mM MgSO $_4$ /1 mM CaCl $_2$ /136 mM NaCl/4.7 mM KCl, pH 7.4). The buffer was replaced with 1 $\mu\text{Ci}/\text{ml}$ 2-deoxy-D-[^3H]glucose in KRPB buffer for 10 min at room temperature. The treated cells were rinsed carefully three times with cold PBS, lysed overnight in 0.8 M NaOH (0.4 ml per well), and neutralized with 13.3 μl of 12 M HCl. Lysate (360 μl) was added to 4 ml of ScintiSafe Plus 50% in a scintillation vial, and the vials were mixed and counted.

RNA and Protein Preparation and Analysis. RNA and protein expression levels were analyzed by quantitative real-time PCR and Western blot analysis as described in ref. 20.

LNO $_2$ Decay. LNO $_2$ decay was induced by incubating 3 μM LNO $_2$ in medium plus serum at 37°C. At different times, samples were removed and analyzed for bioactivity or for LNO $_2$ content by Bligh and Dyer extraction in the presence of 1 μM [^{13}C]LNO $_2$ as an internal standard. Nondecayed LNO $_2$ was quantified by means of triple quadrupole mass spectrometric (MS) analysis (Applied Biosystems/MDS Sciex, Thornhill, Ontario, Canada) as described in ref. 9.

Results

To characterize LNO $_2$ as a potential ligand for a lipid-binding nuclear receptor [e.g., PPAR α , PPAR γ , PPAR δ , androgen receptor, glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor, and retinoic X receptor α (RXR α)], CV-1 reporter cells were cotransfected with plasmids containing the ligand-binding domain for these nuclear receptors fused to the Gal4 DNA-binding domain and the luciferase gene under regulation of four Gal4 DNA-binding elements. LNO $_2$ (1 μM) induced significant activation of PPAR γ (620%), PPAR α (325%), and PPAR δ (221%), with no impact on androgen, glucocorticoid, mineralocorticoid, progesterone, or RXR α receptor activation (Fig. 1A). To further explore PPAR activation by LNO $_2$, CV-1 cells were transiently cotransfected with a plasmid containing the luciferase gene under three PPRE in concert with PPAR γ , PPAR α , or PPAR δ expression plasmids. Dose-dependent activation by LNO $_2$ was observed for all PPARs (Fig. 1B), with PPAR γ showing the greatest response at clinically relevant LNO $_2$ concentrations.

PPAR γ activation by LNO $_2$ rivaled that induced by ciglitazone and rosiglitazone and exceeded that of 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ (21, 22), which only occurred at concentrations three orders of magnitude greater than found clinically (Fig. 1C and *Inset*). Several other reported endogenous PPAR γ activators added in equimolar concentration with LNO $_2$ [LA, conjugated LA (CLA1, CLA2), LPA (16:0, 18:1), azPC, and azPC ester; 1 and 3 μM], displayed no significant activation of PPAR γ reporter gene expression when compared with vehicle control (Fig. 1C) (23–25). LNO $_2$ -mediated PPAR γ activation was inhibited by the PPAR γ -specific antagonist GW9662 and enhanced $\approx 180\%$ by coaddition of the RXR α agonist 9-*cis*-retinoic acid, which facilitates PPRE promoter activation via heterodimerization of activated RXR α with PPAR γ (Fig. 2A).

LNO $_2$ slowly undergoes decay reactions in aqueous solution, displaying a 30- to 60-min half-life (Fig. 2B), yielding NO and an array of oxidation products (data not shown). The activation of PPAR γ paralleled the presence and concentration of the LNO $_2$ parent molecule, as determined by concomitant receptor activation analysis and MS quantitation of residual LNO $_2$ in reaction mixtures undergoing different degrees of aqueous decomposition before addition to CV-1 cells (Fig. 2B). Affirmation that activation of PPAR γ is LNO $_2$ -specific, rather than a consequence of LNO $_2$ decay products, was established by treatment of PPAR γ -transfected CV-1 cells with NO donors and oxidized LA derivatives. Additionally, the fatty acid oxidation products 9-oxo-

Fig. 3. LNO₂ induces CD36 expression in macrophages and adipogenesis of 3T3-L1 preadipocytes. (A) Mouse RAW264.7 macrophages at ~90% confluence were cultured in DMEM with 1% FBS for 16 h and then treated with various stimuli for 16 h as indicated. The PPARγ-specific antagonist GW9662 was added 1 h before the treatment. The cell lysate was immunoblotted with anti-CD36 and anti-β-actin Abs. (B and C) After reaching confluence (2 d), 3T3-L1 preadipocytes were cultured for 14 d and stained by using Oil red O (B) or treated with various stimuli as indicated, and the cell lysate was immunoblotted with anti-PPARγ, anti-aP2, and anti-β-actin Abs (C). (D) LNO₂ increases 2-deoxy-D-[³H]glucose uptake in 3T3-L1 adipocytes. (Left) The dose-dependent effects of LNO₂ on 2-deoxy-D-[³H]glucose uptake in 3T3-L1 adipocytes. (Right) PPARγ-specific antagonist GW9662 was added 1 h before the treatment. 2-deoxy-D-[³H]glucose uptake assay was performed as described in *Materials and Methods*. All experiments were repeated at least three times. Values are expressed as mean ± SD (*n* = 6). Statistical analysis was done by using Student's *t* test (*, *P* < 0.05 vs. vehicle control; #, *P* < 0.05 vs. GW9662 untreated groups). Veh, vehicle; Rosi, rosiglitazone; 15-d-PGJ₂, 15-deoxy-PGJ₂.

the maintenance of tissue homeostasis and the regulation of cell and organ dysfunction. Of relevance, the generation of PPAR-activating intermediates from complex lipids often requires hydrolysis by phospholipase A₂, with the identity of phospholipid *sn*-2 position fatty acid derivatives that serve as PPAR agonists still forthcoming (33, 34). Thus, the fact that ~80% of LNO₂ present in a variety of tissue compartments is esterified encourages the notion that this pool of high affinity PPAR ligand activity will be mobilized upon the phospholipase A₂ activation that occurs during inflammation to regulate the secondary formation of cell signaling molecules.

Comparison of PPARγ-dependent gene expression induced by LNO₂ with that of thiazolidinediones and putative fatty acid- and phospholipid-derived PPARγ ligands affirmed the robust activity of LNO₂ as a PPARγ ligand. Although a number of endogenous lipophilic species are proposed as PPARγ ligands, their intrinsically low binding affinities and *in vivo* concentrations do not support a capability to serve as physiologically relevant signaling mediators. Presently reported endogenous PPARγ agonists include free fatty acids, components of oxidized plasma lipoproteins (9- and 13-oxoODE, azPC), conjugated LA derivatives (CLA1 and CLA2), products of phospholipase hydrolysis of complex lipids (LPA), platelet-activating factor, and eicosanoid derivatives such as the dehydration product of PGD₂, 15-deoxy-Δ^{12,14}-PGJ₂ (14, 22, 23). Herein, we observed minimal or no activation of PPARγ reporter gene expression by 1–3 μM concentrations of these putative ligands, in contrast to the dose-dependent PPAR activation by LNO₂ that, for PPARγ, was significant at clinically relevant concentrations as low as 100 nM. A dilemma exists in that some putative endogenous PPARγ agonists have only been generated by aggressive *in vitro* oxidizing conditions (e.g., Cu-mediated low-density lipoprotein oxidation) and have not been clinically quantified or detected (for instance, azPC and conjugated LA). Other lipid derivatives proposed as PPAR ligands are present in <100 nM tissue concentrations,

orders of magnitude below their binding affinities (1–15 μM), and are not expected to result in significant receptor occupancy and activation *in vivo*. This latter category includes free fatty acids, eicosanoids, 9- and 13-oxoODE, platelet-activating factor, and 15-deoxy-Δ^{12,14}-PGJ₂ (14). For example, although LPA is a notable PPARγ ligand of relevance to vascular, inflammatory, and cell-proliferative diseases, the plasma concentration of LPA is well below 100 nM (35, 36) and its binding affinity for PPARγ has not been established. Also, the *in vivo* downstream vascular signaling actions of LPA are inconsistent with its proposed PPARγ ligand activity (24, 37–39). Future studies using mice genetically deficient for PPARγ and NO synthase isoforms should assist in defining the mechanisms of formation and endogenous PPAR ligand activity of nitrated fatty acids.

In summary, we show that LNO₂ is a high-affinity ligand for PPARs, especially PPARγ, that activates both reporter constructs and cells at physiological concentrations. Fatty acid nitration products, generated by NO-dependent reactions, thus are expected to display broad cell signaling capabilities as endogenous nuclear receptor-dependent paracrine signaling molecules with a potency that rivals thiazolidinediones (Fig. 1C). Present data reveal that LNO₂ mediates cell differentiation in adipocytes, CD36 expression in macrophages, and inflammatory-related signaling events in endothelium (e.g., inhibition of vascular cell adhesion molecule-1 expression and function; data not shown) with an important contribution from PPARγ-dependent mechanisms (Fig. 3). Nitroalkene derivatives of fatty acids thus represent a unique class of receptor-dependent cell differentiation, metabolic, and anti-inflammatory signaling molecules that serve to converge NO and oxygenated lipid redox signaling pathways.

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