

Niemann-Pick Type C2 Deficiency in Human Fibroblasts Confers Robust and Selective Activation of Prostaglandin E2 Biosynthesis*

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Background: NPC2 is a protein that negatively regulates fibroblast activation.

Results: NPC2-null human fibroblasts display induction of cPLA2, COX-2, and mPGES-1 expression that may contribute to the observed robust and selective activation of PGE2 biosynthesis.

Conclusion: NPC2 may regulate the activated fibroblast inflammatory program through modulation of a cPLA2-dependent biosynthetic pathway.

Significance: NPC2 may represent a novel therapeutic tool for the treatment of inflammatory diseases.

Activated fibroblasts, also known as myofibroblasts, are mediators of several major human pathologies including proliferative fibrotic disorders, invasive tumor growth, rheumatoid arthritis, and atherosclerosis. We previously identified Niemann-Pick type C2 (NPC2) protein as a negative regulator of fibroblast activation (Csepegi, C., Jiang, M., Kojima, F., Crofford, L. J., and Frolov, A. (2011) *J. Biol. Chem.* 286, 2078–2087). Here we report that NPC2-deficiency leads to a dramatic up-regulation of the arachidonic acid (AA) metabolic pathway in human fibroblasts. The major enzymes in this pathway, cPLA2 type IVA, COX-2, and mPGES-1, were dramatically up-regulated at both the transcriptional and translational levels. The specific phenotypic changes resulted in a >10-fold increase in the production and secretion of a key modulator of inflammation and immunity, prostaglandin E2. More importantly, AA metabolome profiling by liquid chromatography/tandem mass-spectrometry revealed the very specific nature of prostaglandin E2 up-regulation as the other analyzed AA metabolites derived from the COX-2, cytochrome P450, 5/15-lipoxygenase, and non-enzymatic oxidative pathways were mostly down-regulated. Blocking activity of cPLA2 efficiently suppressed expression of inflammatory cytokines, IL-1 β and IL-6, thereby identifying cPLA2 as an important regulator of the inflammatory program in NPC2-null cells. Altogether, these studies highlight NPC2 as a specific regulator of AA metabolism and inflammation that suggests potential for NPC2 protein or its related signaling in the treatment of inflam-

matory diseases characterized by the presence of activated fibroblasts.

Activated fibroblasts (AFs)³ are increasingly recognized as key effector cells in the development of major human diseases, including cancer (1), tissue fibrosis (2, 3), atherosclerosis (4), and rheumatoid arthritis (5, 6). Originally termed as myofibroblasts due to their expression of α -smooth muscle cell actin, AFs have now been shown to represent a diverse cell population originating from multiple precursors whose functions extend from the governance of connective tissue remodeling (7) to regulation of the innate and adaptive immunity (8, 9). With regard to the latter, AF are known to serve as nonprofessional antigen-presenting cells as well as to modulate immune inflammatory functions by influencing a variety of immune cells through either direct cell-cell contact or by secretion of soluble mediators that include PGE2 (10).

PGE2 production in AF can either be induced by exogenous stimuli such as thrombin (11) and TNF- α (12) or be constitutive in nature as reported for AF from lamina propria (13) and cancer-associated AF (14). In addition to its well known role as a mediator of classical inflammation through its effects on endothelial cells and nociceptive pathways, PGE2 can serve as a potent regulator of innate immunity, suppressing neutrophil and macrophage functions. Indeed, PGE2 has been shown to suppress granulocyte functions (15), inhibit CCL5 expression in activated macrophages (16), reprogram classically activated

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³ The abbreviations used are: AF, activated fibroblast; NPC2, Niemann-Pick type C2; RTK, receptor tyrosine kinase; GFR, growth factor receptor; cPLA2, cytosolic phospholipase A2; COX-1/2, cyclooxygenase-1/2 also known as PTGS1/2 (prostaglandin-endoperoxide synthase 1/2); PG, prostaglandin; PGD2, prostaglandin D2; PGF2 α , prostaglandin F2 α ; 5-, 9-, 11, and 15-HETE, 5-, 9-, 11-, and 15-hydroxyeicosatetraenoic acid; 9,10-DiHOME, (\pm)9,10-dihydroxy-12Z-octadecenoic acid; 14,15-DiHETrE, (\pm)14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid; 9,10-EpOME, (\pm)9,10-epoxy-12Z-octadecenoic acid; 14,15-EpETrE, (\pm)14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid; SF, synovial fibroblast; AA, arachidonic acid; RA, rheumatoid arthritis.

macrophages (M1) into the alternatively activated macrophages (M2) (17), and skew monocyte-to-macrophage differentiation toward M2 (18).

NPC2 protein was originally identified as a human epididymis 1 (HE1), which represents a major secretory protein of epididymal fluid (HE1) (19) and was later assigned to the second NPC disease locus, *NPC2* (20). NPC2 gene encodes ubiquitous, 14–18-kDa protein whose precise physiologic function remains to be elucidated. NPC2 is highly conserved among species and has a strong amino acid sequence homology and striking structural resemblance to the dust mite allergen Der p2 (21) as well as the Toll-like receptor 4 (TLR4) adaptor protein MD-2 (22). Besides its strong presence in epididymal fluid, NPC2 is also found in large quantities in milk, bile, and plasma (21, 23). Because NPC2 is able to bind unesterified cholesterol with a high affinity (24), it was thought to participate, along with NPC1, in regulation of intracellular cholesterol trafficking (25). However, this view has been recently challenged (26, 27). Consistent with this notion, several very important physiologic processes such as hematopoiesis (28), immunity (29), and somatic cell plasticity (30, 31) have been shown to be regulated by NPC2 independently of its cholesterol binding ability.

We have recently reported that human skin fibroblasts with a natural nonsense mutation in the NPC2 gene were characterized by constitutive receptor tyrosine kinase (RTK)/growth factor receptor (GFR) → ERK 1/2 signaling (30). Constitutive RTK/GFR activation has also been observed in mature human adipocytes after silencing NPC2 gene expression by siRNA (31). This evidence points strongly toward RTK/GFR signaling module as a proximal target for the negative regulation by NPC2.

RTK/GFR → ERK1/2 signaling is one of the major driving forces for PGE2 production where it up-regulates the expression and activity of the components in the cPLA2 → COX-1/2 → mPGES-1 → PGE2 biosynthetic pathway (32–34). Furthermore, sustained ERK1/2 activation is required for maximal PGE2 production (35). We, therefore, reasoned that this pathway should be persistently activated in NPC2-null human fibroblasts that bear the AF phenotype (30).

In the present study we confirmed our hypothesis and demonstrated that NPC2 deficiency results in the up-regulation of cPLA2 → COX-1/2 → mPGES-1 biosynthetic axis at both the transcriptional and translational levels, leading to a robust and specific increase in PGE2 production. Furthermore, inhibiting this biosynthetic pathway in NPC2-deficient cells through suppression of cPLA2 activity resulted in a significant reduction of proinflammatory cytokine expression. Altogether, our studies begin to unveil a novel and important role for NPC2 in regulation of the PGE2 biosynthetic pathway that could modulate inflammation and innate immunity.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, glutamine, and penicillin/streptomycin were obtained from Cellgro. cPLA2 specific inhibitor was purchased from Calbiochem (catalog #525143).

Cell Lines—Normal skin fibroblasts (CRL-1474) were obtained from ATCC. NPC2-null human dermal fibroblasts were described elsewhere (36, 37).

Stable overexpression of NPC2 gene in NPC2-deficient synovial fibroblasts (SF) isolated from patients with rheumatoid arthritis (RA-83) was achieved using the respective OmicsLink ORF cDNA lentiviral expression clone from GeneCopoeia. The human NPC2 pseudovirus-containing lentiviral expression construct was produced by the Cincinnati Children's Hospital Research Core Facility. RA-83 cells (30) were transduced with the above construct using Magnetofection Protocol from OZ Biosciences. Stable transducers were selected by culturing cells in the medium supplemented with 3.0–3.5 μ g of puromycin. The human RA-83 cell line stably overexpressing NPC2 gene was termed RA-83/NPC2.

DNA Microarray Assay—Total RNA was isolated from the normal and NPC2-null confluent cells that were cultured under the normal conditions (36, 37) by using Qiagen RNeasy kit following the manufacturer instructions. Samples were hybridized to the human U133A microarray (Affimetrix) by the Alvin Siteman Cancer Center's Multiplexed Gene Analysis Core at the Washington University in St. Louis. Signal intensity ratios of >2-fold (up-regulated) or <2-fold (down-regulated) in at least two trials were considered regulated. Expression of the genes that were undetectable in the wild type cells and were only present in NPC2-null fibroblasts was termed "induced."

Real-time RT-PCR—Total RNA from unelicited normal and NPC2-deficient human skin fibroblasts was quantitatively converted into the single-stranded cDNA by using a High Capacity cDNA Archive kit (Applied Biosystems). The particular genes were detected using the respective TaqMan Gene Expression Assays (Applied Biosystems) on the 7300 Real-time RT-PCR system from the same manufacturer by relative quantitation employing β -glucuronidase as the reference gene.

Western Blot Analysis—It was performed as previously described (30). Briefly, human fibroblasts were cultured under normal conditions as previously described (36, 37), and both the cytosolic and nuclear protein fractions were isolated from the ~90%-confluent cells using nuclear/cytosol fractionation kit from BioVision according to the manufacturer's instructions. cPLA2 and phospho-cPLA2, respectively, were probed with the D49A7 rabbit monoclonal antibody (catalog #5249) and rabbit polyclonal antibody (Ser-505, Cat# 2831) from Cell Signaling. COX-2 was detected using rabbit polyclonal antibody from Cell Signaling (catalog #4842). mPGES-1 was probed with the rabbit polyclonal antibody from Cayman (catalog #160140). β -Tubulin was detected using mouse monoclonal antibody from Santa Cruz (catalog #sc-55529) and served as a loading control. That was followed by the incubation with the Alexa Fluor 680 goat anti-mouse (Molecular Probes) and IRDye800CW goat anti-rabbit (Rockland) affinity-purified IgG. Protein bands were identified on the same membrane by simultaneous, dual-color scanning using the LI-COR Odyssey near-infrared imaging system.

LC/MS/MS Analysis of AA Metabolites—It was carried out as previously described in details (38). Briefly, an Agilent 1200 SL liquid chromatography series (Agilent Corp., Palo Alto, CA) with an Agilent Eclipse Plus C18 2.1 \times 150-mm, 1.8- μ m column was used for the eicosanoid separation. The mobile phase A was water with 0.1% acetic acid, whereas the mobile phase B was composed by acetonitrile/methanol (80/15, v/v) and 0.1%

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acetic acid. Gradient elution was performed at a flow rate of 250 μ l/min. The injection volume was 10 μ l, and the samples were kept at 4 °C in the auto sampler. Analytes were detected by negative MRM mode using a 4000 QTrap tandem mass spectrometer (Applied Biosystems) equipped with an electrospray ionization source (Turbo V). Calibration curves were generated by 10- μ l injections of seven standards containing each analyte, internal standard I (d4-PGE2, d11-14,15-DiHETrE, d8-5-HETE, and d11-11(12)-EpETrE), and internal standard II (1-cyclohexyl-dodecanoic acid urea) for quantification purpose.

Confocal Immunofluorescence Microscopy—Cells grown under normal conditions on coverslips in 24-well cell tissue culture

plate were fixed, permeabilized, and stained with rabbit polyclonal cPLA2 (catalog #sc-438) antibodies from Santa Cruz as previously described (36, 37). That was followed by incubation with the Alexa Fluor 568 goat anti-rabbit antibody (Molecular Probes). Image acquisition was performed on Leica TCS SP5 inverted confocal system.

PGE2 Detection by Enzyme Immunoassay—PGE2 detection in the conditioned medium of SF from RA patient was performed using the PGE2 enzyme immunoassay kit, Monoclonal from Cayman Chemical, following the manufacturer's instructions.

RESULTS

NPC2 Deficiency Confers a Robust and Selective Activation of the PGE2 Biosynthetic Program—We have recently shown that NPC2 deficiency in human fibroblasts resulted in the sustained activation of RTK/GFR \rightarrow ERK1/2 signaling (30). Given that this signaling pathway provides a driving force for activation of the cPLA2 \rightarrow COX-1/2 \rightarrow mPGES-1 \rightarrow PGE2 biosynthetic axis (Fig. 1) (32–34), we hypothesized that the latter should be significantly stimulated in those cells. To confirm our hypothesis, we first probed by DNA microarray analysis the expression of the genes that regulate prostaglandin biosynthesis and signaling (Fig. 1 and Table 1). These data demonstrated either induced or significantly up-regulated expression of the respective genes. We are using term induced to define expression of the genes that were undetectable in the wild type cells and were only present in NPC2-null fibroblasts. To confirm differential expression of specific genes in human fibroblasts, we performed analysis of the respective mRNA transcripts by real-time RT-PCR (Fig. 2). As in this figure, the data showed a coordinate up-regulation of the genes that regulate PGE2 production, including *PLA2G4A* (~60-fold), *PTGS2* (~150-fold), and *PTGES* (~1.3-fold) as well as that of PGE2 signaling membrane receptor, *PTGER4* (~3-fold).

Next, we examined protein expression levels of cPLA2, COX-2, and mPGES-1 by Western blotting of the cytoplasmic and nuclear fractions, recognizing that membrane-associated and soluble proteins are present in the respective fractions (Fig. 3). Under unstimulated culture conditions, cPLA2 and mPGES-1 expression was induced strongly in NPC2-null cells but was undetectable in normal fibroblasts. More importantly, cPLA2 in NPC2-null cells was present in its activated form as shown by its phosphorylation on Ser-505 (Fig. 3). COX-2, on the other hand, displayed a different expression pattern. In

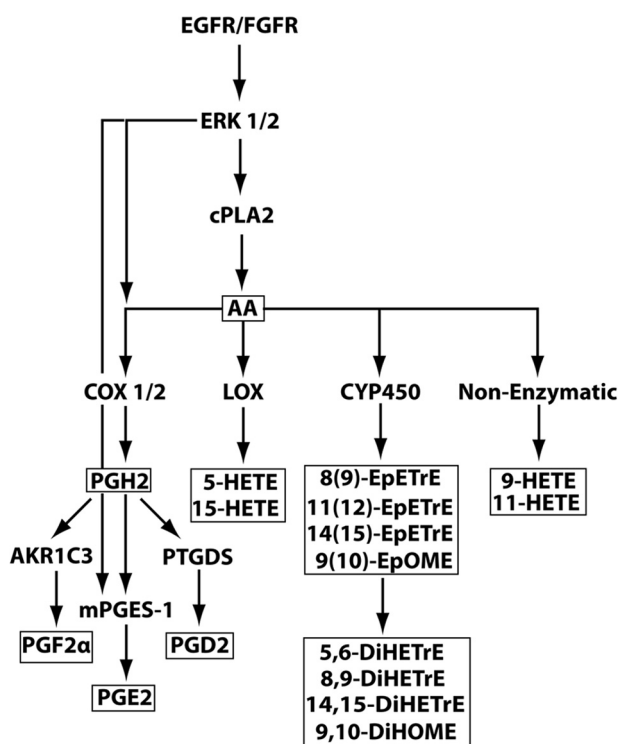


FIGURE 1. Eicosanoid biosynthetic pathway and its activation by RTK/GFR \rightarrow ERK1/2 signaling. Four major branches of the AA metabolic pathway are shown. *COX-1/2*, cyclooxygenase-1/2-dependent branch. *COX-1/2* is also known as prostaglandin-endoperoxide synthase (PTGS) 1/2; *LOX*, lipoxygenase-dependent branch; *CYP450*, cytochrome P450-dependent branch; *Non-enzymatic*, free radical only-dependent branch. *Unboxed abbreviations* depict proteins and biosynthetic pathways. The *boxed symbols* identify AA and its metabolites that were measured in the current study by LC/MS/MS.

TABLE 1

NPC2-deficiency confers activation of a genetic program that controls eicosanoid metabolic pathway and signaling; DNA microarray analysis

Probe set	Gene name	Symbol	Gene expression
Eicosanoid metabolism			
210145_at	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	PLA2G4A	Induced
205128_x_at	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1 (COX-1)	\uparrow 5.3
204748_at	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2 (COX-2)	Induced
210367_s_at	Prostaglandin E synthase	PTGES (mPGES-1)	\uparrow 2.9
211748_x_at	Prostaglandin D2 synthase 21 kDa (brain)	PTGDS	Induced
209160_at	Aldo-keto reductase family 1, member C3 (3 α -hydroxysteroid dehydrogenase, type II)	AKR1C3	\uparrow 10.5
Eicosanoid receptors			
206631_at	Prostaglandin E receptor2 (subtype EP2), 53 kDa	PTGER2 (EP2)	\uparrow 4.7
204897_at	Prostaglandin E receptor4 (subtype EP4)	PTGER4 (EP4)	\uparrow 3.5

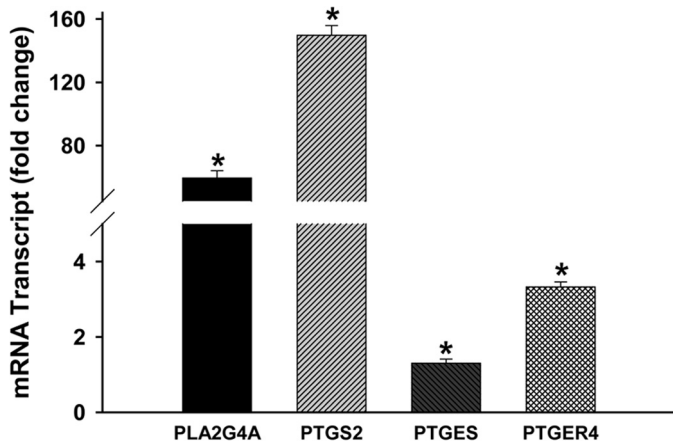


FIGURE 2. Activation of a genetic program that controls eicosanoid metabolic pathway and signaling in unelicited NPC2-fibroblasts; real-time RT-PCR analysis. Total RNA was isolated from unelicited normal and NPC2-deficient human skin fibroblasts and quantitatively converted into the single-stranded cDNA by using a High Capacity cDNA Archive kit (Applied Biosystems). The particular genes were detected using the respective TaqMan Gene Expression Assays (Applied Biosystems) by relative quantitation employing β -glucuronidase as the reference gene. Data were normalized to their respective controls and are shown as the mean \pm S.E. * depicts $p \leq 0.05$ as compared with control ($n = 3-6$).

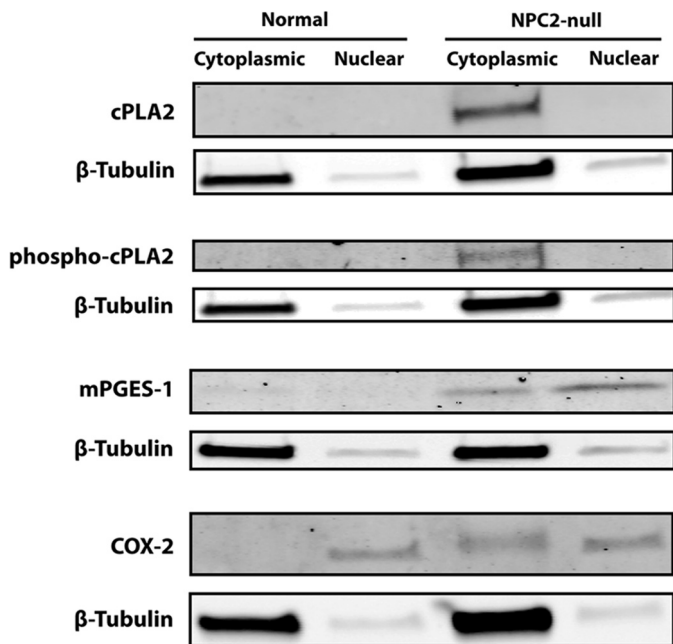


FIGURE 3. Activation of eicosanoid metabolic program in NPC2-null fibroblasts. Protein expression was detected by Western blotting in the cytoplasmic and nuclear protein fractions isolated from unelicited WT and NPC2-null fibroblasts. The respective proteins were labeled with the specific sets of primary/fluorescently tagged secondary antibodies and were identified on the same membrane by simultaneous dual-color scanning using the high resolution LI-COR Odyssey near-infrared imaging system. The phospho-cPLA2 detection was performed after stripping and re-probing a membrane that was originally stained with the cPLA2 antibodies. Quantitative analysis of COX-2 in nuclear protein fractions from control and NPC2-null cells revealed no statistically significant difference in the relative protein expression levels with the respective mean \pm S.E. of 1.22 ± 0.26 ($p = 0.4$, $n = 3$).

NPC2-null cells, COX-2 was present in both the cytoplasmic and nuclear fractions, whereas in normal fibroblasts COX-2 was undetectable in the cytoplasmic fraction and displayed similar expression levels to NPC2-null cells in the nuclear fraction (Fig. 3).

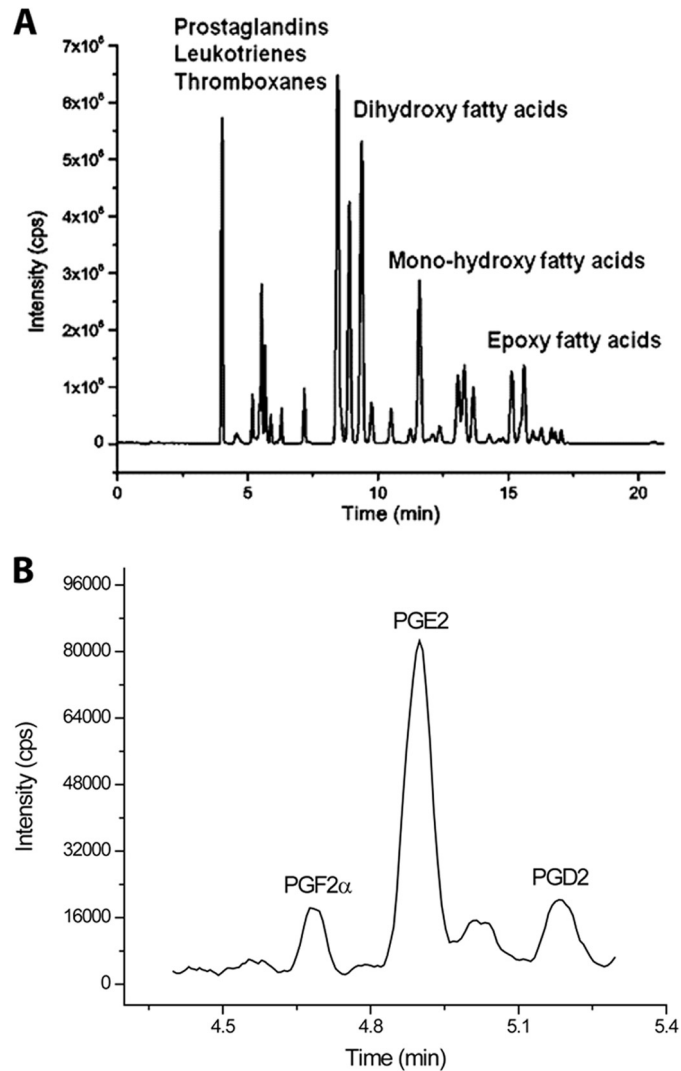


FIGURE 4. Quantitative analysis of selected arachidonic acid metabolites by LC/MS/MS. Lipids were extracted from the conditioned media of near confluent (~90%), unelicited normal, and NPC2-null cells and analyzed by liquid chromatography. *A*, shown is a complete LC chromatogram. *B*, shown is a prostaglandin LC chromatogram.

Altogether, the above data provide strong evidence for constitutive activation of the PGE2 biosynthetic program in NPC2-null fibroblasts. To verify this conclusion, we performed lipidomic profiling of AA metabolites secreted from NPC2-null and normal human fibroblasts by LC/MS/MS. The analysis revealed distinct and detectable levels of prostaglandins, leukotrienes, and thromboxanes as well as mono- and dihydroxy-fatty acids and epoxy-fatty acids (Fig. 4A) with PGE2 being a dominant prostaglandin AA metabolite (Fig. 4B). The specific AA metabolites were further identified and quantified (Fig. 5). As predicted from the transcriptional and translational up-regulation of cPLA2 \rightarrow COX-1/2 \rightarrow mPGES-1 biosynthetic axis in NPC2-null fibroblasts, these cells display a robust (~10-fold) up-regulation of PGE2 production. Unexpectedly, this robust PGE2 up-regulation appeared to be highly specific as the other AA metabolites were either slightly up-regulated (PGD2 and PGF2 α) or mostly down-regulated (Fig. 5). The very modest, in contrast to PGE2, up-regulation of PGD2 and PGF2 α production in the setting of the significant up-regulation of their

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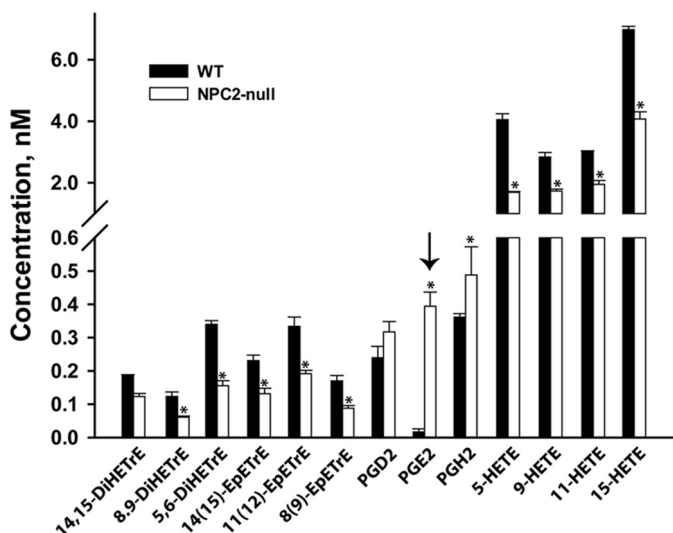


FIGURE 5. Strong and selective up-regulation of PGE2 production in NPC2-null fibroblasts as measured by LC/MS/MS. Lipid analytes isolated from the conditioned media of near confluent (~90%), unelicited normal, and NPC2-null cells were detected by tandem mass spectrometry. Data shown are the mean \pm S.E. * depicts $p \leq 0.05$ as compared with control ($n = 4$). The arrow points toward strongly and selectively up-regulated PGE2 lipid analyte.

respective enzymes, PTGDS and AKR1C3 (Table 1), points toward preferential cPLA2 \rightarrow COX-2 \rightarrow mPGES-1 metabolic coupling in NPC2-null cells (Figs. 1 and 5).

Robust and Selective Up-regulation of PGE2 Biosynthesis in NPC2-null Cells Is Not Controlled by cPLA2 Translocation—It has been previously established that cPLA2 activity is tightly regulated at both the molecular and subcellular levels. The former is more general and includes cPLA2 phosphorylation by ERK1/2 (39) in most of the cell types, whereas the latter is more cell type-specific and includes translocation of the phosphorylated cPLA2 from the cytosol to the perinuclear region (40, 41) and/or nucleus (42) in the immune and endothelial cells. To address a potential cellular mechanism that controls a strong and selective up-regulation of PGE2 production in NPC2-null human fibroblasts, we examined the intracellular localization of cPLA2 in those cells by confocal fluorescence microscopy (Fig. 6). In unelicited normal skin fibroblasts, cPLA2 was virtually undetectable (Fig. 6A), whereas in NPC2-null cells cPLA2 displayed a reticular/punctate cytoplasmic staining pattern (Fig. 6B) that was reported earlier for both unstimulated and stimulated fibroblasts (43, 44). These fluorescence microscopy data correlated well with those of Western blot analysis (Fig. 3) that demonstrated no detectable cPLA2 protein expression in normal fibroblasts along with the exclusive cPLA2 presence in the cytoplasmic fraction of NPC2-null fibroblasts. These findings are consistent with NPC2 regulating cPLA2 activation in human skin fibroblasts at the predominantly molecular level.

cPLA2 Regulates Inflammatory Program in NPC2-null Cells—We have previously shown that NPC2 deficiency in human fibroblasts confers persistent expression and secretion of pro-inflammatory cytokines, such as IL-1 β and IL-6, that was consistent with sustained RTK/GFR \rightarrow ERK1/2 and NF- κ B activation in those cells (30). To further delineate the respective molecular regulatory steps downstream from RTK/GFR \rightarrow

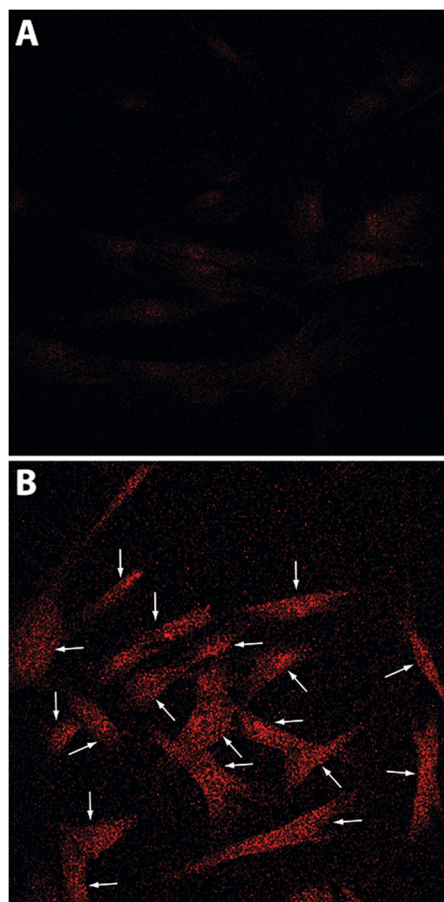


FIGURE 6. Intracellular localization of cPLA2 in unelicited normal (A) and NPC2-null (B) skin fibroblasts as probed by confocal fluorescence microscopy. Cells grown under normal conditions on coverslips in 24-well cell tissue culture plate were fixed, permeabilized, and stained with rabbit polyclonal cPLA2 (catalog #sc-438) antibodies from Santa Cruz. That was followed by the incubation with the Alexa Fluor 568 goat anti-rabbit antibody (Molecular Probes). Image acquisition was performed on Leica TCS SP5 inverted confocal system. The images were taken under the same conditions, and their intensity and contrast were similarly adjusted upward using Adobe Photoshop CS4 Extended software. Arrows point toward individual cells in the confocal section.

ERK1/2 activation and taking into account the constitutive nature of cPLA2 activation as evidenced by its sustained phosphorylation (Fig. 3), we probed the role of cPLA2 in the induction of IL-1 β and IL-6 gene expression by suppressing cPLA2 activity using the cell-permeable, potent, and specific cPLA2 inhibitor CB-525143 (1,2,4-trisubstituted pyrrolidine derivative) (Fig. 7). As shown in this figure, this inhibitor in a dose-dependent manner was able to suppress efficiently both IL-1 β and IL-6 mRNA expression, thereby identifying cPLA2 as an important regulator of the inflammatory program in NPC2-null cells.

NPC2 Overexpression in SF from Patient with RA Suppresses IL-1 β -stimulated PGE2 Production—One of the main maladaptive features of SF in RA is their ability to robustly produce PGE2 upon stimulation with the inflammatory cytokines, such as IL-1 β (45–47). Recently, our group has identified SF from RA patients (SF RA) as being NPC2-deficient, which was indicated by their significantly reduced (ranging from ~2.5 to 5.0-fold) NPC2 mRNA expression as compared with normal human skin fibroblasts (control) (30). Therefore, we reasoned

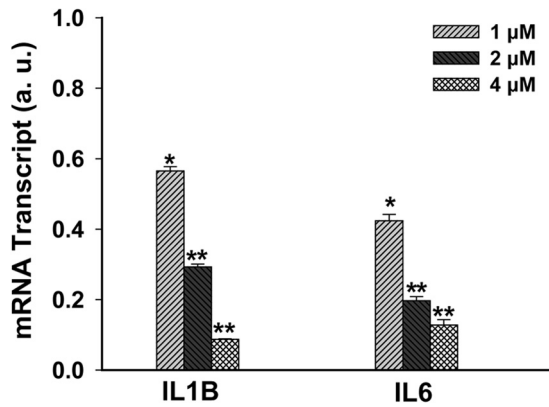


FIGURE 7. cPLA2 activity contributes to the inflammatory cytokine expression in NPC2-null fibroblasts. Cells were treated for 4 h with the indicated concentrations of cPLA2 inhibitor in the DMEM supplemented with 0.1% BSA. IL-1B and IL-6 mRNA levels were probed by real-time RT-PCR. Data were normalized to their respective untreated controls and shown as the mean \pm S.E. * depicts $p \leq 0.05$ as compared with control, and ** depicts $p \leq 0.05$ as compared with the lowest inhibitor concentration ($n = 3$). *a.u.*, arbitrary units.

that the up-regulated PGE2 production by SF in RA could be linked to their NPC2 deficiency. To test this hypothesis as well as to independently verify NPC2 involvement in the negative regulation of PGE2 biosynthesis, we engineered SF RA cell line, RA-83/NPC2, where NPC2 mRNA expression was stably up-regulated by ~ 1.6 -fold as compared with control (Fig. 8A). Yet in comparison to the parental RA-83 cells, the IL-1 β -stimulated PGE2 production in RA-83/NPC2 cells was reduced by ~ 1.6 -fold (Fig. 8B). Altogether, the above data point strongly toward important NPC2 involvement in the negative regulation of PGE2 biosynthesis.

DISCUSSION

The current report provides several important insights into the regulation of inflammation and innate immunity. First, using NPC2-null human skin fibroblasts, we identified NPC2 protein as a novel regulator of AA metabolism that specifically targets the cPLA2 \rightarrow COX-2 \rightarrow mPGES-1 \rightarrow PGE2 biosynthetic pathway (Fig. 1). It appears that NPC2 exerts its function at multiple levels including regulation of cPLA2, COX1/2, and mPGES-1 gene expression as well as the enzyme post-translational modification through phosphorylation, as was seen for cPLA2. In addition, it is quite plausible that mPGES-1 may represent a selective target for NPC2 regulation because in the setting of NPC2 deficiency, which leads to a significant up-regulation of AKR1C3, mPGES-1, and PTGDS that metabolize PGH2 to yield, respectively, PGF2 α , PGE2, and PGD2 (Fig. 1), only PGE2 production was significantly up-regulated, as demonstrated by our lipidomics analysis. Yet the same analysis has revealed the lipoxygenase- and cytochrome P450-dependent as well as non-enzymatic oxidative pathways within the AA metabolic cascade (Fig. 1) were efficiently suppressed in NPC2-null cells as evidenced by a significant reduction in the production of their respective products. Such a pattern of eicosanoid production in the setting of NPC2 deficiency is unique and to our best knowledge has not been previously reported in the literature.

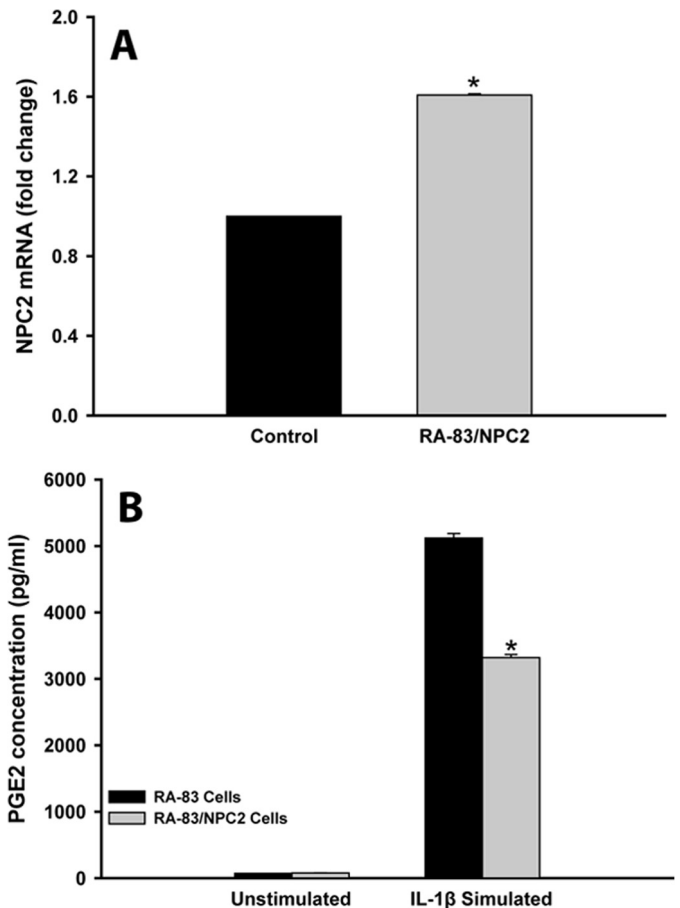


FIGURE 8. NPC2 overexpression in SF RA suppresses IL-1 β -stimulated PGE2 production. A, shown is the NPC2 mRNA level in RA-83/NPC2 cells that stably overexpress NPC2 gene as compared with normal human skin fibroblasts (Control). B, shown is IL-1 β -stimulated PGE2 production in SF RA cells as probed by PGE2 enzyme immunoassay. Data shown are the mean \pm S.E. * depicts $p \leq 0.05$ as compared with RA-83 parental cell line ($n = 3$).

Second, we further delineated a mechanism of NPC2-dependent regulation of the cPLA2-dependent PGE2 biosynthetic pathway in fibroblasts by demonstrating that for cPLA2 this mechanism does not include a subcellular component because cPLA2 activation was not accompanied by its translocation from cytosol to the perinuclear region and/or nucleus as shown earlier for the immune and endothelial cells (40–42). cPLA2 is now considered to be a central enzyme in mediating AA release for eicosanoid production, including PGE2 (48, 49). The most likely mechanism for constitutive cPLA2 activation and subsequent PGE2 production could include, in addition to its Ser-505 phosphorylation, the release of active cPLA2 monomers from inactive intracellular clusters (43, 44). Indeed, it has been previously reported that in fibroblasts, cPLA2 is present in small clusters that were identified as punctuate structures by immunofluorescence and immunogold electron microscopy (43). These cPLA2 clusters were randomly distributed throughout the cytosol and localized in the vicinity of major intracellular membranes, except for the Golgi apparatus. More importantly, upon cell stimulation with EGF or calcium ionophore A23187 that are known to activate cPLA2 and stimulate AA metabolism, the cPLA2 punctate localization pattern did not change (43), but the release of activated cPLA2 monomeric forms from

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inactive clusters and their binding to the nearing intracellular membranes were observed (44). The latter was accompanied by an induction of cPLA2 hydrolytic activity, indicating that the release of active cPLA2 monomers from the existent inactive cPLA2 clusters could be an important mechanism that controls cPLA2 activity in fibroblasts and quite possibly in other non-immune cells (44). Given that very similar punctate cPLA2 structures were observed in NPC2-null fibroblasts by immunofluorescence in the current study and that cPLA2 phosphorylation by ERK1/2 was proposed to be a prerequisite for cPLA2 release from the inactive clusters (44), one may suggest that RTK/GFR → ERK1/2 → cPLA2 phosphorylation axis could be a primary regulatory target for NPC2 within the AA metabolic pathway.

Third, we demonstrated that constitutive cPLA2 activation is responsible for the increased basal production of proinflammatory cytokines in NPC2 null cells. Given that constitutive induction of cPLA2 gene expression and cPLA2 activation through phosphorylation is regulated by sustained RTK/GFR → ERK1/2 signaling (32–34) and that the latter was observed earlier by us in NPC2-null fibroblasts (30), the molecular mechanism of the NPC2-dependent negative regulation of fibroblast activation (30) could be further detailed to include cPLA2 activation step downstream from sustained ERK1/2 signaling.

Fourth, the fact that NPC2 deficiency results in co-stimulated induction of PGE2 (current study) and IL-6 (30) production and secretion could bear very important consequences for the regulation of the innate and adaptive immunity. Indeed, it has been recently reported (18) that the immunosuppressive tumor microenvironment of human cervical carcinoma could be linked to the ability of cancer cells to block dendritic cell differentiation and to skew monocyte-to-macrophage differentiation toward tolerogenic M2 phenotype. As the result, M2 macrophages had a significantly impaired ability to stimulate T cell proliferation and Th1 cell activation, thereby providing, along with the dendritic cell deficiency, a permissive environment for tumor growth. Characteristically, IL-6 and PGE2 had distinct functions in the above process; IL-6 blocks dendritic cell differentiation and PGE2 drives M2 macrophage polarization (18). Finally, using RA-83/NPC2 cells with the stable overexpression of NPC2 gene, we independently confirmed its important involvement in the negative regulation of PGE2 biosynthesis in fibroblasts as well as demonstrated, at least *in vitro*, a potential for the SF RA maladaptive phenotype correction by overcoming their NPC2 deficiency. The latter could provide a basis for the development of a novel therapeutic intervention in RA. In summary, all of these data point toward NPC2 as an important regulator of the phospholipid/AA metabolism, immunity, and inflammation.

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