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The inflammatory response in acyl-CoA oxidase 1 deficiency (pseudoneonatal adrenoleukodystrophy)

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Abstract

Among several peroxisomal neurodegenerative disorders, the pseudoneonatal adrenoleukodys-trophy (P-NALD) is characterized by the acyl-coenzyme A oxidase 1 (ACOX1) deficiency, which leads to the accumulation of very-long-chain fatty acids (VLCFA) and inflammatory demyelination. However, the components of this inflammatory process in P-NALD remain elusive. In this study, we used transcriptomic profiling and PCR array analyses to explore inflammatory gene expression in patient fibroblasts. Our results show the activation of IL-1 inflammatory pathway accompanied by the increased secretion of two IL-1 target genes, IL-6 and IL-8 cytokines. Human fibroblasts exposed to very-long-chain fatty acids exhibited increased mRNA expression of IL-1α and IL-1β cytokines. Furthermore, expression of IL-6 and IL-8 cytokines in patient fibroblasts was down-regulated by MAPK, p38MAPK, and Jun N-terminal kinase inhibitors. Thus, the absence of acyl-coenzyme A oxidase 1 activity in P-NALD fibroblasts triggers an inflammatory process, in which the IL-1 pathway seems to be central. The use of specific kinase inhibitors may permit the modulation of the enhanced inflammatory status.

MESH Keywords Acyl-CoA Oxidase ; deficiency ; genetics ; metabolism ; Cells, Cultured ; Fatty Acids ; pharmacology ; Fibroblasts ; drug effects ; metabolism ; pathology ; Gene Expression Regulation ; drug effects ; Humans ; Immunohistochemistry ; Inflammation ; genetics ; metabolism ; Inflammation Mediators ; metabolism ; Interleukin-1 ; genetics ; metabolism ; Interleukin-6 ; genetics ; metabolism ; Interleukin-8 ; genetics ; metabolism ; Oligonucleotide Array Sequence Analysis ; Osteopontin ; genetics ; metabolism ; Reverse Transcriptase Polymerase Chain Reaction ; Transcriptome

In several peroxisomal disorders, the peroxisomal fatty acid β-oxidation pathway is defective. This may be due to the specific deficiency of an enzyme or transporter involved in peroxisomal β-oxidation or the absence of the complete organelle resulting from a genetic defect in one of the many genes required for proper peroxisome biogenesis and maintenance (1, 2). Pseudoneonatal adreno-leukodystrophy (P-NALD) (OMIM 264470) is a rare, neuroinflammatory, and a neurodegenerative peroxisomal disorder characterized by craniofacial dysmorphism, generalized hypotonia, hepatomegaly, infantile seizures, loss of motor achievements, and white matter demyelination (3–6). P-NALD disease is due to acyl-coenzyme A (CoA) oxidase 1 (ACOX1) deficiency, which leads to a selective impairment of the peroxisomal fatty acid β-oxidation pathway specifically affecting the oxidation of very-long-chain fatty acids (VLCFA). As a consequence, VLCFA accumulate in plasma and tissues (1, 7). ACOX1 catalyzes the α, β-dehydrogenation of a range of acyl-CoA esters, including the CoA-esters of dicarboxylic acids, eicosanoid derivatives, and saturated VLCFA (2, 7, 8). In human and mice, the ACOX1 enzyme is encoded by a single gene, which generates two splice variants, including exon 3a or exon 3b, respectively, leading to the synthesis of two protein isoforms ACOX1a or ACOX1b (2, 9). Although no apparent genotype-phenotype correlation has been established in P-NALD (7), a patient with a single homozygous mutation on exon 3b has also the clinical signs and symptoms of P-NALD (10), thus revealing the substrate specificity of the specific ACOX1 isoforms (2, 8). Mice lacking Acox1 manifest severe inflammatory steatohepatitis with increased intrahepatic H₂O₂ levels and hepatocellular regeneration (11, 12). Progressively, chronic endoplasmic reticulum stress contributes to hepatocarcinogenesis (13), and this steatotic ACOX1 null phenotype can be reversed by expression of the human ACOX1b isoform (8, 13). However, even if they show smaller size and growth retardation when compared with their littermates, Acox1 null mice have no apparent neurological disorder (11, 14). In brain lesions of patients developing the demyelinating form of peroxisomal X-linked adrenoleukodystrophy, oxidative, inflammatory, and apoptotic processes have been described (15–17). In this related peroxisomal disorder, lipid derivatives with an abnormally high proportion of VLCFA residues have been proposed to trigger the initial cascade of the inflammatory demyelination (18, 19). However, the components of this inflammatory process in P-NALD have remained elusive. To explore the inflammatory response in ACOX1 deficiency, we used two patient-derived fibro-blasts for transcriptomic microarray analysis associated with a PCR array screening in an attempt to identify the involved proinflammatory components.
In the present work, we report the expression profiling of inflammatory cytokines in fibroblasts from P-NALD patients. Alterations in the expression of IL-1 pathway were revealed and accompanied by increased secretions of the IL-6 and IL-8. Fibroblasts exposed to VLCFA show increased expression of cytokines mRNA. Signaling pathways involved in the induction of these cytokines were also explored.

**Materials and Methods**

**Cell culture and VLCFA treatment**

Skin fibroblasts were cultured as described (7) and handled according to national and institutional guidelines. Cerotic acid (C26:0) (Sigma-Aldrich, St. Louis, MO) was solubilized in α-cyclodextrine (Sigma-Aldrich). Final concentration of α-cyclodextrine (vehicle) in the culture medium was 1 mg/ml. For fibroblasts treatment, the final concentration of C26:0 was 10 μM.

**Acyl-CoA oxidase activity measurement**

It was performed as described by Oaxaca-Castillo et al. (2).

**Immunostaining, fluorescence microscopy, and Nile red staining**

Immunostaining, fluorescence microscopy, and Nile red staining were achieved as previously described (20).

Microarray analysis (Affymetrix, Santa Clara, CA), cytokines analysis by Cytometric Bead Array Human Inflammation kit (BD Biosciences, Courtaboeuf, France), and PCR array analysis (PAHS-011; SABiosciences-QIAGEN, Courtaboeuf, France) are described in Supplemental Materials and Methods, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

**Results and Discussion**

**Characterization of patient-derived-deficient fibroblasts**

To characterize the deficiency of ACOX1 in P-NALD fibroblasts, the activity of ACOX1 was first measured in cell extracts. As shown in Fig. 1A, weak residual palmitoyl-CoA oxidase specific activity was present in patient 1 fibroblasts, although much reduced, whereas this ACOX1 activity was undetectable in patient 2 fibroblasts. Both patients' fibroblast cells exhibited a strong reduction in the number of peroxisomes per cell, as shown by peroxisomes immunostaining with antibodies against catalase (matrix protein) and 70-kDa peroxisomal integral membrane protein (Fig. 1B). This is accompanied by the enlarged size of peroxisomes as shown by anticatalase immunofluorescence (Fig. 1C). Fibroblasts Nile red staining reveals a transition from the predominance of polar lipids in control fibroblasts (green fluorescence) (Fig. 1C) to an accumulation of neutral lipids in P-NALD fibroblasts (yellow fluorescence) (Fig. 1C). Accumulation of VLCFA in plasma has been previously shown for these patients (7).

**Transcriptomic profiling of inflammatory genes in P-NALD fibroblasts**

To identify proinflammatory genes that are dysregulated in P-NALD/ACOX1-deficient fibroblasts, we used Affymetrix microarray profiling. Transcriptional profiling revealed that a number of genes coding for cytokines and other proinflammatory proteins was up-regulated (≥1.5), including, IL-6, IL-8, and several TNFα family members (3, 8, 9, 10A, 12, and 14) as well as interferon-inducible proteins (Supplemental Table 1). Interestingly, the expression of genes coding for cytokines IL-6, IL-8, and TNFα, which are typically produced by macrophages and by CD4+ T cells Th1, has also been found to be increased in multiple sclerosis and cerebral forms of X-adrenoleukodystrophy lesions (15). On the other hand, several cytokines and chemokine mRNA are strongly down-regulated in P-NALD fibroblasts, including chemo-kine (C-X-C motif) ligand (CXCL)14 and CXCL12 genes, which have been shown to participate in the regulation of cell or tissues homeostasis (21, 22).

**Alterations of the IL-1β pathway in P-NALD fibroblasts**

To define a specific inflammatory pathway activated in ACOX1 deficiency, PCR array (SABiosciences), containing 84 key genes mediating the inflammatory response and which include several genes deregulated in our transcriptomic profiling, was used to determine the profile of reverse-transcribed RNA from the two patients derived fi-broblasts compared with the control fibroblasts. Table 1 shows results for genes significantly regulated in both patients. Based on the 2^−ΔΔCT analyses of three PCR arrays (n = 3) for each fibroblasts sample, 14 genes were strikingly and similarly regulated in ACOX1-deficient fibro-blasts for both patients (cut-offs, −1.5-fold ± gene fold expression ±1.5-fold). Absence of ACOX1 activity, which leads to VLCFA accumulation, triggered mRNA up-regulation of IL-1α, IL-1β, IL-1R1, IL-1RN, IL-17C, secreted phosphoprotein 1 (SPP1), chemokine (C-C motif) receptor type 1 (CCR1), chemokine (C-C motif) ligand (CCL)3, CCL7, CAAT/enhancer binding protein β (CEBPβ), and Toll-interacting protein (TOLLIP) (1.65- to 15-fold) and down-regulation of CXCL14, CCL26, and CXCL5 (~1.92- to −50-fold). Remarkably, all these regulated genes are connected to the IL-1 pathway. Activation of this pathway is triggered by the binding of the IL-1α/IL-1β heterodimer to IL-1R1 (23). Correspondingly, Table 1
shows that IL-1α, IL-1β, and IL-1R1 mRNA are significantly induced in P-NALD fibroblasts. Thus, IL-1, which is recognized as a proinflammatory cytokine (24), is known to control the expression of other inflammatory genes, including TNFα and interferon through a well-defined transduction signaling pathway (24). Intriguingly, the expression of IL-1RN, an IL-1 receptor antagonist, which modulates the inflammatory responses (23), was induced as well (Table 1). It is noteworthy that IL-1RN is also induced in patient serum developing a neurological disorder, such as schizophrenia (25). We cannot exclude that IL-1RN induction may contribute to the attenuation of the inflammatory stress during P-NALD progression by antagonizing IL-1 activity and thus preserving immune homeostasis (23). Furthermore, another cytokine transcript IL-17C was increased more than 2-fold in both patients derived fibroblasts (Table 1). It is a homologue gene of IL-17, which is increased in autoimmune diseases, such as multiple sclerosis (26). Thus, IL-17C may participates in P-NALD-fibroblasts to the release of both IL-1β and TNFα (27).

As shown in Table 1, the SPP1 (also called osteopontin) mRNA is highly induced (at least 4-fold) in ACOX1-null fibroblasts. Reportedly, SPP1 expression is induced by IL-1α or IL-1β as well (28, 29). SPP1 is an extracellular glycoprotein, belonging to the integrin superfamily (30). This two-sided mediator acts in a context-dependent manner as a neuroprotectant (31) or as triggering the neuronal toxicity (32) and has been reported in several neurodegenerative diseases, such as multiple sclerosis, Parkinson's disease, and Alzheimer's disease (32). Interestingly, in P-NALD fibroblasts beside the induction of cytokine mRNA, the expression of several chemokine transcripts (CCL3, CCL7, CCL26, CCR1, CXCL5, and CXCL14) is strongly modified as well (Table 1). Transcripts of both CCR1 and its chemokine ligands CCL3 (Rantes/macrophage inflammatory protein 1α) and CCL7 (monocyte chemoattractant protein-3) were highly induced in P-NALD fibroblasts. CCR1 and its ligands play a critical role in the recruitment of inflammatory cells to neurological lesions (33, 34). Hence, infusions of several cell lines with IL-1α or IL-1β, including Caco-2, hepatoma, smooth muscle, or astrocytes cell lines (35–38), display enhanced synthesis of CCL3 and/or CCL7, which may interact with its CCR1 receptor. Thus, induction of CCR1 and its ligands in P-NALD-fibroblasts may reflect a common inflammatory response as reported in many neurodegenerative diseases (34, 39).

Interestingly, the increased expression of CEBPβ (2.25-fold) and TOLLIP (mean 2.8-fold) constitutes an additional argument of the activation of the IL-1 inflammatory pathway in P-NALD-fibroblasts (Table 1 and Supplemental Table 1). Hence, enhanced synthesis of CCL3 ligand (Table 1) through the activation IL-1 pathway (as cited above) is dependent on the transcriptional activation of CCL3 gene promoter by CEBPβ (40). Furthermore, TOL-LIP, which constitutes an important component of IL-1R signaling pathway (41), can limit the production of pro-inflammatory cytokines (42) by controlling the magnitude of IL-6 and TNFα in response to IL-1β (43).

According to our transcriptomic profiling results (Supplemental Table 1) and using cytometric bead array analysis, we show in Fig. 2 that the secretions of IL-6 and IL-8 cytokines were strongly induced in P-NALD fibroblasts, whereas secretion of TNFα was not significantly changed (data not shown). Thus, ACOX1 deficiency in P-NALD fibroblasts leads to the activation of IL-1 inflammatory pathway and enhanced synthesis of its target genes, IL-6 and IL-8 (Fig. 2).

From the 84 genes present in PCR array, only three chemokine genes (i.e. CCL26, CXCL5, and CXCL14) exhibited a similar down-regulation in the two patients derived fibroblasts (Table 1). The CCL26 (or Eotaxin-3) is a strikingly decreased chemokine gene in P-NALD-fibroblasts (~3.5- to ~14-fold) (Table 1). This may be correlated to the induction of CCL3, revealing an autocrine mechanism involving CCL3, which selectively down-regulates CCL26 (44). Two other transcripts encoding chemokine ligands were highly decreased in P-NALD fibroblasts, and both belong to the CXCL family. CXCL5 (also called epithelial-derived neutrophil-activating peptide 78) is down-regulated in P-NALD fibroblasts (Table 1) and also in plasma of patients with chronic liver disease and serves as biomarker of necroinflammation and liver fibrosis (45). Hence, P-NALD patients are known to develop hepatomegaly and liver fibrosis (7). Although CXCL14 deficiency has been linked to the attenuation of obesity and brain control of behavior feeding (46). Decreased expression of both CXCL5 and CXCL14 (Table 1) may reflect the dysregulation of lipid metabolism, thus impacting the inflammatory process during P-NALD disease progression.

**Inflammatory response of fibroblasts to increased VLCFA-cerotic acid concentration**

The increase in the VLCFA levels precede largely the white matter demyelination in P-NALD and the neuroinflammatory response in childhood X-linked adrenoleukodystrophy as well (15, 18, 19). Although it is well known that both P-NALD and X-linked adrenoleukodystrophy are associated with the accumulation of VLCFA (1, 8), the direct role of VLCFA in the induction of inflammatory process still is, however, merely speculative (18). To try and understand this possible relationship, we treated control fibroblasts with the cerotic C26:0 fatty acid. Figure 3 shows the time-course expression of cytokines (IL-1α, IL-1β, and IL-6) and ACOX1b, the ACOX1 isoform involved in C26:0-β-oxidation (2, 8), transcripts in fibroblasts exposed to 10 μM C26:0 during 48 h. As shown in Fig. 3, enhanced cytokines mRNA expression, particularly IL-1α and IL-1β, was evident already between 6 and 12 h, showing a sequential and similar induction with a maximum at 12 h. A return to the control level of both cytokine mRNA at 18 h is concomitant to a delayed ACOX1b mRNA expression hit (Fig. 3). By contrast, 6 h later (a 24-h time course), the expression levels of IL-1α and IL-1β mRNA increased at 24 and 48 h, whereas at the opposite, ACOX1 transcripts were reduced again and stay under the control threshold at 48 h of VLCFA treatment. Thus, C26:0-VLCFA seems to regulate concomitantly and sequentially, in a divergent manner, both cytokines and ACOX1 mRNA levels. This sequential regulation in fibroblasts is probably linked to the fact that cytokines, such IL-1β, are able to increase
accumulation of VLCFA through inhibition of the peroxisomal β-oxidation of C26:0-cerotic acid by an unknown mechanism (19). This may install a vicious circle, in which C26:0 fatty acid triggers earlier increase of mRNA cytokines, which down-regulate peroxisomal β-oxidation leading to the accumulation of VLCFA. The latter in turn promotes the reinduction of cytokine transcripts during a second late phase.

**Signaling pathway involved in cytokines expression**

To explore the transduced signaling associated with IL-1 pathway activation in P-NALD fibroblasts, we used several known kinase inhibitors and evaluate by cytometry the level of both IL-6 and IL-8 cytokines. In the light of the activation of IL-1 pathway in P-NALD/ACOX1-deficient fibroblasts, induced IL-6 is mostly addressed to the medium (Fig. 4A). By using PD 98059, a selective noncompetitive inhibitor of the MAPK kinase (MAPKK), we have shown the inhibition of secreted IL-6. This result was confirmed by P-NALD fibroblasts exposure to another MAPKK inhibitor, U0126 (Fig. 4A). Likewise, SB 203580, a highly specific inhibitor of p38 MAPKK, decreased IL-6 secretion as well. Similarly, PD 98059, U0126, and SB 203580 molecules inhibited IL-8 expression in P-NALD fibroblasts (Fig. 4). On the other hand, treatment with SP600125 compound, a selective Jun kinases (JNK) inhibitor, exhibited differential effects on IL-6 and IL-8 secretions by decreasing only IL-8 secretion (Fig. 4, A and B). Regarding the activation of IL-1 pathway in P-NALD fibroblasts, the induction of IL-8 seems to be dependent on the activation of p38 MAPK and JNK kinase. Hence, IL-1 transduction cascade through these kinases has been shown for both IL-8 and CCL3 (47). In addition, the implication of nuclear factor κB signaling pathway is not excluded, because C/EBPβ-dependent transcriptional induction of chemokines by IL-1 is triggered through the activation of p38 MAPK and inhibitor of κB kinase (40). Accordingly, we also reported (Supplemental Table 1) that the mRNA increase of TNF receptor-associated factor 6, which is known as an IL-1 control relay, functions as signal transducer of inhibitor of κB kinase (48).

**Conclusions**

Although precise role of VLCFA accumulation in P-NALD demyelination remains to be determined, their ability to induce an inflammatory response adds further evidence to the role of peroxisomal β-oxidation in the maintenance of cellular homeostasis. Therefore, the reported results in the present report highlight that in P-NALD, ACOX1 deficiency is associated with significant alterations in the inflammatory response leading to the activation of IL-1 pathway. Such activation is triggering the induction of both IL-6 and IL-8 cytokines mostly through MAPK and p38 MAPKK, in addition to the possible role of JNK kinase in IL-8 induction. Our results also suggested a feed-forward mechanism leading to an additional down-regulation of peroxisomal VLCFA β-oxidation by the produced cytokines, which may aggravates the inflammatory picture in P-NALD. These results open a way to explore the modulation of kinase pathway in an attempt to reduce the inflammatory process in this orphan disease.

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**Footnotes:**

Disclosure Summary: The authors have nothing to disclose.

**Abbreviations**

ACOX1 : Acyl-CoA oxidase 1
C26:0 : cerotic acid
CEBP : CAAT/enhancer binding protein β
CCL : chemokine (C-C motif) ligand
CCR1 : chemokine (C-C motif) receptor type 1
CoA : coenzyme A
CXCL : chemokine (C-X-C motif) ligand
JNK : Jun kinase
MAPKK : MAPK kinase
P-NALD : pseudoneonatal adrenoleukodystrophy
SPP1 : secreted phosphoprotein 1
TOLLIP : Toll-interacting protein
VLCFA : very-long-chain fatty acid

**References:**


Characterization of P-NALD patient's fibroblasts. A, ACOX1 activity measured in both patients' (1 and 2) fibroblasts. Enzymatic activity of ACOX1 was measured using palmitoyl-CoA as substrate (2). B, Immunostaining of fibroblasts (control, patient 1 and patient 2 fibroblasts) by catalase, a peroxisomal marker, reveals high number of peroxisomes in control cells and low number of peroxisome in P-NALD patient 1 fibroblasts. C, Immunostaining of control (a) and P-NALD (b) fibroblasts by anticatalase reveals enlarged peroxisome size in patient 1 P-NALD fibroblasts (b). Nile red staining of control (c) and P-NALD (d) fibroblasts. The green color indicates the predominance of polar lipids in control cells, whereas the yellow staining of deficient fibroblasts reveals an accumulation of neutral lipids. Microscope images magnifications, ×100. Scale bar, 10 μm. PMP70, 70-kDa peroxisomal integral membrane protein.
FIG. 2
IL-6 and IL-8 cytokine secretion in the culture medium obtained from the control and P-NALD fibroblasts. 1.2 × 10^6 cells were seeded in 10-cm Petri dishes and cultured in DMEM supplemented with 10% fetal calf serum at 37°C with 5% CO₂; 24 h after seeding, fibroblasts were rinsed three times with PBS and incubated in DMEM without serum for 18 h. Culture media were collected and analyzed by cytometric bead array as described in Materials and Methods. Values are mean ± SD. Fib, Fibroblasts.

FIG. 3
Time-course fold inductions of cytokines and ACOX1b mRNA in human control fibroblasts treated with C26:0 at 10 μM in α-cyclodextrin at final concentration of 1 mg/ml. 1.2 × 10^6 cells were seeded in 10-cm Petri dishes and cultured in DMEM complemented with 10% fetal calf serum at 37°C with 5% CO₂; 24 h after seeding, fibroblasts were rinsed three times with PBS solution and incubated in DMEM with α-cyclodextrin (1 mg/ml) as control or with α-cyclodextrin (1 mg/ml) supplemented with C26:0 at 10 μM. Cells were collected at the indicated time point by trypsination. Values are mean ± SD. Total RNA isolated from treated fibroblasts were analyzed by RT-quantitative PCR using gene-specific primers as described in Materials and Methods.
FIG. 4
Regulation of IL-6 (A) and IL-8 (B) cytokines in P-NALD fibroblasts by kinases inhibitors. P-NALD fibroblasts were treated with the indicated concentration of kinase inhibitors for 24 h. Culture media and fibroblasts were collected separately. Cells were washed in PBS solution. The media and the cell pellet were deep frozen at −80°C until analysis. Values are mean ± SD. Statistical significance of higher mean signal intensity (**, P < 0.01; *, P < 0.05) compared with the control. MEK, MAP kinase or extracellular signal-regulated kinase.
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Values indicate fold change in P-NALD fibroblast obtained using the Excel analysis tool (SABiosciences), which includes descriptive statistics.

<sup>a</sup> P < 0.1.
<sup>b</sup> P < 0.01.
<sup>c</sup> P < 0.001.