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Mutations associated with a congenital form of ichthyosis (NCIE) inactivate the epidermal lipoxygenases 12*R*-LOX and eLOX3

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Abstract

Non-bullous congenital ichthyosiform erythroderma (NCIE) is one of the main clinical forms of ichthyosis. Genetic studies indicated that 12*R*-lipoxygenase (12*R*-LOX) or epidermal lipoxygenase-3 (eLOX3) was mutated in six families affected by NCIE [F. Jobard, C. Lefèvre, A. Karaduman, C. Blanchet-Bardon, S. Emre, J. Weissenbach, M. Özgüc, M. Lathrop, J.F. Prud'homme, J. Fischer, Lipoxygenase-3 (*ALOXE3*) and 12(*R*)-lipoxygenase (*ALOX12B*) are mutated in non-bullous congenital ichthyosiform erythroderma (NCIE) linked to chromosome 17p13.1, Hum. Mol. Genet. 11 (2002) 107–113.], but the impact of these mutations on LOX function has not been defined. To explore this, we overexpressed the wild-type or mutated enzymes in *E. coli* and COS7 cells and then analyzed the essential catalytic properties. We showed recently that human eLOX3 is a hydroperoxide isomerase (hepoxilin synthase) that converts the product of 12*R*-LOX, 12*R*-hydroperoxyeicosatetraenoic acid (12*R*-HPETE) to a specific epoxyalcohol. Using incubations with [^{14}C]-labeled substrates and HPLC analyses, we found that the naturally occurring mutations totally eliminate the lipoxygenase activity of 12*R*-LOX and the hydroperoxide isomerase activity of eLOX3. We further demonstrate that the 12*R*-LOX/eLOX3-derived 8*R*-hydroxy-11*R*,12*R*-epoxide is converted by an epoxide hydrolase in COS7 cells and in human keratinocytes to a single isomer of 8,11,12-trihydroxyeicosa-5,9,14-trienoic acid. Taken together, the results support the hypothesis that 12*R*-LOX, eLOX3, and perhaps an epoxide hydrolase function together in the normal process of skin differentiation, and that the loss of function mutations are the basis of the LOX-dependent form of NCIE.

Keywords: Lipoxygenase; Ichthyosis; Mutation; 12*R*-LOX; Epoxyalcohol; Hepoxilin; Epoxide hydrolase; Skin; Keratocyte

1. Introduction

Of the six lipoxygenases (LOX) in the human genome, 15-LOX-2, 12*R*-LOX and eLOX3 form a subgroup with preferential expression in epithelial tissues [1]. The three epithelial lipoxygenases are located as a gene cluster on human chromosome 17p13.1, and the proteins share about 50% amino acid identity. A common physiological role of these epithelial lipoxygenases was suggested in the regulation or modulation of normal proliferation and differentiation of epithelial cells and keratinocytes [1]. 12*R*-LOX is expressed almost exclusively in skin. Synthesis of its product, 12*R*-HETE, is up-regulated in psoriasis while it is almost undetectable in normal human skin [2–5]. 15-LOX-2 appears to modulate the differentiation of prostate epithelial cells and acts as a negative regulator of the cell cycle; its expression tends to be lost in prostate cancer [6,7]. Changes in lipoxygenase expression in these tissues imply an important role for these enzymes in the regulation of cellular proliferation and differentiation.

A recent genetic study links mutations in the coding sequence of eLOX3 or 12*R*-LOX genes to the development of

Abbreviations: NCIE, non-bullous congenital ichthyosiform erythroderma; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; KETE, ketoeicosatetraenoic acid; LOX, lipoxygenase; eLOX3, epidermal lipoxygenase type 3; H(P)ETE, hydro(pero)xyeicosatetraenoic acid; RP-HPLC, reversed-phase high pressure liquid chromatography; TMS, trimethylsilyl

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an inherited skin disease, non-bullous congenital ichthyosiform erythroderma (NCIE) [8]. NCIE represents the first example in which mutations in the coding region of a LOX gene have been associated with a disease. However, the functional impact of these mutations on enzyme activities has not been defined. Since mutations in one or the other enzyme are associated with the same phenotype, the implication is that 12*R*-LOX and eLOX3 function in the same metabolic pathway [8]. 12*R*-LOX normally oxygenates arachidonic acid to the specific hydroperoxide product 12*R*-HPETE [9,10]. eLOX3, however, does not catalyze a conventional lipoxygenase reaction. We recently reported that eLOX3 exhibits hydroperoxide isomerase (hepoxilin synthase) activity [11]. It preferentially transforms the 12*R*-LOX-derived product, 12*R*-HPETE, into a specific epoxyalcohol product, 8*R*-hydroxy-11*R*,12*R*-epoxyeicosa-5*Z*,9*E*,14*Z*-trienoic acid. This provides biochemical support for the concept of the two enzymes working together. We also have shown that the LOX-products 12*S*-HPETE and 15*S*-HPETE are converted to specific epoxyalcohol products of related structure, albeit with lower catalytic efficiency [11].

To explore whether the mutations reported in NCIE patients may be associated with alteration of 12*R*-LOX and eLOX3 functionality, in the current study we have mutated the corresponding amino acids in 12*R*-LOX and eLOX3. After overexpressing the wild-type and mutant proteins in *E. coli* and COS7 cells, we have characterized the essential catalytic properties. Our preliminary report and similar findings by another research group were presented recently [12,13]. We also describe the further enzymatic transformation of the epoxyalcohol product of eLOX3 to a specific trihydroxy derivative, related isomers of which have been detected before in human epidermal fragments [14,15].

2. Methods

2.1. Materials

[1-^{14}C]arachidonic acid was purchased from PerkinElmer Life Sciences. [1-^{14}C]15*S*-HPETE was prepared by reaction of [1-^{14}C]arachidonic acid with soybean lipoxygenase type V (Sigma, St. Louis, MO). 12*R*-HPETE was prepared by arachidonic acid autoxidation as described previously [11]. [1-^{14}C]12*R*-HPETE was prepared by reaction of [1-^{14}C]arachidonic acid with human 12*R*-LOX [9].

2.2. Plasmids and site-directed mutagenesis

The cDNAs for human 12*R*-LOX and eLOX3 were subcloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA). Site-directed mutagenesis was carried out using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Site-directed mutants were selected by sequencing.

2.3. Transfection and harvesting of COS7 cells

Subconfluent COS7 cells in 100-mm dishes were transfected with 7 μg of wild-type and mutant human 12*R*-LOX and eLOX3 plasmids or vector control using FuGENE 6 (Roche Molecular Biochemicals). Forty-eight hours after transfection, cells were harvested and washed with phosphate-buffered saline, and sonicated for 5 s in incubation buffer (100 mM KH_2PO_4, 200 mM NaCl, pH 6.5). Cell lysates were assayed for protein content using Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA).

2.4. Western blot analysis

Aliquots of cell lysates from each sample (20 μg total protein) were boiled in sample buffer and separated on 10% SDS-PAGE followed by transferring to a nitrocellulose membrane. The primary antibody used in 12*R*-LOX detection was rabbit antiserum raised against the human 12*R*-LOX. To analyze eLOX3 expression, the membranes were probed using a primary rabbit antiserum raised against human 15-LOX-2, which will also detect human eLOX3 with sufficient sensitivity to allow evaluation of its expression level in the current experiments. The blots were developed with alkaline phosphatase-conjugated donkey anti-rabbit IgG antibodies.

2.5. Activity assays

Aliquots of cell lysates from each sample (normalized to a similar amount of wild-type and mutant enzymes) were used in the incubations. The total incubation volume was 200 μl. For 12*R*-LOX activity assays, the cell lysates were incubated with 25 μM [1-^{14}C]arachidonic acid at 37 °C for 45 min. For eLOX3 activity assay, the cell lysates were incubated with 25 μM [1-^{14}C]15*S*-HPETE or [1-^{14}C]12*R*-HPETE at 37 °C for 45 min. After the incubation, protein was removed by precipitation with 500 μl of methanol and 250 μl of methylene chloride and subsequent centrifugation. After evaporation of the organic solvents, the products were recovered by extraction using a 100-mg Oasis HLB cartridge (Waters) essentially as described by Powell [14]. Product analysis was performed by RP-HPLC using a Waters Symmetry C18 5-μm column (0.46×25 cm) eluted at a flow rate of 1 ml/min with methanol/water/acetic acid (90:10:0.01, by volume) for incubation with 12*R*-LOX or methanol/water/acetic acid (80:20:0.01, by volume) for eLOX3. Peaks were monitored using an Agilent 1100 diode array detector. To detect radiolabeled products, a Packard A100 Flo-One Radiomatic liquid scintillation detector was connected to the Agilent 1100 diode array detector. The incubations with 15*S*-HPETE and 12*R*-HPETE were repeated twice with identical results.

For incubations of the 8*R*,11*R*,12*R*-epoxyalcohol with COS7 cells and keratinocytes, a 100-mm plate (90%