Very-Long-Chain Fatty Acids Activate Lysosomal Hydrolases in Neonatal Human Skin Tissue

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Key Words

Peroxisomes · Lysosomes · Catalase · Hydrolases ·
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Abstract

Objective: The aim of this study was to examine the in vitro effect of peroxisomal dysfunction on lysosomal enzymes, the autophagic machinery in the cell, in order to understand the mechanisms of pathogenesis of peroxisomal disorders. **Materials and Methods:** Foreskin samples were obtained immediately after circumcision of 1- to 2-day-old infants at the Maternity Hospital, Kuwait. Skin tissues were cleaned, cut into slices of 1–2 mm² in size and treated with lignoceric acid (1–20 µg/ml), a very-long-chain fatty acid (VLCFA), in the presence or absence of 1–5 mM aminotriazole (ATZ). A battery of lysosomal enzymes were assayed following treatment of dermal tissue with VLCFA or ATZ. **Results:** Treatment of skin slices with lignoceric acid significantly increased ($p < 0.001$) the enzymic activities of acid lipase, acid phosphatase, α -glucosidase, α -galactosidase, N-acetyl- α -D-glucosaminidase (NAGA) and N-acetyl- α -D-galactosaminidase (NAGTA). ATZ (1–5 mM), an inhibitor of key peroxisomal enzyme catalase, also markedly increased the enzymic activities of acid phosphatase, α -glucosidase (23%)

and α -galactosidase (18%) without any significant effect on NAGA or NAGTA. Western blot analysis further revealed that both VLCFA and ATZ significantly increased the protein expression of lysosomal enzymes, β -galactosidase and β -glucuronidase. **Conclusion:** Experimental dysfunction of peroxisomes mimicked by elevated VLCFA or ATZ-mediated catalase inhibition significantly increased the activities of lysosomal hydrolases in human dermal tissue, suggesting that activation of the lysosomal system could be one of the factors responsible for cellular damage during pathogenesis of peroxisomal diseases.

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Introduction

Peroxisomes are known to carry out a number of important cellular functions such as synthesis of plasmalogens, β -oxidation of very-long-chain fatty acids (VLCFAs) and detoxification of reactive oxygen species [1–3]. The importance of peroxisomes in human health was highlighted with the recognition of a number of inherited metabolic disorders associated with dysfunction of this sub-cellular organelle [4, 5]. Accumulation of VLCFAs, lack of plasmalogens and deficient activities of peroxisomal

enzymes are well-documented features of peroxisomal biogenesis disorders [6, 7]. Most of the peroxisomal disorders including the single enzyme defect, X-linked adrenoleukodystrophy are neurodegenerative [8, 9]; however, the pathogenic mechanisms associated with this group of inherited diseases are not clear. Lysosomal hydrolases, which play an important role in various pathophysiological events, have recently been shown to participate in the neurodegenerative process in Alzheimer's disease [10, 11]. The lysosomal system participates in cellular catabolism either by digesting the exogenous material taken up by endocytosis or by breaking down the endogenous material separated from other intracellular components through autophagy. Lysosomal hydrolases are also known to be excreted by exocytosis to degrade extracellular matrix components such as glycosaminoglycans and proteoglycans [12]. Extracellular matrix is vital for structural and functional integration of cells in various tissues, and its degradation through activation of lysosomal enzymes [13, 14], such as α -glucosidase, α -galactosidase, N-acetyl- α -D-glucosaminidase (NAGA) and N-acetyl- α -D-galactosaminidase (NAGTA), may be responsible for tissue damage during pathological states. This study was carried out to examine the effect of lignoceric acid, a VLCFA, on lysosomal hydrolases in order to understand the mechanisms of cellular and tissue degeneration associated with peroxisomal diseases.

Materials and Methods

Materials

Bovine serum albumin, penicillin/streptomycin, fetal bovine serum and lignoceric acid were purchased from Sigma Chemical Company (St. Louis, Mo., USA). Dulbecco's minimum essential medium (DMEM):Ham's F-12 (1:1) and trypsin-EDTA were from GIBCO (Grand Island, N.Y., USA). Substrates used in lysosomal enzyme assays were obtained from Sigma. Monoclonal antibodies raised against lysosomal glucosidase and glucuronidase proteins were used in this study (MONOSAN, Uden, The Netherlands). Electrophoresis reagents were purchased from Bio-Rad (Richmond, Calif., USA). All other chemicals and reagents were of highest quality and obtained from Sigma and Calbiochem (La Jolla, Calif., USA).

Methods

Preparation of Dermal Tissue and Treatment with Experimental Agents. Foreskin tissues from 1- to 2-day-old babies were obtained immediately after circumcision at a local maternity hospital and processed for experiments. Dermal tissue was washed repeatedly with Eagle's MEM supplemented with 5% fetal calf serum, 5 U/ml penicillin G sodium and 5 μ g/ml streptomycin, and cut into small slices. Dermal tissue slices were then incubated at 37°C for 24 h in DMEM F-12 in the presence or absence of varying concentrations

(1–5 mM) of aminotriazole (ATZ; inhibitor of catalase) and/or VLCFA (1–20 μ g/ml of lignoceric acid). After each incubation, the medium was removed and stored at –20°C, and tissue was homogenized in 0.25 M sucrose (pH 7.4) buffer.

Assay of Lysosomal Enzymes and Catalase. Enzyme activities of lysosomal hydrolases, acid phosphatase, acid lipase, α -glucosidase, α -galactosidase, NAGA and NAGTA were assayed in the tissue homogenates using 4-methyl-umbelliferone (4-MeU)-linked or *p*-nitrophenol-linked substrates as described elsewhere [16]. Briefly, enzyme reaction was started by adding a known amount of tissue protein to the reaction mixture that consisted of 50 mM sodium acetate buffer, pH 4.5, 0.05% Triton X-100 and 0.05 mM substrate (4-MeU- α -glucoside or 4-MeU- α -galactoside or *p*-nitrophenyl-NAGA or *p*-nitrophenyl-NAGTA). Enzyme reaction was carried out at 37°C for 15–30 min and then stopped by adding either 0.2 M glycine buffer, pH 10.5, or 0.1 N sodium hydroxide to the reaction mixture tube. The release of 4-MeU or *p*-nitrophenol following substrate hydrolysis by the lysosomal enzyme was measured using Hoefer's TKO 100 fluorometer or GBC spectrophotometer, respectively. Catalase, a key peroxisomal enzyme, was assayed by the method of Baudhuin et al. [15] using hydrogen peroxide as enzyme substrate.

Western Blot Analysis. Tissue homogenates were prepared following each experiment and SDS-polyacrylamide gel electrophoresis was performed using Laemmli's sample buffer. Equal amounts of proteins for each sample were loaded onto the gel. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred onto nitrocellulose and immunoblotted with antibodies against different lysosomal enzymes. Protein bands were visualized using horseradish peroxidase-labeled secondary antibody and ECL reagents.

Data Analysis

Specific activities of lysosomal hydrolases were calculated for each experiment and data were analyzed for statistical significance using Student's *t* test.

Results

ATZ (1–5 mM) significantly inhibited ($p < 0.001$) the enzymic activity of catalase, resulting in a significant increase ($p < 0.01$) in the enzymic activity of acid phosphatase, a key lysosomal marker enzyme as shown in figure 1. Lignoceric acid, a biochemical characteristic of peroxisomal dysfunction, also significantly ($p < 0.01$) increased the enzymic activities of lysosomal hydrolases, acid phosphatase and acid lipase as shown in figure 2. Activities of lysosomal enzymes involved in degradation of complex glycoconjugate molecules were also assayed, and figure 3 depicts that the enzymic activity of α -glucosidase was significantly ($p < 0.01$) increased following treatment with ATZ and/or lignoceric acid. Enzymic activity of α -galactosidase (fig. 4), another hydrolytic enzyme for complex glycosaminoglycan molecules, was also markedly elevated ($p < 0.01$) by experimental conditions of peroxisomal

Fig. 1. Enzyme activities of catalase (open bars) and acid phosphatase (dark bars) in human dermal tissue following treatment with 1–5 mM of ATZ. Results shown are means $\pm$ SD of specific enzyme activities (percent of control) from five different experiments. Control specific activity of acid phosphatase is 14.28 ± 0.81 $\mu\text{mol/h/mg}$ protein.

Fig. 2. Specific enzyme activities (as percent of control) of acid lipase (open bars) and acid phosphatase (dark bars) in human dermal tissue following treatment with lignoceric acid (1–20 $\mu\text{g/ml}$). Values are means $\pm$ SD of five different experiments. Control specific activity of acid lipase is 4.21 ± 0.26 $\mu\text{mol/h/mg}$ protein.

dysfunction; however, ATZ and VLCFA did not synergize each other's effect on enzymic activities of lysosomal hydrolases. Figures 5 and 6 show that enzymic activities of NAGA and NAGTA were not influenced by inhibition of catalase, whereas lignoceric acid significantly ($p < 0.01$) enhanced the activities of both lysosomal hydrolases, NAGA and NAGTA. Though VLCFA and/or ATZ enhanced the activities of various lysosomal enzymes, none of these lysosomal proteins leaked out of the tissues as no activity could be detected for any of these lysosomal hydrolases in the incubation media. In order to assess whether the observed effects of catalase inhibition and/or lignoceric acid in this study had a transcriptional and/or translational regulation, Western blot anal-

ysis was performed, and figure 7 shows that both ATZ and lignoceric acid significantly (2- to 3-fold) increased the protein expression of lysosomal hydrolases, β -galactosidase and β -glucuronidase. Overall, lignoceric acid was observed to be a versatile and highly potent modulator of lysosomal hydrolases, whereas inhibition of catalase resulted in selective activation of lysosomal enzymes. Further, the lack of synergism between the effects of VLCFA and catalase inhibition on lysosomal hydrolases suggests that two different mechanisms might be involved in the observed activation of the lysosomal hydrolytic machinery during the pathogenesis of peroxisomal disorders.

Fig. 3. Enzyme activity (as percent of control) of α -glucosidase in human dermal tissue following treatment with ATZ, 1 mM (ATZ 1) or 5 mM (ATZ 5) in the presence or absence of lignoceric acid (C:24). Results shown are means $\pm$ SD of specific enzyme activities (as percent of control) from five different experiments. Control represents tissue without ATZ or C:24 treatment. Control specific activity of α -glucosidase is $9.41 \pm 0.18 \mu\text{mol/h/mg protein}$.

Fig. 4. α -Galactosidase activity in human dermal tissue treated with ATZ, 1 mM (ATZ 1) or 5 mM (ATZ 5) in the presence or absence of lignoceric acid (C:24). Control specific activity of α -galactosidase is $12.4 \pm 1.8 \mu\text{mol/h/mg protein}$. Results shown are means $\pm$ SD of specific enzyme activities (as percent of control) from five different experiments.

Discussion

Peroxisomal disorders are a relatively new category of autosomal inherited metabolic diseases, but represent a deadly class of genetic disorders that are commonly associated with developmental delay, hypotonia, failure to thrive and neurological impairment [4, 17]. Due to a defect in the assembly of the organelle or import of proteins into the organelle, peroxisomal functions are impaired in patients with peroxisomal diseases. Elevated levels of VLCFAs due to impaired β -oxidation, and increased oxidative stress due to deficiencies of peroxisomal enzymes like catalase are believed to cause cellular pathology in

peroxisomal disorders [18, 19]. Though the biochemical defects linked with peroxisomal disorders have been identified, the molecular mechanisms of pathogenesis that lead to cellular and tissue degeneration are unknown. Upregulation of lysosomes, the subcellular organelles that carry out degradation of several types of complex biomolecules, has recently been suggested to play a role in the pathogenesis of neurodegenerative disorders like Alzheimer's disease [10, 20]. Activation of lysosomal hydrolases, such as acid phosphatase, acid lipase, α -glucosidase and α -galactosidase, by ATZ or lignoceric acid as observed in this study suggests that the lysosomal system may be involved in the pathogenesis of peroxisomal dis-

Fig. 5. Effect of ATZ, 1 mM (ATZ 1) or 5 mM (ATZ 5) on enzyme activity of NAGA with or without lignoceric acid (C:24) treatment. Control values for NAGA are $5.48 \pm 0.21 \mu\text{mol/h/mg protein}$. Results shown are means $\pm$ SD of specific enzyme activities (as percent of control) from five different experiments.

Fig. 6. NAGTA activity in human dermal tissue following treatment with ATZ, 1 mM (ATZ 1) or 5 mM (ATZ 5), and/or lignoceric acid (C:24). Control NAGTA activity is $4.62 \pm 0.33 \mu\text{mol/h/mg protein}$. Results shown are means $\pm$ SD of specific enzyme activities (as percent of control) from five different experiments.

eases as well. Stimulating effects of VLCFA and/or ATZ on lysosomal enzymes as observed in this study are intriguing in terms of molecular mechanisms of the pathogenesis of peroxisomal diseases. Reactive oxygen species are known to affect several cellular functions and activation of lysosomal enzymes by hydrogen peroxide or superoxide anions is documented to contribute towards cellular pathology [21, 22]. Inhibition of catalase by ATZ would be expected to raise the intracellular levels of hydrogen peroxide, thus, the observed activation of lysosomal enzymes by ATZ in this study is most likely due to increased cellular oxidative stress. Though free fatty acids or fatty acid esters are believed to affect the lysosomal system either through signal transduction mechanisms involving PKC or by destabilizing the lysosomal membranes [23], the effect of VLCFAs on lysosomal enzymes has remained unexplored. This study shows for the first time that VLCFAs lead to activation of the lysosomal

Fig. 7. Immunoblot detection of lysosomal enzymes, β -galactosidase and β -glucuronidase, in human dermal tissue treated with lignoceric acid (lane 2) or 5 mM ATZ (lane 3) or both (lane 4). Control is shown in lane 1.

system, thereby suggesting another key mechanism for the pathogenesis of peroxisomal disorders. A selective and nonsynergistic increase in lysosomal enzyme activities by ATZ and lignoceric acid as observed in this study indicates that VLCFAs and oxidative stress may be up-

regulating the lysosomal system through different and independent mechanisms. Both ATZ and lignoceric acid increase the enzyme activities of acid phosphatase, α -galactosidase and α -glucosidase; however, the activation of NAGA and NAGTA is only specific to lignoceric acid. Pathological conditions associated with degradation of connective tissue or extracellular matrix have earlier been shown to exhibit increased activities of lysosomal enzymes [24, 25]. In this in vitro study, the lignoceric acid-mediated increase in activities of NAGA and NAGTA, two lysosomal hydrolases mainly responsible for the breakdown of glycosaminoglycans of the extracellular matrix, would further suggest a possible degradation of intercellular connective tissue during peroxisomal dysfunction in vivo. Our findings that lignoceric acid significantly enhances the activities of lysosomal enzymes, which carry out degradation of extracellular matrix components and other complex intracellular molecules, further underscores the role of lysosomal hydrolases in the

pathogenesis of disorders of peroxisomal fatty acid oxidation such as X-linked adrenoleukodystrophy. Future studies should, however, reveal if the activities of lysosomal hydrolases are elevated in the tissues of peroxisomal disorder patients and whether such induction of the lysosomal system is at the transcriptional level.

Conclusions

An increased activity of lysosomal hydrolases by the presence of elevated VLCFAs is an important finding with regard to understanding the cellular and molecular mechanisms of the pathogenesis of peroxisomal dysfunction. Activation of the lysosomal system by the events of peroxisomal defect may destabilize tissue organization through increased autophagy-, endocytosis- or exocytosis-mediated degradation of complex biomolecules and cause tissue damage such as neurodegeneration.

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