Abnormality in catalase import into peroxisomes leads to severe neurological disorder

PEROXISOMAL DISORDERS AND STROKE

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Peroxisomal disorders are lethal inherited diseases caused by either defects in peroxisome assembly or dysfunction of single or multiple enzymatic function(s). The peroxisomal matrix proteins are targeted to peroxisomes via the interaction of peroxisomal targeting signal sequences 1 and 2 (PTS1 or PTS2) with their respective cytosolic receptors. We have studied human skin fibroblast cell lines that have multiple peroxisomal dysfunctions with normal packaging of PTS1 and PTS2 signal-containing proteins but lack catalase in peroxisomes. To understand the defect in targeting of catalase to peroxisomes and the loss of multiple enzyme activities, we transfected the mutant cells with normal catalase modified to contain either PTS1 or PTS2 signal sequence. We demonstrate the integrity of these pathways by targeting catalase into peroxisomes via PTS1 or PTS2 pathways. Furthermore, restoration of peroxisomal functions by targeting catalase-SKL chimera (a catalase fused to the PTS1 sequence) to peroxisomes indicates that loss of multiple functions may be due to their inactivation by H2O2 or other oxygen species in these catalase-negative peroxisomes. In addition to enzyme activities, targeting of catalase-SKL chimera to peroxisomes also corrected the in situ levels of fatty acids and plasmalogens in these mutant cell lines. In normal fibroblasts treated with aminotriazole to inhibit catalase, we found that peroxisomal functions were inhibited to the level found in mutant cells, an observation that supports the conclusion that multiple peroxisomal enzyme defects in these patients are caused by H2O2 toxicity in catalase-negative peroxisomes. Moreover, targeting of catalase to peroxisomes via PTS1 and PTS2 pathways in these mutant cell lines suggests that there is another pathway for catalase import into peroxisomes and that an abnormality in this pathway manifests as a peroxisomal disease.

MATERIALS AND METHODS

We investigated human skin fibroblasts (Rh and Sm cells) from patients with Zellweger-like clinical features and multiple peroxisomal enzymatic deficiencies but found that catalase was mislocalized to the cytosol. These cell lines have morphologically intact peroxisomes with normal levels of acyl-CoA oxidase, a PTS1 protein, and 3-keto acyl-CoA thiolase, a PTS2 protein, but patients with this disorder appear to have multiple enzyme deficiencies (21). In this article, we report that the multiple peroxisomal enzyme defects in these patients is caused by the absence of catalase in peroxisomes because multiple peroxisomal functions were normalized when catalase was reintroduced into peroxisomes via PTS1 pathway. These observations suggest that in humans targeting of catalase to peroxisomes is mediated by a pathway that is independent of PTS1R or PTS2R. Moreover, these studies also show that the absence of catalase in peroxisomes results in the inactivation of multiple peroxisomal functions that gives a phenotype very similar to Zellweger syndrome in which cells lack peroxisomes.

Abbreviations: PTS, peroxisomal targeting signal; PTS1R and PTS2R, PTS1 and PTS2 receptors, respectively; Chat, chloramphenicol acetyltransferase; DHAP-AT, dihydroxyacetone phosphate acetyltransferase; DMA, dimethylacetate; ATZ, aminotriazole; ROS, reactive oxygen species.

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purchased from Sigma. [1-14C]Lignoceric acid was synthesized from n-tricosanol bromide and K214CN as described (22).

[2,3-3H]Phytanic acid was synthesized from [2,3-3H]dihydrophytol as described (23). Antibodies against catalase and chloromphenicol acetyltransferase (ChAT) were purchased from Biodiesgn International (Kennebunkport, ME) and 5 Prime–3 Prime, Inc., respectively.

**Generation of Human Catalase Expression Constructs.** To construct vectors for expression of full-length catalase (pFS1), normal catalase was subcloned into the KpnI–NotI site of pcDNA3 (Invitrogen). Plasmid pFS2 with SKL at carboxyl terminus of normal catalase was generated by PCR using primers 5'-TCGAGTTTTAGAGATTTGCTTTC-3' followed by a stop codon and 5'-CGCACGCTATGCTGACAC-3' and cloned in pcDNA3. This spans positions –8 to +1,651 of the normal catalase transcript with additional nucleotides for SKL at the carboxyl terminus. For construction of the vector for expression of normal catalase with PTS2 signal (pFS3), a PTS2 fragment was generated by annealing 5'-AGCATGATTCGCTCAGACATCTACAG-3' and 5'-ATTCCGGCGACCGAACAGATC-3' and a double-stranded fragment was obtained by a polymerizing reaction with the Klenow fragment of DNA polymerase I. The EcoRI/SacII-digested PTS2 fragment was then ligated to SacII/NotI-cleaved catalase from pZEM vector (Promega) and EcoRI/NotI-digested pcDNA3. The pFS4 vector construct of pcDNA3 expressing catalase from pZEM vector (Promega) and EcoRI/NotI-cleaved catalase was confirmed by DNA sequencing.

**Transfection of the Cells.** Exponentially growing cells of 60–80% confluence were transfected with vector constructs by using Lipofectin as described by the manufacturer (Life Technologies). Briefly, 2 μg of plasmid DNA was diluted in 100 μl of serum-free medium per 35-mm dish. Lipid was prepared by diluting 10 μl of Lipofectin in 100 μl of serum-free medium, and then 0.8 ml of serum-free medium was added to the DNA–lipid complex. Cells were washed with serum-free medium immediately before transfection, overlaid with 1 ml of DMEM supplemented with 15% fetal calf serum. The transient expression experiments, the cells were used 24, 48, and 72 h after transfection. The stable transformants were selected by incubating the cells with G418 antibiotic immediately before transfection, overlaid with 1 ml of Lipofectin in 100 ml for 2 weeks. The transient and stable transformants were tested in the following biochemical and morphological studies.

**Cell Culture and Studies of Intracellular Distribution of Catalase.** Normal human fibroblasts, mutant cells, and transient transformants of catalase into catalase-negative peroxisomes of Mutant Cells. Previous and present studies from our laboratory showed that in these cells both PTS1 and PTS2 signaling pathways were functional but catalase activity remained localized in the cytoplasm instead of peroxisomes (ref. 21, Fig. 1, and Table-1). These observations indicated that the lack of targeting of catalase to peroxisomes may be either due to a mutation in the catalase nucleotide or an alteration in the import machinery for catalase. To test the first hypothesis, we sequenced the transcript for catalase in full in the mutant cells. However, despite our extensive study, no mutation was found in the catalase transcript from these cells (data not shown). A recent study reported that KANL at the carboxyl-terminal end of human catalase is the targeting sequence for import of catalase into peroxisomes (15). To determine further that the abnormality in these cells was in the import of catalase, we constructed a vector (pFS4) to express ChAT fused to KANL (ChAT-KANL) and transected mutant (Fig. 1A) and normal (Fig. 1B) cells. The subcellular distribution of ChAT-KANL was analyzed by immunocytochemical staining with anti-ChAT antibody. Interestingly, ChAT-KANL was targeted to peroxisomes in control cells, but we were unable to detect punctate fluorescence of peroxisomes in mutant cells, indicating that some factor required for catalase import was missing in the mutant cells.

**RESULTS**

Expression and Targeting of Catalase Chimeras (Catalase-PTS1 and PTS2-Catalase) into Catalase-Negative Peroxisomes of Mutant Cells. Previous and present studies from our laboratory showed that in these cells both PTS1 and PTS2 signaling pathways were functional but catalase activity remained localized in the cytoplasm instead of peroxisomes (ref. 21, Fig. 1, and Table-1). These observations indicated that the lack of targeting of catalase to peroxisomes may be either due to a mutation in the catalase nucleotide or an alteration in the import machinery for catalase. To test the first hypothesis, we sequenced the transcript for catalase in full in the mutant cells. However, despite our extensive study, no mutation was found in the catalase transcript from these cells (data not shown). A recent study reported that KANL at the carboxyl-terminal end of human catalase is the targeting sequence for import of catalase into peroxisomes (15). To determine further that the abnormality in these cells was in the import of catalase, we constructed a vector (pFS4) to express ChAT fused to KANL (ChAT-KANL) and transected mutant (Fig. 1A) and normal (Fig. 1B) cells. The subcellular distribution of ChAT-KANL was analyzed by immunocytochemical staining with anti-ChAT antibody. Interestingly, ChAT-KANL was targeted to peroxisomes in control cells, but we were unable to detect punctate fluorescence of peroxisomes in mutant cells, indicating that some factor required for catalase import was missing in the mutant cells.

To decipher further that the abnormality is in the targeting of catalase into peroxisomes, we constructed vectors that express normal catalase (pFS1), chimeric catalase containing PTS1 (pFS2), or PTS2 (pFS3). The mutant cells, transected with pFS1, pFS2, and pFS3 expressing normal catalase, catalase-SKL, and PTS2-catalase, respectively, were analyzed by immunocytochemistry for catalase-containing peroxisomes. The staining with anti-catalase antibody detected a punctate pattern of peroxisomes in pFS2- (Fig. 1D) and pFS3- (Fig. 1E)
1F)-transfected cells, whereas no catalase-containing peroxisomes were detected in pFS1-transfected cells (Fig. 1E) even though catalase activity in these cells increased by 3-fold (Fig. 2A). These studies and the cytosolic distribution of ChAT-KANL clearly demonstrate that the cytoplasmic localization of catalase is due to a defect in the import machinery of catalase into peroxisomes.

**Distribution of Catalase and the Catalase-SKL Chimera Expressed in Mutant Cells.** The mutant cells transfected with pFS1 and pFS2 expressing catalase and catalase-SKL chimeric protein, respectively, were tested for the expression of catalase activity. In transient and stable transfection, the particulate-bound catalase activity increased linearly in pFS2-transfected cells (Table 1), but there was no such tendency in cells transfected with pFS1, which expresses normal catalase protein, even though total catalase activity increased (Fig. 2A and B). Specific activity of particulate-bound catalase in stable transformants expressing the chimeric catalase-SKL was also increased but not in cells transfected with pFS1 (Fig. 2B).

**Catalase-SKL Chimera in Stable Transformants Restored Peroxisomal Activities.** To test the hypothesis that the observed multiple enzyme abnormalities (e.g., β- and α-oxidation of fatty acids and synthesis of plasmalogens) were due to the inactivation of enzymes by excessive H2O2 accumulation, we tested peroxisomal functions in transient and stable transformants. Interestingly, the peroxisomal activities increased as the catalase targeted to peroxisomes increased in transient transfection with pFS2 (Table 1). Next, we examined the recovery of functions in stable transformants. Oxidation of lignoceric acid is severely affected in Rh and Sm cells (Fig. 2C) and transfection of these cells with pFS2 restored this enzyme activity.

Table 1. Peroxisomal function of transiently transfected mutant cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Peroxisomal function</th>
<th>Without transfection</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
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<tbody>
<tr>
<td>Rh (pFS2)</td>
<td>Catalase (particulate bound)</td>
<td>7.85 ± 1.48</td>
<td>14.90 ± 3.95</td>
<td>18.90 ± 2.54</td>
<td>27.46 ± 3.72</td>
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<td></td>
<td>Lignoceric acid oxidation</td>
<td>52.80 ± 3.39</td>
<td>72.40 ± 2.12</td>
<td>115.30 ± 9.89</td>
<td>137.26 ± 3.16</td>
</tr>
<tr>
<td></td>
<td>DHAP-AT</td>
<td>1.02 ± 0.07</td>
<td>1.15 ± 0.06</td>
<td>1.58 ± 0.09</td>
<td>2.56 ± 0.08</td>
</tr>
<tr>
<td>Sm (pFS2)</td>
<td>Catalase (particulate bound)</td>
<td>6.60 ± 1.73</td>
<td>16.80 ± 8.48</td>
<td>21.00 ± 3.67</td>
<td>28.82 ± 4.85</td>
</tr>
<tr>
<td></td>
<td>Lignoceric acid oxidation</td>
<td>46.70 ± 4.24</td>
<td>72.05 ± 2.47</td>
<td>111.55 ± 11.52</td>
<td>136.10 ± 4.94</td>
</tr>
<tr>
<td></td>
<td>DHAP-AT</td>
<td>0.89 ± 0.05</td>
<td>1.42 ± 0.06</td>
<td>1.52 ± 0.07</td>
<td>2.32 ± 0.12</td>
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</table>

Rh and Sm mutant cells were transfected with pFS2 expressing the catalase-SKL chimera. The cells were also transfected simultaneously with vector alone and pFS1 construct that expresses normal catalase. No improvement was noticed with only vector and pFS1-transfected cells (data not shown). Activities of particulate-bound catalase, lignoceric acid oxidation, and DHAP-AT are expressed in milliunits/mg, pmol per h per mg, and nmol per h per mg of protein, respectively. These values in normal cells were 77.59 ± 5.82, 513 ± 22.6, and 10.03 ± 0.14, respectively.
activity in both Rh and Sm cells but not in pFS1-transfected cells expressing normal catalase (Fig. 2C). Phytanic acid is oxidized in peroxisomes (30) and was reduced to 10–15% in these mutant cells compared with normal activity, indicating a partially active peroxisomes (21). The function of phytanic acid oxidation was also restored to 80% of the normal values by introduction of catalase-SKL into peroxisomes but not in cells transfected with pFS1 expressing normal catalase (Fig. 2D).

The early steps of plasmalogen biosynthesis occur in peroxisomes (1, 27). Activity of DHAP-AT, the first enzyme in the plasmalogen biosynthesis pathway, was reduced severely in these mutant cells compared with normal activity, indicating a partially active peroxisomes (21). The function of phytanic acid oxidation was also restored to 80% of the normal values by introduction of catalase-SKL into peroxisomes but not in cells transfected with pFS1 expressing normal catalase (Fig. 2D).

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Next we tested whether the normalization of enzymatic activities would also correct the in situ levels of metabolites (e.g., VLC fatty acids and plasmalogens) after targeting catalase to catalase-negative peroxisomes. As shown in Fig. 3, the steady-state level of VLC fatty acids (C26:0 and C24:0) and their ratios (C26:0/C22:0 and C24:0/C22:0) were normalized in cells transfected with pFS2 but not in cells transfected with pFS1. Steady-state level of plasmalogens was also normalized in mutant cells transfected with pFS2 but not with
pFS1 (Fig. 4). Thus, the normalization of peroxisomal enzymatic activities and normalization of steady-state level of metabolites of peroxisomes after targeting catalase to catalase-negative peroxisomes may be due to inactivation of these enzymes by excessive accumulation of H₂O₂, produced by oxidases present in catalase-negative peroxisomes.

H₂O₂ Inhibits Peroxisomal Activities in Normal Fibroblast Cells. To support our hypothesis, we investigated the effect of endogenously produced H₂O₂ on peroxisomal functions by inhibition of catalase with aminotriazole (ATZ) (42) in cultured control skin fibroblasts. Normal fibroblast cells were treated with various concentrations of ATZ for 24 h and their peroxisomal activities were measured. All three major peroxisomal activities were reduced severely in parallel with inactivation of catalase (Fig. 5A). In a parallel study, we treated normal cells with a constant concentration of ATZ for various periods. The enzymatic activities were found to be reduced drastically within the first 24 h of treatment (Fig. 5B). To investigate whether the loss of fatty acid oxidation in ATZ-treated cells was due to any impairment of substrate/cofactor transport, we examined the oxidation of palmitic acid, a mitochondrial function, and oxidation of lignoceric acid, a peroxisomal function, in intact and homogenized cells. ATZ inhibited the β-oxidation of lignoceric acid but not of palmitic acid, indicating that the loss of peroxisomal fatty acid oxidation in ATZ-treated cells is not due to alteration in fatty acids/cofactors transport properties of cells. These studies further support the conclusion that excessive H₂O₂ may be responsible for the observed loss of peroxisomal functions in mutant cells that lack catalase in peroxisomes.

**DISCUSSION**

Fundamental peroxisomal functions, such as α- and β-oxidation of fatty acids and plasmalogen biosynthesis are defective in disorders of peroxisomal biogenesis (1–3), in pseudo-Zellweger syndrome (31), in Zellweger-like syndrome (32), and in pseudo-infantile refsum disease (33). Recently, we reported patients with clinical features very similar to disorders of peroxisomal biogenesis (21, 34). The biochemical features of these patients differ from those of pseudo-Zellweger syndrome (31), Zellweger-like syndrome (32), and pseudo-infantile refsum disease (33) reported previously. Peroxisomes from skin fibroblasts of these mutant cells were morphologically intact (refs. 21 and 34 and Fig. 1). The PTS1 and PTS2 pathways of protein import were also functional (Fig. 1). Thus, the studies reported in this article clearly demonstrate that multiple peroxisomal enzymatic deficiencies in these patients were due to abnormality in targeting of catalase into peroxisomes. These conclusions are based on the following observations. (i) Targeting of catalase via PTS1 pathway into peroxisomes corrected multiple enzymatic defects. (ii) Inhibition of catalase in normal fibroblast reduced enzymatic activity to levels that were similar to those found in the mutant cells.

Peroxisomal proteins are synthesized on free polysomes and posttranslationally targeted to existing peroxisomes via peroxisomal targeting signals. Catalase is a major peroxisomal enzyme. The peroxisomal targeting signal for catalase differs from classical PTS1 consensus sequence. The PTS1 signal has an absolute requirement of a basic residue, lysine, at position −2 from the carboxyl terminus in mammals (4–6). Whereas in catalase asparagine is present at this position (15). The PTS for catalase is KANL and the lysine at position −4 from the carboxyl terminus is required for targeting of catalase to peroxisomes, but when the arginine at position −2 was replaced with different amino acids, there was little or no effect on targeting of catalase into peroxisomes (15). The targeting of PTS1-containing proteins is mediated by a cytoplasmic receptor (PTS1R). This receptor binds PTS1-signal-containing proteins in the cytosol and delivers them to peroxisomal membranes via its interaction with the cytoplasmic SH3-domain-containing peroxisomal membrane protein Pex13p (35–37). In addition to the abnormality in targeting of PTS1 proteins, the targeting of PTS2-signal-containing proteins and catalase into peroxisomes was defective in the Pex13 mutant. These observations suggest that Pex13p may be the common translocation machinery for catalase and other proteins with PTS1 or PTS2 signals into peroxisomes (35–37). Studies described in this article clearly show that PTS1R or PTS2R is not involved in the targeting of catalase into peroxisomes. This conclusion is based on the following observations. (i) The PTS1 and PTS2 pathway of protein import into peroxisomes are normal in these mutant cells; however, catalase with an intact KANL sequence is not translocated into peroxisomes (Figs. 1A and 2A). (ii) ChAT fused to KANL at the carboxyl terminus was targeted to peroxisomes in normal cells but not in mutant cells (Fig. 1A and B). (iii) Overexpression of normal catalase could not increase the particulate-bound catalase in mutant cells (Fig. 2A and B), whereas the catalase chimera with the PTS1 signal catalase-SKL was efficiently targeted into peroxisomes (Fig. 1D) with an increase in particulate-bound catalase activity (Fig. 2B).

Peroxisomes were named because of their association with the conversion of H₂O₂ to H₂O and to O₂, but they also have many other functions. Peroxisomes are estimated to consume between 5% and 20% of the total cellular oxygen in liver (39, 40). More than 90% of the oxygen consumed by mitochondria is converted to H₂O and rest to O₂.
whereas the oxygen consumed by peroxisomes is quantitatively converted to \( \text{H}_2\text{O}_2 \) and possibly a small amount is converted to \( \text{O}_3 \) (40). These reactive oxygen species (ROS) are the normal byproducts of cellular metabolism and are accordingly kept in check by cellular defenses provided by antioxidant enzymes (1, 40). These antioxidant enzymes provide protection to the cell against ROS (\( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \)) by detoxifying them at the site (compartments, organelle, and membrane) at which they are generated because ROS with the exception of \( \text{H}_2\text{O}_2 \) are not expected to diffuse away from the site of generation because of their highly reactive nature and short half-life. Lack of or deficiency in the detoxification of ROS could result in the oxidative injury, which includes oxidative modification of proteins, lipids, and nucleic acids. The possible role of catalase in protection of peroxisomal enzymes is also supported by loss of peroxisomal functions and catalase activity as a result of ischemia–reperfusion injury (41) and inactivation of its function when purified peroxisomes were treated with exogenous \( \text{H}_2\text{O}_2 \) (42). These observations indicate that excessive \( \text{H}_2\text{O}_2 \), produced by oxidases, by itself or \( \text{OH}^\cdot \), which may react with \( \text{H}_2\text{O}_2 \) (42), may react with \( \text{H}_2\text{O}_2 \) to generate highly reactive \( \text{OH}^\cdot \) in peroxisomes, thus leading to inactivation of peroxisomal functions. The \( \text{H}_2\text{O}_2 \)-induced toxicity in cells with catalase-negative peroxisomes may not induce significant toxicity in the cytoplasm because even if some of the excessive \( \text{H}_2\text{O}_2 \) diffuses out of peroxisomes, it may be detoxified by GPX and by the mistargeted catalase in the cytoplasm in these mutant cells. The redox in peroxisomes is maintained by antioxidant enzymes that are present in peroxisomes, and an alteration in the enzyme system that produces or degrades ROS can result in change in redox in peroxisomes (40, 44). Thus, we propose that the lack of catalase in peroxisomes results in an alteration in peroxisomal redox that results in inactivation of multiple enzyme activities in peroxisomes, because these enzymatic deficiencies are corrected by targeting of catalase to peroxisomes in these mutant cells.

In summary, experiments described in this study provide evidence for a mechanism to transport catalase into human peroxisomes. The catalase import pathway is independent of PTS1R and PTS2R pathways and impairment in this pathway delocalizes catalase to the cytosol. In the absence of particulate-bound catalase the accumulated \( \text{H}_2\text{O}_2 \) suppresses the activities of many peroxisomal matrix proteins leading to a peroxisomal disease with severe neurological malfunctioning. This is the first description of such a disease.

We thank Mrs. Swarupa Pahan for technical help and Dr. Avtar K. Singh for reviewing the manuscript and valuable advice. This work was supported by grants from the National Institutes of Health (NS-22576 and NS-34741).