Increased Apoptosis in Cystinotic Fibroblasts and Renal Proximal Tubule Epithelial Cells Results from Cysteinylation of Protein Kinase Cδ

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Cystinosis is a rare genetic disease characterized by defective lysosomal cystine transport and increased lysosomal cystine. How lysosomal cystine causes the lethal nephropathic phenotype is unknown. It was shown recently that cultured fibroblasts and renal proximal tubule epithelial cells whose lysosomes are cystine-loaded display a two-fold or greater increase in apoptosis after both intrinsic and extrinsic stimuli. The mechanism for the increased apoptosis is unknown. Protein kinase Cδ (PKCδ) is a proapoptotic protein kinase that has been shown *in vitro* to be activated *via* cysteinylation. This report now shows that PKCδ forms disulfide bonds specifically with cystine that is released from lysosomes in cultured fibroblasts and renal proximal tubule epithelial cells during apoptosis. PKCδ in cystinotic fibroblasts and renal proximal tubule epithelial cells have a four- to six-fold greater association with its substrate, lamin B, and a 2.5-fold increase in specific activity after TNF-α exposure. Both RNA inhibition and chemical inhibition of PKCδ resulted in a significant decrease in apoptosis in cystinotic cells but not in normal cells. It is proposed that abnormally increased apoptosis plays a role in evolution of the cystinotic phenotype.

J Am Soc Nephrol 17: 3167–3175, 2006. doi: 10.1681/ASN.2006050474

Cystinosis is an autosomal recessive lysosomal storage disorder caused by defects in *CTNS*, the gene that encodes cystinosin (1). Cystinosin is the lysosomal membrane transporter for the disulfide amino acid cystine; hence, cystine accumulates in lysosomes of cystinotic patients (2).

Patients with the nephropathic form of cystinosis are of normal length and weight at birth, but have the onset of short stature and failure to thrive during the first year of life. Patients display renal abnormalities, including the swan-neck deformity (a thinning of the proximal tubule), the renal Fanconi syndrome, and end-stage renal failure, by 10 yr of age if untreated. Although the molecular defect in cystinotic tissues has been well delineated, it is not known how lysosomal cystine, which is isolated from the cytosol, causes this fatal disease (2).

Apoptosis is a set of characteristic morphologic and physiologic changes that lead to cell death (reviewed in reference [3]). During apoptosis, lysosomes become permeabilized, and their contents enter the cytosol in a controlled manner (4), with concomitant translocation of cathepsins B and D (lysosomal cysteine and serine proteases, respectively) to the cytosol (5,6). Lysosomes permeabilized by digitonin and atractyloside have been shown to cleave procaspases 1, 3, and 11 (7,8). Cathepsins leaked from lysosomes during apoptosis cleave the mitochondrial membrane transition pore protein Bid to its active form (4,9), suggesting that lysosomal permeabilization occurs upstream from mitochondrial cytochrome C release and before an irrevocable commitment to apoptosis (10).

Shared domains in the protein kinase C (PKC) family include a C-terminal catalytic domain, an N-terminal regulatory region, and a variable region (11). A cysteine-rich domain found in the N-terminal region may account for the sensitivity of PKCδ to cysteinylation (12). Overexpression of PKCδ inhibits cell growth (13,14), and cleavage of PKCδ by caspase-3 results in a 40-kD kinase-active fragment, which alone can induce apoptosis (reviewed in reference [15]). PKCδ has multiple nuclear substrates, including Lamin-B, a nuclear support protein that must be phosphorylated before it can be cleaved (16).

PKCδ activity can be regulated by cysteinylation, displaying a 2.5-fold increase in activity *in vitro* after incubation with cystine or other oxidative compounds, including diamide, oxidized glutathione, and $(Cys\text{-}Gly)_2$ (17,18). Addition of dithiothreitol (DTT) abolishes the increase in activity (17,18). We hypothesized that lysosomal cystine release early in apoptosis cysteinylates and thereby activates PKCδ, leading to the observed increase in apoptosis in cystinotic cells.

Materials and Methods

Tissue Culture

Cystinotic fibroblasts (GM00304) and normal fibroblasts (GM05565) were purchased from Coriell Cell Repositories (Camden, NJ) and cultured in MEM with Earl's salts, supplemented with 10% FBS, 1% glutamine, and 1% PFS. Line GM00304 was derived from a skin biopsy of a 24-wk fetal male proband. Line GM05565 was derived from a 3-yr-old boy and was the suggested control for the GM00304 cell line. GM00304 were used because they are one of the few available cell types that are derived from patients with cystinosis and display native lyso-

Received May 15, 2006. Accepted August 12, 2006.

Published online ahead of print. Publication date available at www.jasn.org.

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ISSN: 1046-6673/1711-3167

somal cystine accumulation, permitting the effect of lysosomal cystine on apoptosis to be investigated in the absence of other compounds. They are an essential control for the use of cystine dimethylester (CDME) in studies in noncystinotic cells. Their response to apoptotic stimuli has been characterized by us previously (19), complementing the experiments performed with renal proximal tubule epithelial (RPTE) cells.

Nontransformed RPTE cells were purchased from Cambrex Biosciences (East Rutherford, NJ). Transformed cystinotic RPTE cells were a gift from Dr. W. Gahl (National Institutes of Health, Bethesda, MD). These cells, derived from the urine of a patient with nephropathic cystinosis, were characterized by Racusen *et al.* (20,21). They are the only renal cystinotic line known to us. Human kidney-2 (HK-2) cells were used (American Type Culture Collection, Manassas, VA) as a control for the transformed cystinotic RPTE cells.

All RPTE cells were cultured in renal epithelial growth medium, made according to the manufacturer's instructions (Cambrex). All cells were passaged with trypsin (0.05%) and were cultured in a 95% air/5% CO_2 Thermo Forma incubator (Waltham, MA) at 37°C.

Cystine Binding Protein Assay for Intracellular Cystine

Cystine binding protein (CBP) was purchased from Riverside Scientific (Bainbridge Island, WA), and the competitive protein binding assay was performed as described previously (22). The results are expressed as nanomoles of cystine per 10^6 cells.

Studies of Apoptosis

TNF-α (30 ng/ml) + Actinomycin D (2.5 μg/ml), anti-Fas antibodies (500 ng/ml) + Actinomycin D (2.5 μg/ml), and ultraviolet (UV) light (70 mJ) followed by 16 h of incubation were used as stimuli in apoptosis experiments. Cells were stained with CaspACE (Promega, Madison, WI), a cell-permeable, FITC-conjugated form of the caspase inhibitor VAD-fmk, and analyzed by FACS using a Beckman Coulter Epics Elite machine (Fullerton, CA).

Methylester Synthesis

Whereas CDME is commercially available, methylesters of other compounds used are not. They were synthesized using the method of Steinherz *et al.* (23,24). Samples were analyzed by thin-layer chromatography to assess completeness of esterification (data not shown).

Immunocytochemistry

Normal and cystinotic RPTE cells were plated on chamber slides, washed twice in PBS, fixed in 4% formalin (30 min), and permeabilized with 0.5% Triton X-100 (30 min). Cells were incubated in blocking buffer (PBS [pH 7.2] and 3% BSA) for 1 h, then washed twice in PBS. Incubation with primary antibodies anti–cathepsin B (Santa Cruz Biotechnologies, Santa Cruz, CA) and anti–cytochrome C (Abcam, Cambridge, MA) for 2 h was performed, followed by washing and incubation with secondary antibody (Invitrogen, Carlsbad, CA) for 2 h. Slides were viewed using a Zeiss confocal microscope (Thornwood, NY).

Immunoprecipitation

Cells were harvested in lysis buffer (1× PBS, 0.1% SDS, 0.1% Triton X-100, 0.1% octylphenoxy poly(ethyleneoxy)ethanol, branched [IGEPAL], 1 mM EDTA, 1.5 μg/ml chymotrypsin, 0.8 μg/ml thermolysin, 1 mg/ml papain, 1.5 μg/ml pronase, 1.5 μg/ml pancreatic extract, and 0.002 μg/ml trypsin). Protein was quantified by the bicinchonic acid method, and lysates were incubated for 16 h with rotation at 4°C with primary antibody. Protein a/g–conjugated beads (30 μl; Pierce Biotechnology, Rockford, IL) were added, and the solution was incubated with

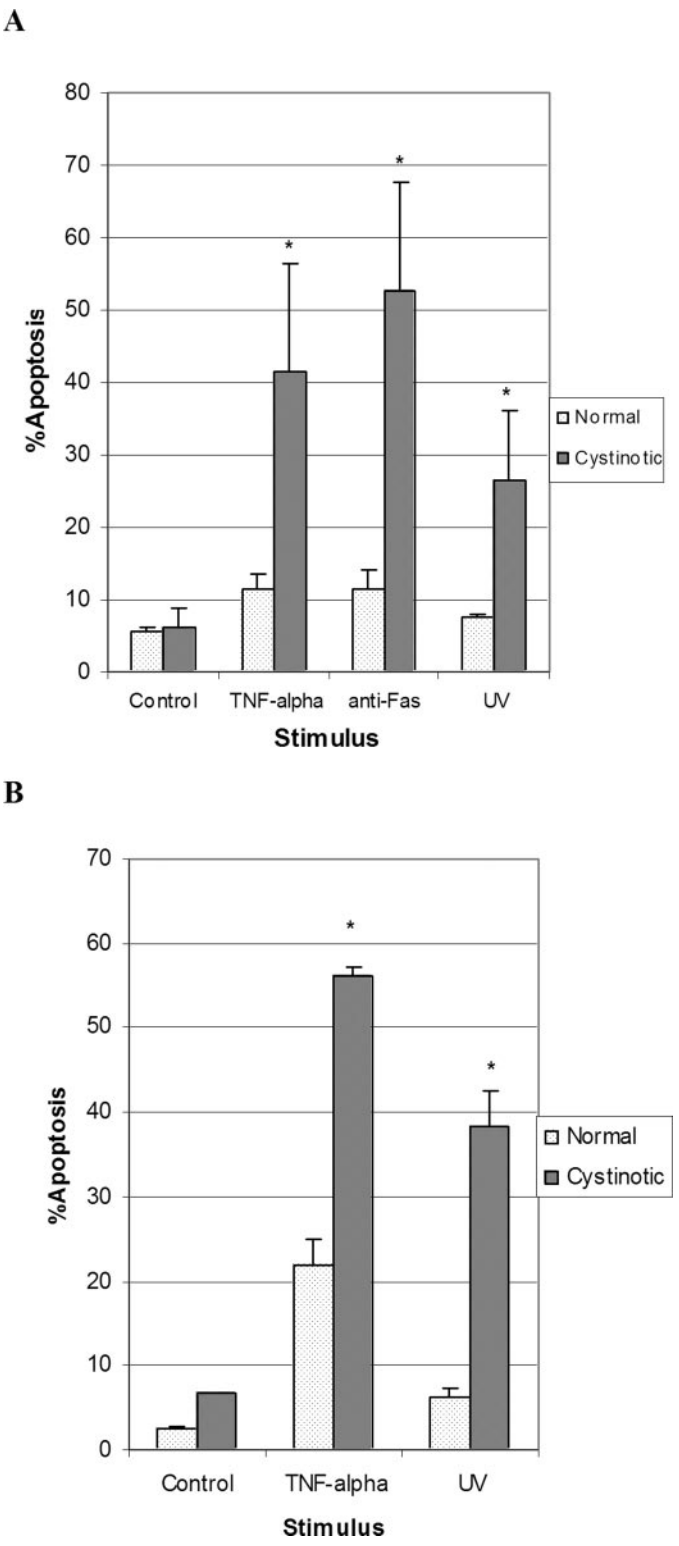

Figure 1. Apoptosis in normal and cystinotic fibroblasts and renal proximal tubule epithelial (RPTE) cells. Cystinotic and normal fibroblasts (A) and RPTE cells (B) were treated with TNF-α + Actinomycin D (ActD), anti-Fas antibodies + ActD (fibroblasts only), or ultraviolet (UV) light as described in Materials and Methods, then stained with CaspACE and analyzed for apoptosis by FACS. *$P < 0.05$ for cystinotic *versus* normal cells. Experiments were performed in triplicate.

Table 1. Effect of cystine analogs on apoptosis in cultured RPTE cells[a]

Compound	Structure	% Apoptosis (± SD)
Control	—	6.4 ± 3.1
Cystine	$HOOCNH_2CHCH_2$-**S-S**-CH_2CHNH_2COOH	7.4 ± 5.9
Cystine dimethylester	H_3C-$OOCNH_2CHCH_2$-**S-S**-CH_2CHNH_2COO-CH_3	72.2 ± 21.4
Cystamine	NH_2-CH_2CH_2-**S-S**-CH_2CH_2-NH_2	10.4 ± 6.8
Djenkolic acid	$HOOC$-NH_2CHCH_2-**S**-CH_2-**S**-CH_2CHNH_2-$COOH$	4.2 ± 2.0
Djenkolic acid dimethylester	H_3C-$OOCH$-NH_2CHCH_2-**S**-CH_2-**S**-CH_2CHNH_2-$COOCH_3$	7.9 ± 4.0
Penicillamine disulfide	$HOOC$-$NH_2C(CH_3)_2CH_2$-**S-S**-$C(CH_3)_2CHNH_2$-$COOH$	5.7 ± 3.4
Penicillamine disulfide dimethylester	H_3C-OOC-$NH_2C(CH_3)_2CH_2$-**S-S**-$C(CH_3)_2CHNH_2$-$COOCH_3$	6.6 ± 2.6

[a]Cultured human renal proximal tubule epithelial (RPTE) cells were treated with 0.5 mM cystine or the cystine analogs indicated (16 h). Cells were stained with CaspACE and analyzed *via* FACS. % Apoptosis indicates the percent apoptosis induced by each compound (±SD). Sulfur atoms are in boldface.

rotation for 2 h at 4°C. The beads were then washed three times in lysis buffer, and protein was eluted by boiling in Laemmli buffer.

Western Blotting

Samples were electrophoresed, blotted onto nitrocellulose, and incubated in blocking buffer (PBS + 0.1 mM TWEEN-20 + 5% nonfat dry milk) for 2 h with rocking. Membranes were incubated in primary antibody for 1 h (in blocking buffer), washed three times in wash buffer (PBS + 0/1% TWEEN-20), and incubated with secondary antibody for 1 h. Membranes were developed using enhanced chemiluminescence.

^{35}S-CDME Labeling of PKCδ in Cultured Cells

^{35}S-CDME was synthesized from ^{35}S-cystine as described previously (22,24). The ^{35}S-CDME was analyzed by thin-layer chromatography, and the CDME band was removed and analyzed using a Beckman Coulter scintillation counter. This count was divided by the total number of counts in the lane to give the purified percentage yield (>85%; data not shown). Cystinotic and normal RPTE cells were pretreated with 5×10^6 CPM/ml ^{35}S-CDME (1 h), then apoptosis was induced with TNF-α in the absence of ^{35}S-CDME (control cells were incubated for 16 h in medium minus TNF-α). Cells were harvested in lysis buffer (see Immunoprecipitation), and equal amounts of cell protein were immunoprecipitated with antibodies against PKCδ. The immunoprecipitate was electrophoresed, then blotted onto a nitrocellulose membrane. The membrane was exposed to autoradiographic film at −80°C, and Western blot was performed on the membrane for identification of PKCδ.

PKCδ In Vitro *Assay*

PKCδ was assayed *in vitro* by the method of Ward *et al.* (17,25). PKCδ (Invitrogen) was incubated with 0 to 0.5 mM L-cystine (as assessed by CBP assay) for 30 min followed by microdialysis (30 min) to rid the sample of excess cystine. Samples were analyzed on a Beckman Coulter scintillation counter.

PKCδ Assay in Cultured Cells

Cystinotic and normal RPTE cells were harvested in assay lysis buffer (10 mM Tris [pH 7.4], 1% glycerol, 1 mM EDTA, 1.5 μg/ml chymotrypsin, 0.8 μg/ml thermolysin, 1 mg/ml papain, 1.5 μg/ml pronase, 1.5 μg/ml pancreatic extract, and 0.002 μg/ml trypsin) and immunoprecipitated with anti-PKCδ. Protein a/g beads were resuspended in 25 μl of PBS, 5 μl of which was used for protein determination (*via* Western blot and densitometry), and the assay (Promega PepTag Assay) was performed on the remaining 20 μl, according to the manufacturer's instructions. The resulting gel was photographed, scanned, and analyzed by a densitometry program (Scanalytics, Fairfax, VA). The result is expressed as a ratio of phosphorylated substrate to PKCδ to yield an activity per protein unit.

Inhibition of PKCδ

Small interfering RNA (siRNA) to sequences in the gene for PKCδ (PRKCD1 sense GGCCAAGGUGUUGAUGUCUtt and antisense AGACAUCAACACCUUGGCCtg) were purchased from Ambion (Austin, TX), along with "silencer" negative control siRNA (siRNA 19-mer scrambled sequences). Cystinotic and normal fibroblasts were transiently transfected with 60 pmol of either control or PKCδ siRNA using 5 μl of Lipofectamine 2000 (Invitrogen) per 0.25 ml for 6 h in serum-free medium, followed by incubation in normal medium for 48 h. Alternatively, PKCδ was inhibited using 12-O-tetradecanoylphorbol-13-acetate (TPA; 10 μM, 6 h) (26).

Statistical Analyses

Statistical analyses were performed using the two-tailed *t* test and SPSS (SPSS, Chicago, IL). Data sets were determined to be significantly different at $P < 0.05$.

Results

Normal fibroblasts (cystine content <0.01 nmol cystine/10^6 cells) demonstrated a baseline apoptosis rate of 5.7%, which rises to 11.5% after TNF-α exposure, 11.5% after anti-Fas antibodies, and 7.6% after UV light. Cystinotic fibroblasts (cystine content 3.4 nmol cystine/10^6 cells) have an apoptosis rate of 6.1% at baseline, which rises to 41.4% after TNF-α, 52.6% after anti-Fas, and 26.4% after UV light (Figure 1A). The values for normal fibroblasts were significantly lower ($P < 0.05$) than the values for cystinotic fibroblasts after all stimuli.

Transformed cystinotic RPTE cells (2.4 nmol cystine/10^6 cells) displayed a basal apoptosis rate of 6.7%, which rose to 56.1% after TNF-α and 38.2% after UV light. HK-2 cells (0.05 nmol cystine/10^6 cells) displayed an apoptosis rate of 2.4% (baseline), which rose to 21.9% after TNF-α and 6.3% after UV light. $P < 0.05$ for cystinotic RPTE *versus* HK-2 cells after TNF-α or UV treatment (Figure 1B).

Structural congeners of L-cystine were purchased and their

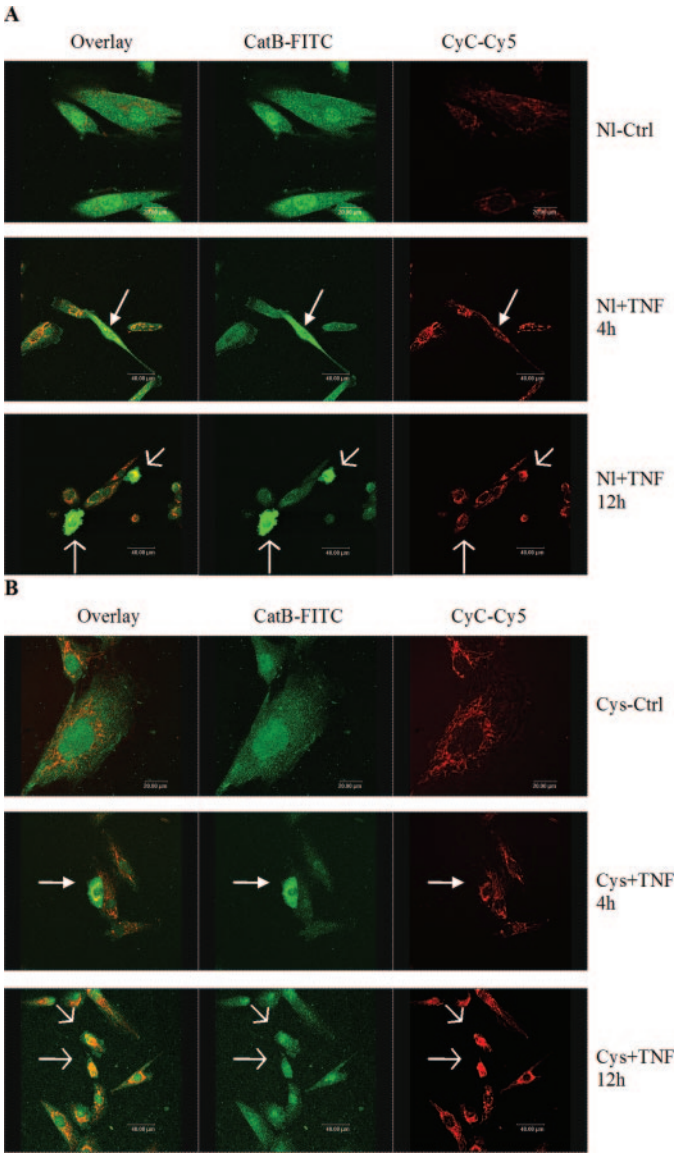

Figure 2. Loss of mitochondrial integrity of lysosomes and mitochondria in normal (A) and cystinotic (B) RPTE cells after TNF-α exposure. After TNF-α (60 ng/ml for 4 or 12 h), cathepsin B was visualized using a FITC-conjugated secondary antibody (green), and cytochrome C was visualized using a Cy5-labeled antibody (red). Closed arrow points indicate cells in which lysosomes are permeabilized but mitochondria are not. Open-pointed arrows indicate cells in which both lysosomes and mitochondria are permeabilized.

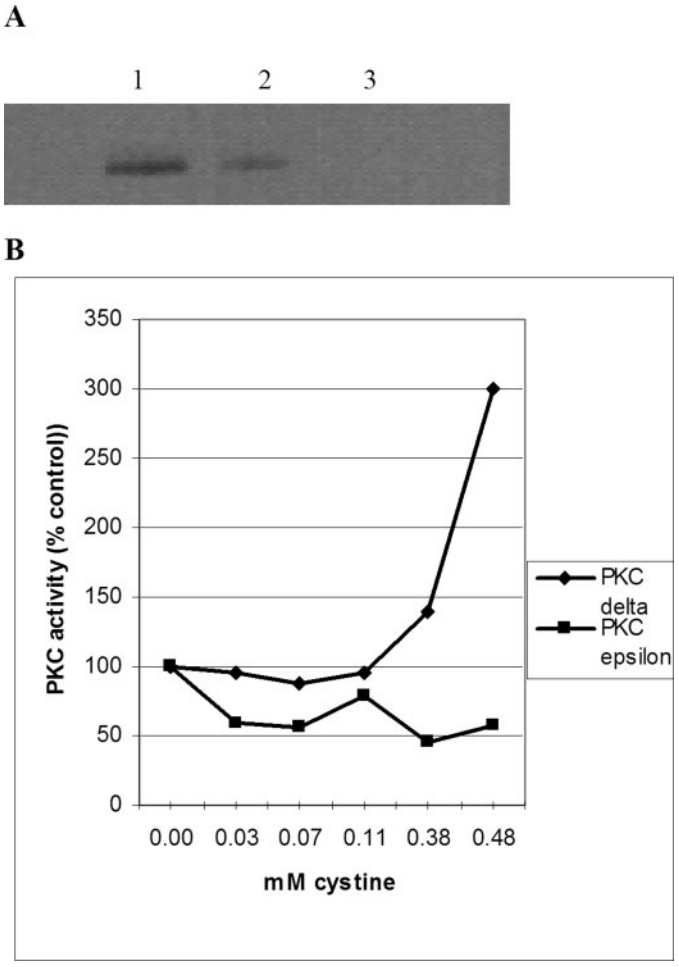

Figure 3. (A) ^{35}S-cystine labeling of protein kinase Cδ (PKCδ) *in vitro*. PKCδ was incubated with ^{35}S-cystine *in vitro* for 30 min (lane 1), in ^{35}S-cystine + mercaptoethanolamine (MEA) (1 mM, lane 2), or ^{35}S-cystine + dithiothreitol (DTT) (1 mM, lane 3). Samples were electrophoresed, blotted onto nitrocellulose membrane, and exposed to autoradiography film for 24 h. (B) The effects of cystine concentration on PKCδ and PKCε activity *in vitro*. Purified PKCδ and PKCε were incubated with the indicated concentration of cystine (30 min) as determined by cystine binding protein assay, then activity was assayed as described in Materials and Methods.

methylesters were prepared in order to assess structural specificity of these compounds on the apoptosis rate. These esters are lysosomotropic, resulting in intralysosomal localization of the parent compound as a result of the action of lysosomal hydrolases (22,27). Cells were treated with cystamine to determine the effect of a noncystine cytosolic disulfide on apoptosis. Djenkolic acid is a congener of cystine with a methyl group between the sulfur atoms; therefore, disulfide exchange reactions are precluded. Penicillamine disulfide (β,β′-dimethylcysteine) is similar to cystine but is methylated at the β carbon. Normal RPTE cells treated with each compound showed no significant increase in basal apoptosis levels (6.4%) after 16 h except for CDME, which caused a significant increase in apoptosis over the basal level (72.2%; $P < 0.05$). The specificity of lysosomal cystine (produced by CDME exposure) as opposed to cytosolic disulfide in inducing apoptosis demonstrates that disulfide exposure alone is insufficient to induce apoptosis at these concentrations in these cells (Table 1).

To assess the point at which lysosomal permeabilization occurs in relation to mitochondrial permeabilization, we performed immunostaining for cathepsin B, a lysosomal protease, and for cytochrome C, a mitochondrial protein found in the cytosol after mitochondrial permeability transition, in normal and cystinotic RPTE cells. At baseline, cathepsin B (green)

localizes as a punctate perinuclear pattern that co-localizes with lysosomes (detected by lysotracker red; data not shown). Cytochrome C staining (red) also is discrete, indicative of mitochondrial localization. By 4 h after induction of apoptosis, cathepsin B becomes spread diffusely throughout the cytosol, but cytochrome C staining remains elongated, rod-shaped, and discrete. At 12 h, the red cytochrome C staining is dispersed, indicating that loss of mitochondrial integrity occurs downstream of lysosomal permeability (Figure 2).

Incubation of pure PKCδ with ^{35}S-cystine *in vitro* followed by electrophoresis and nitrocellulose blotting yielded a radioactive band at 80 kD, the molecular weight of PKCδ. This band was absent or diminished upon preincubation with reductant (DTT or cysteamine), demonstrating the disulfide character of the bond between labeled cystine and PKCδ (Figure 3A).

Purified PKCδ and PKCε were preincubated with L-cystine *in vitro* at 0 to 0.5 mM for 30 min, followed by an activity assay. PKCδ displayed markedly increased phosphorylation of the PKC-specific substrate [ser25]PKC(19-31) when preincubated with L-cystine, in contrast to PKCε, which showed no increase in activity (Figure 3B).

Lysosomal ^{35}S-cystine specifically labels PKCδ *via* a mixed disulfide reaction after TNF-α stimulus in cultured cells *in vivo*. ^{35}S-CDME was synthesized, and both cystinotic and normal RPTE cells were exposed to ^{35}S-CDME and then treated with TNF-α in culture medium minus ^{35}S-CDME. PKCδ was immunoprecipitated from the cells, electrophoresed, blotted onto a nitrocellulose membrane, and exposed to autoradiographic film. PKCδ is *not* labeled when TNF-α is absent (Figure 4, lanes 1 and 5), indicating that apoptosis induction and lysosomal permeabilization are required for labeling, but PKCδ is labeled with ^{35}S-cystine after TNF-α exposure. It is well established that amino acid methylesters are lysosomotropic and that CDME treatment causes accumulation of cystine in the lysosome (23,27). Labeling of the lysosomal pool of cystine with ^{35}S-CDME and the subsequent demonstration of PKCδ labeling from that pool demonstrate that PKCδ is cysteinylated by lysosomal cystine after apoptosis is induced by TNF-α in cultured cells. The effect is ablated by DTT exposure after immunoprecipitation, demonstrating the disulfide nature of this bond (Figure 4).

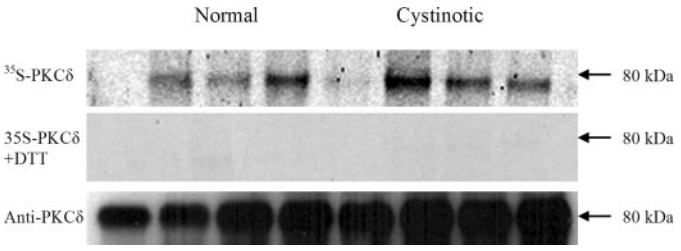

Figure 4. Labeling of PKCδ *in vivo* in normal and cystinotic RPTE cells by lysosomal cystine. Normal (lanes 1 to 4) and cystinotic (lanes 5 to 8) RPTE cells were incubated with ^{35}S-cystine dimethylester (CDME) for 1 h, then treated with TNF-α in ^{35}S-CDME–free medium for 1 h (lanes 2 and 6), 2 h (lanes 3 and 7), or 16 h (lanes 4 and 8) or incubated in ^{35}S-CDME–free medium for 16 h without TNF-α (lanes 1 and 5). Cells were harvested and PKCδ was immunoprecipitated. Precipitates were electrophoresed, blotted onto nitrocellulose membrane, and exposed to autoradiographic film for 24 h. A Western blot using anti-PKCδ antibodies ensured that the absence of signal in the non–TNF-α–treated cells was not due to absence of PKCδ.

PKCδ activity is increased in cystinotic RPTE cells and fibroblasts compared with normal RPTE cells and fibroblasts. The activity of PKCδ was assessed in two ways: By co-immunoprecipitation with lamin B and by a PKC assay using a fluorescent and colorimetric substrate. PKCδ was immunoprecipitated from normal and cystinotic fibroblasts and RPTE cells after TNF-α treatment. The immunoprecipitate was then probed with anti–lamin B and anti-PKCδ antibodies. A greater amount of lamin B was recovered from the cystinotic TNF-α–treated fibroblast immunoprecipitates than in the normal (Figure 5A). Similarly, a greater amount of lamin B co-immunoprecipitated with PKCδ in the cystinotic TNF-α–treated RPTE cells (Figure 5B) than in the normal RPTE cells.

To directly assess PKCδ activity in normal and cystinotic fibroblasts and RPTE cells, we performed immunoprecipitation

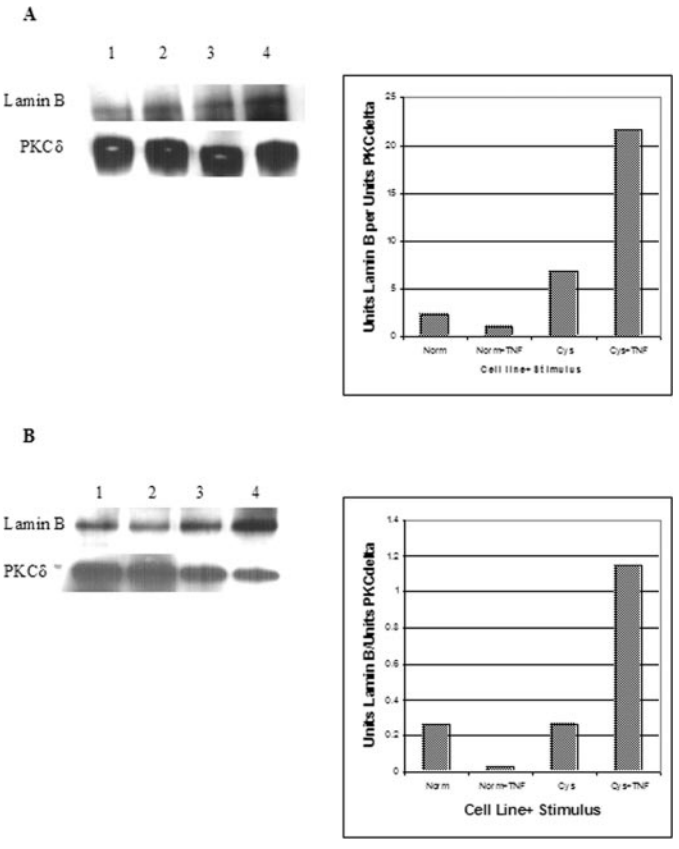

Figure 5. PKCδ and lamin B co-immunoprecipitation in normal and cystinotic fibroblasts and RPTE cells. PKCδ was immunoprecipitated from normal and cystinotic fibroblasts (A) or RPTE cells (B). Western blots were performed with anti-PKCδ or anti–lamin B antibodies. Lane 1, normal; lane 2, normal+TNF-α; lane 3, cystinotic; lane 4, cystinotic+TNF-α. The resulting blots were analyzed using the Scanalytics densitometry program, and the ratios of lamin B to PKCδ are shown in the bar graphs.

using anti-PKCδ antibodies. PKC activity was assessed in the immunoprecipitate, and a Western blot was performed to quantify the amount of immunoprecipitated PKCδ. The resulting gel and blot were analyzed by densitometry, and the ratio of PKCδ enzymatic activity to the amount of PKCδ as determined by Western blot was calculated for each sample. All samples were normalized to the normal control condition to control for differences in the length of exposure time between Western blots. A greater than three-fold increase (7.2 to 28.2 arbitrary units) in PKCδ activity in TNF-α–treated cystinotic fibroblasts was found (Figure 6A). In cystinotic RPTE cells, the activity of PKCδ was more than four-fold higher than normal (1.2 to 5.2 arbitrary units; Figure 6B). All assays were performed in triplicate, and bar graphs represent the mean and SD of each condition. In fibroblasts, $P = 0.05$ for control *versus* cystinotic and $P = 0.02$ in control *versus* cystinotic in RPTE cells.

RNA inhibition of PKCδ decreased the apoptosis rate after TNF-α from 40.2 to 32.7% ($P > 0.05$) in normal fibroblasts and from 78.2 to 51.5% ($P < 0.05$) in cystinotic fibroblasts (Figure 7). The cells were compared with cells treated with control siRNA (see Materials and Methods).

Depletion of PKCδ by treatment with TPA replicated the siRNA results. Normal fibroblasts treated with TPA showed a decrease in apoptosis from 15.6 to 11.8% ($P > 0.05$). Cystinotic fibroblasts, however, showed a decrease in apoptosis from 26.0 to 15.8% ($P < 0.05$; Figure 8). These data, in combination with the inhibition of PKCδ by siRNA, indicate that PKCδ plays an important role in lysosomal cystine–enhanced apoptosis.

Discussion

We previously reported an increase in apoptosis in normal cells when they are loaded with CDME, mimicking the cystine loading found in the lysosomes of cystinotic cells (28). Cystinotic RPTE cells demonstrate a two- to six-fold increase of apoptosis over normal cells. The increased apoptosis in cystinotic cells normalizes when those cells are depleted of lysosomal cystine by treatment with cysteamine (26); therefore, the presence of lysosomal cystine correlates with an increased incidence of apoptosis. A plot of cystine content *versus* the apoptosis rate in normal and cystinotic fibroblasts forms a rectangular hyperbola with a K_m of approximately 0.2 nmol cystine/mg protein (close to the lysosomal cystine content of normal fibroblasts [2]) and a correlation coefficient of 0.79 (29).

In nephropathic patients, renal symptoms are the first to appear and have the greatest impact on patient health. RPTE cells demonstrate a two- to six-fold increase of apoptosis in cystinotic RPTE cells over normal cells. The increased incidence of apoptosis is specific for lysosomal cystine, because equal concentrations of similar disulfides and/or their methylesters do not induce apoptosis in normal RPTE cells. This specificity may be secondary to steric hindrance of the other molecules or unknown effects.

All experiments using the apoptosis inducer TNF-α or anti-Fas antibodies also used Actinomycin D at a concentration that inhibits transcription by >90% (30) (see Materials and Methods); therefore, a transcriptional increase in apoptosis effectors as a result of lysosomal cystine is unlikely to be involved in lysosomal cystine–enhanced apoptosis.

Apoptotic stimuli lead to lysosomal permeabilization and translocation of cathepsins B and D into the cytosol (4–9). A rapid release of cystine into the cytosol during this permeabilization phase could result in a locally altered redox potential and/or cysteinylation of critical thiols that may sensitize the cells to or trigger the apoptotic response. This hypothesis is tenable if lysosomal permeabilization precedes loss of mitochondrial integrity, the irreversible step in apoptosis (10). As-

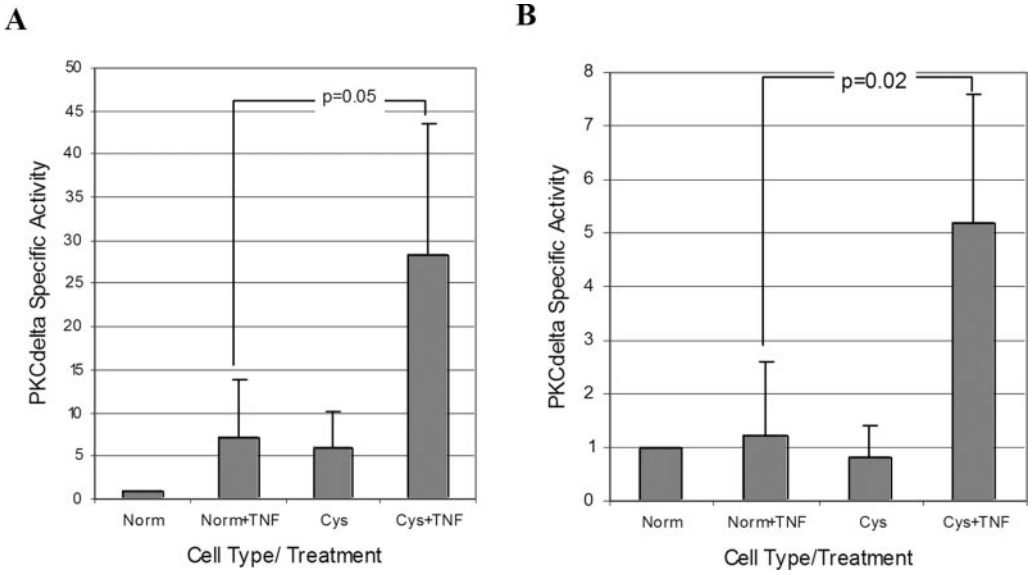

Figure 6. PKCδ activity in normal and cystinotic fibroblasts and RPTE cells. PKCδ was immunoprecipitated from normal or cystinotic fibroblasts (A) and normal or cystinotic RPTE cells (B) after treatment with TNF-α. PKCδ activity was measured in the immunoprecipitate. PKCδ activities were normalized to the values acquired from densitometric scans of the corresponding Western blot, and the ratios are shown. The experiment was performed in triplicate.

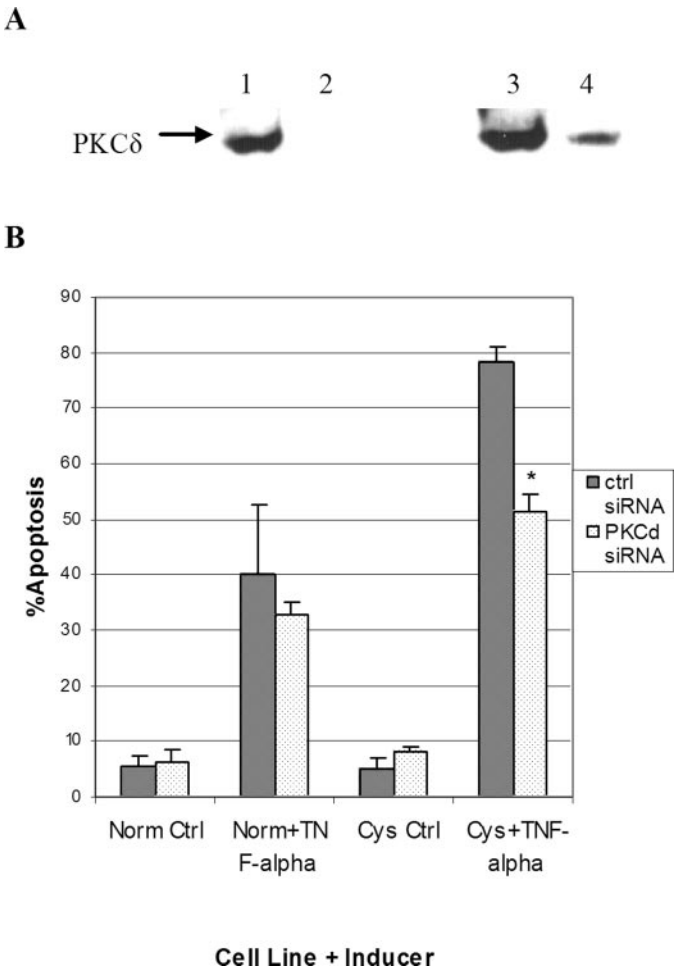

Figure 7. The effect of PKCδ inhibition by small interfering RNA (siRNA) on apoptosis in normal and cystinotic fibroblasts. (A) Normal (lanes 1 and 2) and cystinotic (lanes 3 and 4) fibroblasts were treated with 0.2 nmol/ml control siRNA (lanes 1 and 3) or PKCδ siRNA (lanes 2 and 4) for 6 h. Cells were incubated in serum-containing medium for 72 h, then harvested for protein. Cell lysates were electrophoresed and blotted onto nitrocellulose membrane, and the membrane was probed with anti-PKCδ. (B) Fibroblasts were pretreated with siRNA to PKCδ as described, then treated with TNF-α. Apoptosis was assessed in triplicate using CaspACE dye followed by FACS analysis. $*P < 0.05$.

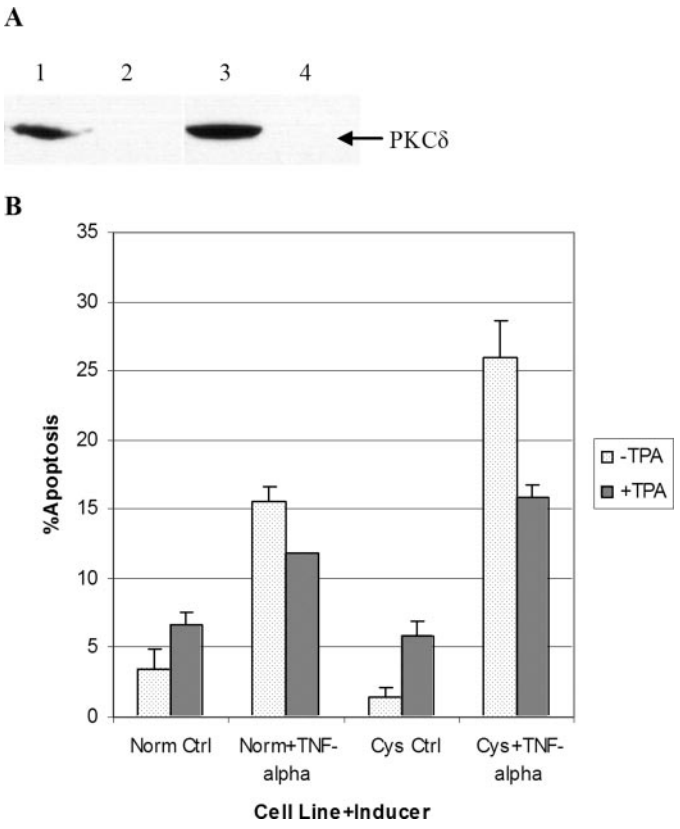

Figure 8. The effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on apoptosis in normal and cystinotic fibroblasts. (A) Normal (lanes 1 and 2) and cystinotic (lanes 3 and 4) fibroblasts were treated with TPA (10 μM) for 6 h and analyzed by anti-PKCδ antibodies. Lanes 1 and 3 are controls; lanes 2 and 4 are treated with TPA. (B) Apoptosis was assessed after TNF-α treatment with and without prior TPA inhibition of PKCδ. Cells were stained and analyzed as described. $*P < 0.05$. The experiment was performed in triplicate.

sessment of the location of cathepsin B and cytochrome C during apoptosis demonstrates that lysosomal cathepsin B loss occurs upstream of mitochondrial cytochrome C release (Figure 2). This finding was confirmed by Western blots of lysosomal and mitochondrial proteins in the cytosolic fraction over time (data not shown).

^{35}S-cystine labels PKCδ *via* mixed disulfide formation *in vitro*, and cystine increases the activity of purified PKCδ six-fold *in vitro* (Figure 3). ^{35}S-CDME labels PKCδ in cultured normal and cystinotic RPTE cells only after TNF-α stimulus, supporting a role for lysosomal permeabilization in lysosomal cystine–enhanced apoptosis and validating the reaction of PKCδ with lysosomal cystine (Figure 4). DTT ablates this effect, demonstrating that PKCδ is labeled in this process *via* disulfide bond.

Co-immunoprecipitation of PKCδ with lamin B shows that the PKCδ in apoptotic cystinotic fibroblasts and RPTE cells binds six- to seven-fold more lamin B than the PKCδ in normal fibroblasts and RPTE cells, consistent with an increase in PKCδ activity in these cells. Furthermore, direct assay of PKCδ that immunoprecipitated from normal and cystinotic fibroblasts and RPTE cells demonstrated a five-fold increase in specific activity in cystinotic cells compared with normal cells (Figures 5 and 6).

Inhibition of PKCδ is a way to assess the importance of this protein in the process of lysosomal cystine–enhanced apoptosis. RNA inhibition diminishes the production of selected proteins at the level of translation (31). Use of targeted siRNA against PKCδ decreases apoptosis in cystinotic fibroblasts while having no significant effect on normal fibroblast apoptosis (Figure 7). TPA is known initially to increase PKCδ activity. However, over longer exposure times, this compound causes ubiquitination and degradation of PKCδ (26). Our data show that TPA dampens apoptosis that is induced by TNF-α in cystinotic fibroblasts but has a lesser effect on normal fibroblasts (Figure 8).

PKCδ is likely to be a primary effector in the lysosomal cystine–enhanced apoptosis pathway, which supports the hypothesis that cystine is released from the lysosome during early apoptosis, leading to cysteinylation of PKCδ. PKCδ is a critical proapoptotic protein that is capable of phosphorylating substrates involved in nuclear disintegration, DNA damage repair, and membrane symmetry (16,32,33). Cysteinylation leads to increased PKCδ activity in cystinotic cells undergoing apoptosis, making them less likely to be rescued from cell death (Figure 9).

We hypothesize that the presence of lysosomal cystine amplifies the apoptotic cascade, causing cystinotic cells to commit to apoptosis, when under normal conditions they would not. This leads to generalized hypocellularity and the cystinotic phenotype, including the Fanconi syndrome and glomerular failure. The retinopathy that is found in patients with cystinosis may be due to increased retinal apoptosis, and the short stature may be due to increased apoptosis in the growth plates of long bones. The later pathologic findings (myopathy, neuropathy, diabetes, and ESRD [34]) also may be attributed to aberrantly increased apoptosis.

It is unlikely that PKCδ is the only compound affected by lysosomal cystine. Cystine is a small molecule and therefore is likely to form disulfide bonds promiscuously with all proximate unhindered thiols.

An increased incidence of apoptosis has been hypothesized in the oligodendrocytes in Krabbe's disease, the "psychosine hypothesis" (35–37). Psychosine (β-galactosphingosine) may act extracellularly to activate the c-Jun N-terminal kinase pathway or to induce mitochondrial membrane permeability (33,38), as opposed to cystine, which is released from an intracellular location. The neurologic symptoms of other lysosomal storage diseases, including Tay-Sachs, Sandhoff (39), and Niemann Pick C diseases (40), also are thought to derive from increased neuronal apoptosis.

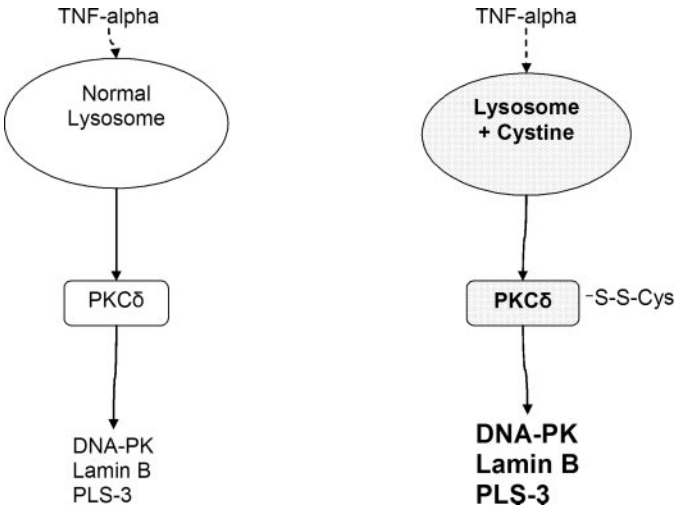

Figure 9. Proposed mechanism of the action of lysosomal cystine on PKCδ. In normal cells (left), lysosomes do not release cystine and PKCδ activity is not increased. Lysosomal cystine in cystinotic cells (right) is released during apoptosis and increases PKCδ activity, which in turn phosphorylates multiple targets and promotes apoptosis. Normal cells thus may be rescued from the apoptotic pathway, whereas a cystinotic cell under the same conditions proceeds to apoptosis.

Recent mouse studies have shown that systemic increases in apoptosis are responsible for much of the aging phenotype (41). This may account for the premature aging characteristic of cystinotic patients (2).

Enhanced sensitivity to apoptosis as a result of increased lysosomal cystine offers insight into the role of lysosomes in apoptosis, as well as new perspectives in understanding the pathophysiology of cystinosis. Enhanced sensitivity is observed after triggers that stimulate both intrinsic and extrinsic pathways, suggesting a central point of action for lysosomal cystine.

Further study of the pathway(s) by which cellular perturbations result in modulation of the apoptotic cascade may lead to a clearer understanding of the regulation of apoptosis and a better understanding of the development of the cystinotic phenotype.

Acknowledgments

We gratefully acknowledge the support of the Cystinosis Research Foundation, the Cystinosis Research Network, and Dr. William A. Gahl for contribution of the cystinotic RPTE cell line.

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