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Original Contribution

Modulation of CTNS gene expression by intracellular thiols

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ABSTRACT

The cysteine/cystine (Cys/CySS) couple represents one of the major cell thiol/disulfide systems and is involved in the regulation of several metabolic pathways and the cell redox state. Nephropathic cystinosis (NC) is an autosomal recessive disease characterized by renal cellular dysfunction due to mutations in the CTNS gene, which encodes cystinosin, a CySS lysosomal transporter. To analyze the mechanisms involved in cell damage in NC, we have investigated the effects of CTNS gene overexpression or inhibition on cell thiol/disulfide systems and vice versa. Overexpression of the CTNS gene had no remarkable effect on intracellular Cys/CySS and GSH/GSSG redox state. Silencing the CTNS gene increased cell CySS and Cys and decreased cell GSH and GSSG and increased mildly the redox state of the Cys/CySS-couple. Extracellular CySS and Cys deprivation for 48 h caused an oxidation of the Cys/CySS (73 mV) and GSH/GSSG (100 mV) redox couples and increased CTNS mRNA levels by 1.9 ± 0.2-fold ($p<0.001$). Conversely, a reduced cell environment associated with a GSH/GSSG reduction from -250.1 ± 3.10 to -330.6 ± 4.70 mV ($p<0.001$) and a Cys/CySS reduction from -167.0 ± 11.30 to -240.0 ± 8.17 mV ($p<0.005$) was associated with a 40% decrease in CTNS mRNA levels ($p<0.05$). By regression analysis, CTNS gene expression was correlated with intracellular Cys level and with Cys/CySS redox state.

The CTNS gene encodes cystinosin, a cystine (CySS) transporter that is localized mainly in lysosomal membranes [1]. Recently, a second CTNS isoform, termed cystinosin-LKG, was shown to be targeted to the plasma membrane and to small cytosolic vesicles, in addition to lysosomes [2]. Mutations in the CTNS gene cause cystinosis, an inherited autosomal recessive disease that is characterized by early renal tubular dysfunction in its most common and severe form, also termed nephropathic cystinosis (NC). If not treated with cysteamine, patients with NC rapidly develop chronic renal failure and a variety of symptoms related to cystine accumulation in other tissues, including eyes, endocrine organs, muscles, nerves, brain, and liver [3].

Loss of cystinosin activity causes accumulation of CySS in lysosomes, which has an impact on key metabolic pathways leading to cell dysfunction. For the most part, these pathways have not been fully elucidated. Current evidence, obtained using in vitro models of cystinosis, point to abnormal cysteinylation of proapoptotic kinases, altered cell redox state, and decreased glutathione (GSH) synthesis, most of which impair mitochondrial activity, although mitochondria do not seem to be primarily compromised in cystinosis [4–11].

Cytosolic cysteine (Cys) originates primarily from the reduction of CySS by the reduced/oxidized glutathione (GSH/GSSG) redox couple. When needed, cells can also generate Cys from metabolic pathways, including the pyroglutamate cycle and the transulfuration pathway, from protein breakdown in lysosomes, or by direct uptake of CySS from the extracellular milieu [12,13].

Regulation of cell Cys levels is essential for the modulation of several metabolic pathways including protein and GSH synthesis, cell redox state, and redox-based signaling [14].

Cystinosin may therefore play a pivotal role in cell homeostasis, by regulating cell CySS transfer from lysosomes and from other cell compartments or from the extracellular milieu by means of its LKG isoform.

To further analyze the role of cystinosin in cells, we have experimentally modified the expression levels of the CTNS gene to monitor its effects on cell thiols and, in a reverse set of experiments, have modified cell thiols and redox state to investigate their effects on CTNS gene expression.

Materials and methods

Cell culture and reagents

Human kidney-2 cells (HK-2; ATCC CRL-2190) were grown in DMEM–F12 medium (Invitrogen Life Technologies, Carlsbad, CA, USA)

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supplemented with 5% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin, 10 μg/ml insulin from bovine pancreas, 5.5 μg/ml human transferrin (substantially iron-free), and 5 ng/ml sodium selenite (ITS). When specified, DMEM-F12 medium was replaced by Cys-, CySS-, and methionine-free DMEM. Cells were grown in a humidified atmosphere with 5% CO_2 at 37°C. Unless otherwise specified all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Measurement of intracellular reactive oxygen species (ROS)

Generation of ROS was detected with the 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) fluorescent dye. Briefly, after addition of H_2DCF-DA (1 μM), cells were incubated for 30 min at 37°C in the dark, detached by trypsin–EDTA, washed twice with phosphate-buffered saline (PBS), and immediately analyzed by flow cytometry (excitation, 488 nm; emission, 530 nm; BD LSR II System; BD Biosciences, San Jose, CA, USA). Ten thousand data points were collected for each sample and analyzed with CellQuest software (BD Biosciences).

Measurement of intracellular GSH/GSSG and Cys/CySS levels

Cell pellets were suspended in ice-cold PBS in the presence or in the absence of 10 mM *N*-ethylmaleimide, which blocks reduced thiol residues, and sonicated with a VIBRA-CELL sonicator (Sonics & Materials, Newtown, CT, USA). After protein precipitation with 10% sulfosalicylic acid, cell homogenates were centrifuged at 15,000 g for 10 min at 4°C. The acid-soluble fraction was derivatized with monobromobimane and CySS, Cys, GSSG, and GSH contents were determined by high-performance liquid chromatography as reported in [15], with slight modifications. Protein concentrations were measured by the Bradford method.

Calculation of redox potentials

Theoretical redox potentials (E_h) of the GSH/GSSG and Cys/CySS couples were calculated using the Nernst equation, $E_h = E_0 + RT/nF \ln([\text{disulfide}]/[\text{thiol}]^2)$, where E_0 is the standard potential for the redox couple (−264 mV for $GSSG/GSH^2$, −250 mV for $CySS/Cys^2$), R is the gas constant, T is the absolute temperature, n is the number of transferred electrons (e.g., 2), and F is Faraday's constant [16]. Calculations were made assuming a dilution volume for Cys/CySS and GSH/GSSG of 5 μl/mg of protein.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from HK-2 cells using TRIzol reagent (Invitrogen Life Technologies). mRNA was reverse-transcribed into cDNA using a first strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. CTNS and GAPDH amplicons were obtained by PCR adding the following components: 2.5 U *Taq* DNA polymerase, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Invitrogen Life Technologies), and 0.5 μM each primer (Sigma–Aldrich). Human CTNS and GAPDH primers were selected using the Oligo 6.71 primer analysis software (Molecular Biology Insights, Cascade, CO, USA). A common region of the two CTNS isoforms was amplified by using the following primers: CTNS forward primer, 5′-CCCTGAAGCTCGTAGAGAAATG-3′, and CTNS backward primer, 5′-GGAGGAACATCTGCAGGAGG-3′. Isoform-specific amplicons were obtained by using of the following pair of primers: forward primer, 5′-CCCTGAAGCTCGTAGAGAAATG-3′, and backward, 5′-TCAGCCCTTCAAGCTGCTTGC-3′. This PCR yields two different amplicons corresponding to the CTNS isoforms. PCR conditions for CTNS amplification were as follows: 94°C for 5 min; then 94°C for 45 s, 60°C for 45 s, 72°C for 75 s for 35 cycles; and 72°C for 10 min.

GAPDH primers were the following: GAPDH forward primer, 5′-CTGCACCACCAACTGCTTAG-3′, and GAPDH backward primer, 5′-AGGTCCACCACTGACACGTT-3′. PCR conditions for GAPDH amplification were as follows: 94°C for 5 min; then 94°C for 45 s, 55°C for 45 s, 72°C for 45 s for 25 cycles; and 72°C for 10 min.

PCR products were resolved on a 1.5% agarose gel with GelRed staining (Biotium, Hayward, CA, USA). Band intensities were measured with the ImageJ 1.38× software (NIH, http://rsb.info.nih.gov.ij) using digital photographs of gels. CTNS mRNA was quantified using the ratio between CTNS and GAPDH mRNA expression levels and expressed as relative values.

SDS–PAGE and immunoblot analysis

Samples were washed in ice-cold PBS, then boiled 5 min in Laemmli reducing sample buffer and resolved by 8% SDS–PAGE, transferred to nitrocellulose, Western blotted for cystinosin (Abnova, Taipei City, Taiwan) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and visualized using ECL.

Overexpression of CTNS gene

The full-length CTNS and CTNS-LKG coding regions were amplified by PCR using the following primers: CTNS/CTNS-LKG forward primer, 5′-AAACTCGGATCCATGATAAGGAATTGGCTG-3′; CTNS backward primer, 5′-AAACCACTCGAGCTAGTTCAGCTGGTCATACCC-3′; and CTNS-LKG backward primer, 5′-AAACCACTCGAGTCAGCCCTTCAAGCTGCTTGC-3′. CTNS and CTNS-LKG amplicons were cloned into the pcDNA3.1 expression vector (Invitrogen Life Technologies) between the *Xho*I and the *Bam*HI sites (Fermentas, Hanover, MD, USA). Plasmids were thereafter transiently transfected in HK-2 cells using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's protocol. Control experiments were performed by transfection of the pcDNA3.1 empty plasmid. Four independent experiments were performed.

CTNS gene silencing

Selection of small interfering RNAs (siRNAs), based on the CTNS gene sequence and production of a SMARTpool reagent, was performed by Dharmacon, Inc. (Lafayette, CO, USA). CTNS SMARTpool siRNA (50 pmol/μl) was transiently transfected into HK-2 cells using Lipofectamine 2000 (Invitrogen Life Technologies) in accordance with the manufacturer's protocol. RT-PCR and Western blotting were performed after 72 h, to assess silencing effects. Intracellular levels of GSH/GSSG and Cys/CySS were measured when cystinosin protein expression was decreased by about 50%. The siRNA used in these experiments inhibited the expression of both CTNS isoforms. For controls, mock expression of a nonspecific siRNA pool provided by the vendor was performed. Three independent experiments were performed.

Statistical analysis

Data are expressed as means ± SEM and were analyzed with the SSPS for Windows 11.5 software (SPSS, Chicago, IL, USA). Significant differences between means were assessed by *t* test. Regression analysis was performed on mean values obtained from different experiments, as indicated. All results are two-tailed and considered significant for probabilities lower than 0.05.

Results

Effects of CTNS gene overexpression and CTNS gene inhibition on thiol/disulfide systems

To determine the influence of cystinosin on major cellular thiol/disulfide systems, HK-2 cells were transiently transfected with the

pcDNA 3.1 expression vector containing the CTNS or the CTNS-LKG coding sequences and incubated at 37°C for 48 h. CTNS or CTNS-LKG expression levels were analyzed by RT-PCR and Western blot. As reported in Fig. 1, intracellular concentrations and redox potentials of GSH/GSSG and Cys/CySS were not significantly modified in transfected cells overexpressing either of the two isoforms, despite significant increase in cystinosin levels.

On the other hand, when both CTNS isoforms were silenced for 72 h by approximately 50% with a CTNS siRNA pool (50 pmol/μl), intracellular Cys and CySS levels increased from 10.47 ± 0.2 to 29.56 ± 0.72 nmol/mg protein ($p<0.001$) and from 0.38 ± 0.02 to 2.01 ± 0.05 nmol/mg protein ($p<0.001$), respectively, whereas GSH and GSSG levels decreased from 195.3 ± 1.62 to 137.3 ± 1.6 nmol/mg protein ($p<0.001$) and from 12.09 ± 0.19 to 6.06 ± 0.3 nmol/mg protein ($p<0.001$), respectively. The redox potential of the GSH/GSSG couple was similar to that of control cells transfected with mock siRNA, whereas silenced HK-2 cells showed a small, but significant, increase in their Cys/CySS redox potential, from -164.7 ± 0.4 to -176.7 ± 3.74 ($p<0.01$), as illustrated in Fig. 2.

Modulation of CTNS gene expression by thiol deprivation, inhibition of GSH synthesis, or induction of oxidative stress

To study the contribution of cell thiols on the regulation of CTNS gene expression, HK-2 cells were incubated in thiol-free medium (Cys-, CySS-, and methionine-free) for 24 and 48 h. CTNS expression levels were analyzed by RT-PCR.

Under these conditions, intracellular Cys and GSH levels decreased after 48 h from 10.4 ± 1.4 to 3.3 ± 0.9 nmol/mg protein ($p<0.001$) and from 195.3 ± 46.4 to 27.0 ± 4.0 nmol/mg protein ($p<0.001$), respectively, whereas ROS production increased by 3.6 ± 0.5-fold ($p<0.001$). This correlated with a significant oxidation of the Cys/

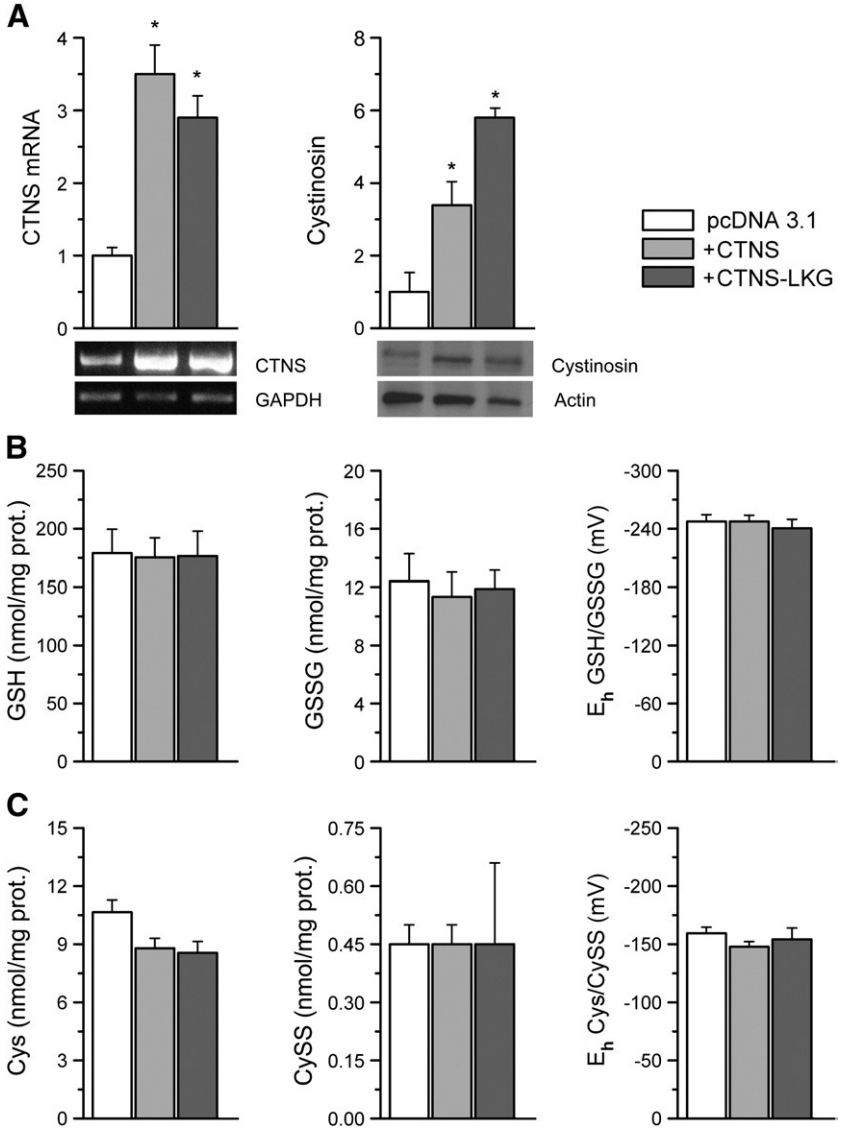

Fig. 1. Effects of CTNS gene overexpression on thiol/disulfide systems. HK-2 cells were transiently transfected with pcDNA3.1-CTNS (+CTNS) or pcDNA3.1-CTNS-LKG (+CTNS-LKG) and incubated for 48 h. (A) mRNA and protein expression levels were analyzed by RT-PCR and Western blot. Quantitative analysis of two isoforms was performed by normalization to GAPDH for RT-PCR products and actin for proteins. (B) Intracellular GSH and GSSG levels and redox potential of GSH/GSSG couple under basal (pcDNA 3.1) and CTNS or CTNS-LKG overexpression conditions. (C) Intracellular Cys and CySS levels and redox potential of Cys/CySS couple under basal (pcDNA 3.1) and CTNS or CTNS-LKG overxpression conditions. Data are expressed as means ± SEM of four independent experiments. *$p<0.001$ versus basal condition. *Abbreviations used:* GSH, reduced glutathione; GSSG, oxidized glutathione; Cys, cysteine; CySS, cystine; E_h, redox potential.

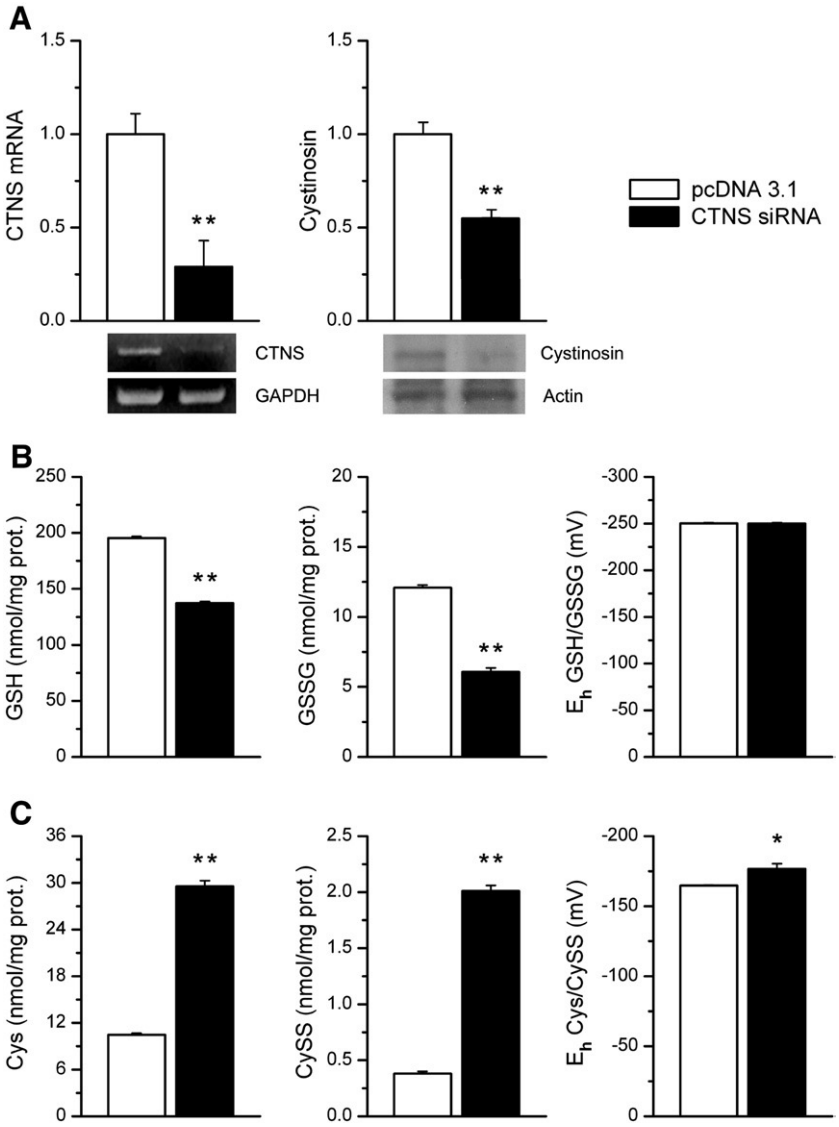

Fig. 2. Effects of CTNS gene inhibition on thiol/disulfide systems. HK-2 cells were transiently transfected with CTNS SMARTpool siRNA (50 pmol/μl). After 72 h, mRNA and protein levels were analyzed by RT-PCR and Western blot, respectively. (A) Quantitative analysis of two isoforms was performed by normalization to GAPDH for RT-PCR products and actin for proteins. (B) Intracellular GSH and GSSG levels and redox potential of GSH/GSSG couple under basal (mock) and CTNS gene inhibition conditions. (C) Intracellular Cys and CySS levels and redox potential of Cys/CySS couple under basal (mock) and CTNS gene inhibition conditions. Data are expressed as means ± SEM of three independent experiments. *$p<0.01$, **$p<0.001$ versus basal condition.

CySS (73 mV on average) and GSH/GSSG redox couples (100 mV on average) and with a progressive increase, by 1.9 ± 0.2-fold ($p<0.001$), of the CTNS mRNA expression levels (Fig. 3).

To further analyze these data, HK-2 cells were treated for 24 and 48 h with 100 μM L-buthionine sulfoximine (BSO), a selective inhibitor of the γ-glutamylcysteine synthetase, which blocks GSH biosynthesis. As expected, treatment with BSO caused a severe depletion of intracellular GSH from 195.3 ± 5.6 to 16.0 ± 0.6 nmol/mg protein ($p<0.001$) at 48 h. This was associated with a marked increase in intracellular ROS (from 1.0 ± 0.1- to 5.1 ± 0.4-fold, $p<0.001$) and oxidation of the GSH/GSSG redox couple (from −267.8 ± 4.1 to −117.2 ± 4.2 mV, $p<0.001$) at 48 h. Conversely, no substantial changes were observed in the Cys/CySS redox balance. BSO treatment showed a modest increase in CTNS mRNA after 48 h (1.35 ± 0.1-fold, $p<0.01$) compared to baseline levels (Fig. 3).

The above data suggest that cell GSH levels do not constitute a primary stimulus for the CTNS gene activation. To test the hypothesis that CTNS transcription or stabilization of its mRNA could be driven by cell oxidation, HK-2 cells were treated with 50 μM *tert*-butylhydroperoxide (t-BHP), an oxidant agent that acts similar to hydrogen peroxide and is neutralized in cells by glutathione peroxidase.

As illustrated in Fig. 3, intracellular ROS increased on average by 2.5 ± 0.2-fold ($p<0.001$) after 48 h of t-BHP treatment. In parallel, GSH levels increased moderately (from 195.3 ± 14.0 to 238.0 ± 26.3 nmol/mg protein), and Cys concentration decreased from 10.35 ± 1.5 to 8.74 ± 1.3 nmol/mg protein ($p<0.001$). The E_h of both thiol couples was not significantly modified, whereas CTNS mRNA expression was moderately stimulated (Fig. 3).

As mentioned above, two isoforms of the CTNS gene have been identified. To assess whether regulation of CTNS gene expression involves primarily one of these isoforms, RT-PCR was performed using isoform-specific primers. The lysosomal CTNS isoform was expressed in a ratio of approximately 4:1 in comparison with the CTNS-LKG isoform. The relative proportion between the two isoforms remained constant under the various experimental conditions that were tested (Fig. 4). When all the results obtained under the various experimental conditions reported above were pooled together, regression analysis showed that CTNS gene expression correlated with intracellular Cys level ($r^2 = 0.85$; $p<0.001$) and with

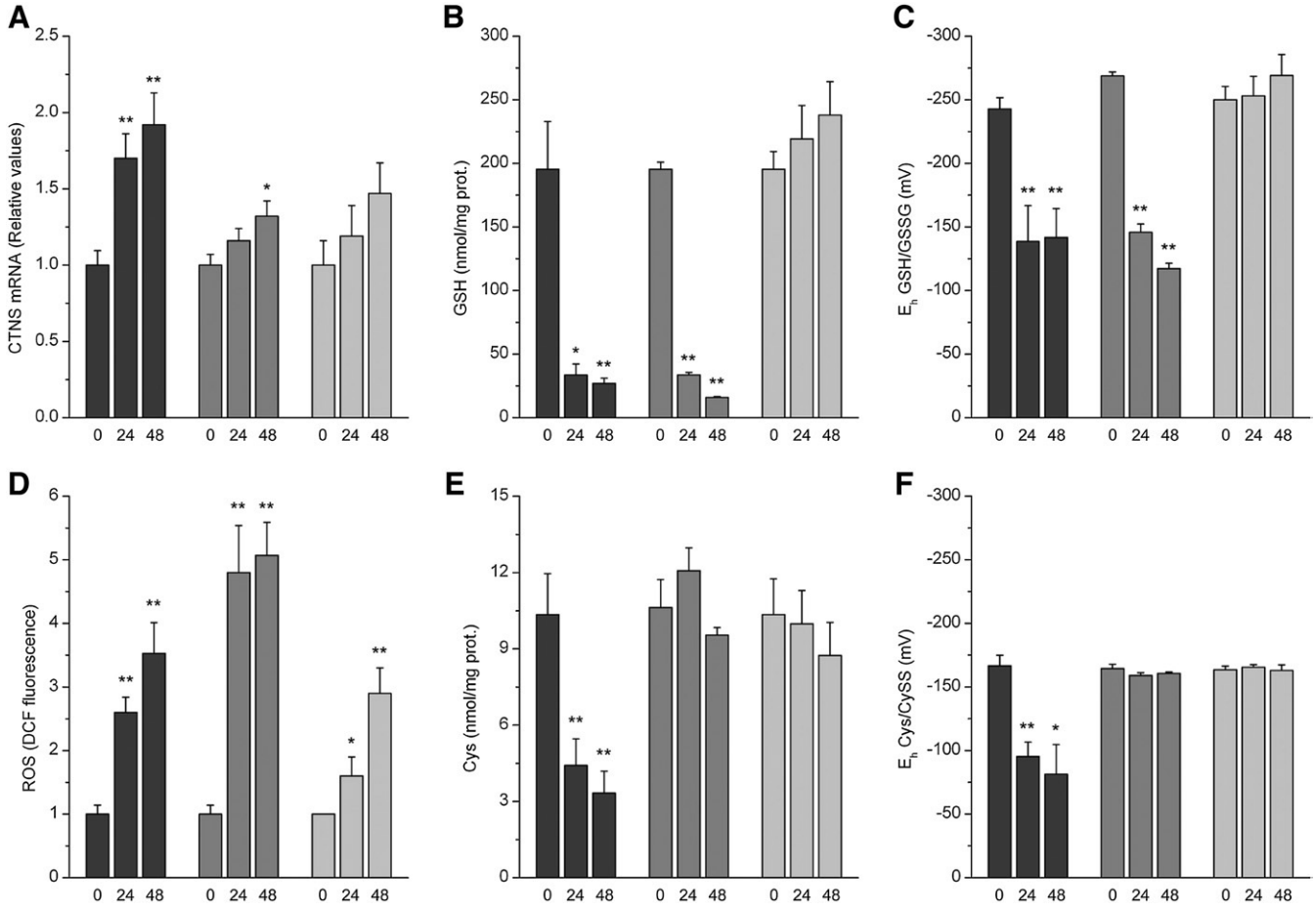

Fig. 3. Effects of thiols on CTNS gene expression. HK-2 cells were incubated in thiol-free medium (black bars) or were treated with 100 μM BSO (dark gray bars) or 50 μM t-BHP (light gray bars) for 24 and 48 h as indicated on the *x* axis. (A) CTNS mRNA expression levels were normalized to GAPDH mRNA expression. (B) GSH level was measured by HPLC and (C) the Nernst equation was used to calculate redox potential for the GSH/GSSG redox couple. (D) Flow cytometry analysis was used to assess ROS production measured as fluorescence of dichlorofluorescein. (E) Cys level was measured by HPLC and (F) the Nernst equation was used to calculate redox potential for the Cys/CySS redox couple. Data are expressed as means ± SEM of eight independent experiments. *$p<0.01$, **$p<0.001$ versus untreated cells. *Abbreviations used:* ROS, reactive oxygen species; BSO, L-buthionine sulfoximine; t-BHP, *tert*-butylhydroperoxide; and same as for Fig. 1.

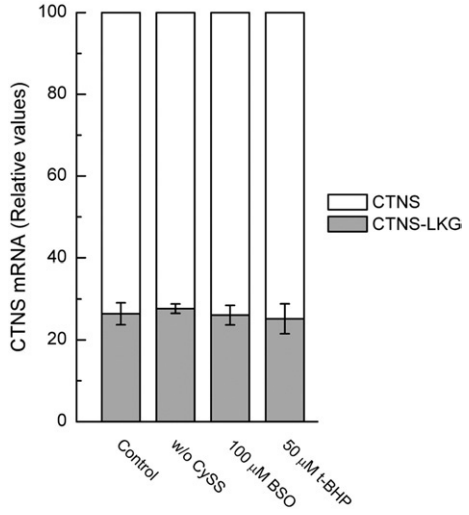

Fig. 4. Relative expression of CTNS and CTNS-LKG isoforms. HK-2 cells were treated with the same conditions that are described for Fig. 2. CTNS-LKG expression levels account for approximately 1/4 of the total CTNS transcripts. Data are expressed as means ± SEM of four independent experiments.

the Cys/CySS redox potential ($r^2=0.66$, $p<0.01$; Supplementary Table 1).

Modulation of CTNS gene expression by modifying intracellular thiols

To further test whether CTNS mRNA expression was influenced by a changes in intracellular Cys levels or by the redox state of the Cys/CySS couple, HK-2 cells were treated for 24 h with 0.5 mM L-cystine dimethyl ester dihydrochloride (CDME), a lysosomal CySS-loading agent, or with 0.5 mM cysteamine, a CySS-depleting agent. CDME treatment resulted in a 1.4 ± 0.24-fold increase in CTNS mRNA ($p<0.05$), which was associated with a very significant oxidation of the GSH/GSSG (from −250.3 ± 1.47 to −174 ± 0.63 mV, $p<0.001$) and Cys/CySS (from −164.6 ± 2.46 to −86 ± 1.38 mV, $p<0.001$) redox couples. Cysteamine treatment induced a mild but significant oxidation of the GSH/GSSG (from −250.3 ± 1.47 to −238.2 ± 1.03 mV, $p<0.01$) and Cys/CySS (from −164.6 ± 2.46 to −144.1 ± 3.86 mV, $p<0.02$) redox couples without significant changes in CTNS mRNA (Fig. 5).

Treatment of HK-2 cells with compounds that reduce the Cys/CySS and GSH/GSSG redox couples, such as *N*-acetyl-L-cysteine (NAC) and glutathione ethyl ester (GSHOEt), was performed to test whether increasing cell thiols or their redox potentials inhibited CTNS mRNA expression. For this purpose, HK-2 cells were incubated with 10 mM NAC or 2 mM GSHOEt in DMEM or in thiol-free DMEM for 48 h.

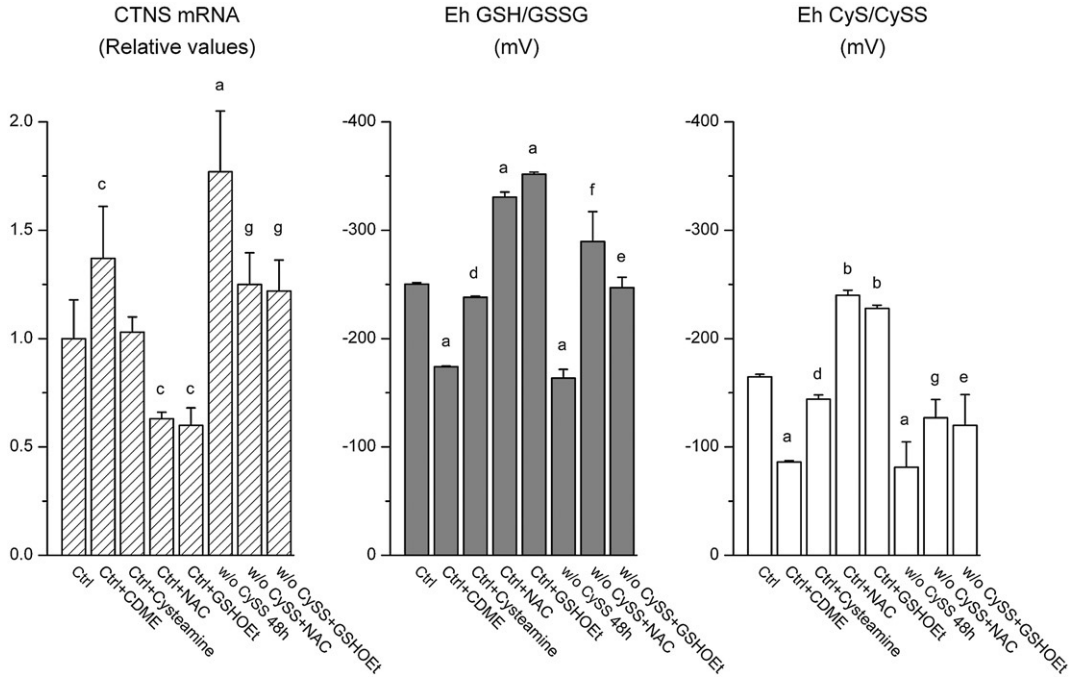

Fig. 5. Effects of altered redox state on CTNS mRNA expression. Exponentially growing cells in complete medium (Ctrl) were supplemented with 0.5 mM CDME or 0.5 mM cysteamine for 24 h. 10 mM NAC or 2 mM GSHOEt was added to exponentially growing cells in complete medium or in thiol-free medium for 48 h. CTNS mRNA expression levels were normalized to GAPDH and the redox states of GSH/GSSG (gray bars) and Cys/CySS (white bars) redox couples were measured under these growth conditions. Data are expressed as means ± SEM of three independent experiments. [a]$p<0.001$ versus control; [b]$p<0.005$ versus control; [c]$p<0.05$ versus control; [d]$p<0.02$ versus control; [e]$p<0.001$ versus without CySS 48 h; [f]$p<0.002$ versus without CySS 48 h; [g]$p<0.05$ versus without CySS 48 h. *Abbreviations used:* CDME, L-cystine dimethyl ester dihydrochloride; NAC, *N*-acetyl-L-cysteine; GSHOEt, glutathione ethyl ester.

As shown in Fig. 5, CTNS mRNA levels significantly decreased by approximately 40% ($p<0.05$) in HK-2 cells grown for 48 h in DMEM containing NAC or GSHOEt, compared to control condition. This effect was associated with a significant change in the GSH/GSSG and Cys/CySS redox state. After NAC treatment both thiol redox couples were more reduced. The GSH/GSSG E_h changed from -250.1 ± 3.10 to -330.6 ± 4.70 mV ($p<0.001$) and the Cys/CySS Eh changed from -167.0 ± 11.30 to -240.0 ± 8.17 mV ($p<0.005$). Similarly, GSHOEt promoted a shift toward a more reduced state in GSH/GSSG (from -250.1 ± 3.1 to -351.7 ± 1.89 mV, $p<0.001$) and in Cys/CySS (from -167.0 ± 11.3 to -227.8 ± 3.04 mV, $p<0.005$; Fig. 5).

Finally, incubation of HK-2 cells with 10 mM NAC or 2 mM GSHOEt for 48 h in thiol-free DMEM rescued significantly GSH and Cys levels and their redox couples in comparison to cells grown in thiol-free DMEM (Fig. 5). Specifically, NAC decreased on average by 23% the CTNS mRNA levels that were induced after incubation in thiol-free medium and restored changes in the redox balance from -163.5 ± 8.0 to -289.6 ± 27.6 mV ($p<0.002$) for the GSH/GSSG couple and from -81.3 ± 23.4 to -126.9 ± 16.9 mV ($p<0.02$) for the Cys/CySS couple (Fig. 5). Similarly, addition of GSHOEt to a thiol-free medium decreased on average the CTNS mRNA content by 25% and restored in part the GSH/GSSG redox state (from -163.5 ± 8.0 to -247.1 ± 9.5 mV, $p<0.001$) and the Cys/CySS redox state (from -81.3 ± 23.4 to -119.9 ± 28.4 mV, $p<0.001$; Fig. 5).

The data referring to GSH, GSSG, Cys, and CySS concentrations and shown as E_h values in Fig. 5 are collected in Supplementary Table 2.

Discussion

Cys acts in cells as a sulfur switch and is consequently involved in several redox-based signaling pathways. In extracellular fluids, the Cys/CySS couple has a redox potential of approximately −80 mV with a Cys:CySS ratio of approximately 1:5, indicating that Cys is rapidly oxidized into CySS [16]. Conversely, the E_h of the Cys/CySS redox couple decreases to approximately −160 mV in the cytosol, as CySS is reduced into Cys through electron transfer from the other major cell thiol/disulfide systems, mainly GSH/GSSG [17]. These systems are kept in a reduced state by several thiol reductases [16,18,19].

All members of the thiol/disulfide cascade, which includes other molecules such thiol residues on proteins and cysteinyl-glycine, are intrinsically linked by the hierarchical gradient of their E_h and by a complex loop of biosynthesis interactions that influence their concentrations. GSH, for example, reduces CySS into Cys, which in turn is a rate-limiting substrate for GSH synthesis [20].

Cell Cys concentrations need therefore to be tightly regulated to prevent, among others, inhibition of key metabolic pathways caused by Cys scarcity, or nonspecific cysteinylation of proteins and other molecules caused by excessive intracellular Cys levels.

Until now, the role of cystinosin has been primarily restricted to a resident CySS transporter that allows for CySS efflux from lysosomes. This role is substantiated by seminal works demonstrating impaired CySS efflux from lysosomes purified from cystinotic cells and, more recently, by direct demonstration that mutated cystinosin proteins expressed in the plasma membrane can transport CySS in the presence of a proton gradient [21,22].

The present results significantly modify this concept, by demonstrating that cystinosin expression is regulated.

In addition, these results also begin describing cell stimuli that might modulate the expression of cystinosin. Specifically, we have shown that variations in the intracellular Cys/CySS redox state is a major stimulus that modifies CTNS mRNA synthesis or stabilization. A similar regulation has already been demonstrated for other amino acid carriers, including CySS transporters [23]. The x_C system, for example, is a highly specific plasma membrane glutamate/CySS exchanger, allowing CySS influx into the cell [24]. This transporter participates in the regulation of intracellular GSH levels and its activity is stimulated by electrophilic agents, such as diethyl maleate, oxygen, and bacterial lipopolysaccharides, or by CySS deprivation [25–27].

The complexity of the cell redox state and the interactions between the Cys/CySS and the GSH/GSSG redox couples raises the possibility that cells can also sense other stimuli.

In our experimental settings, a moderate increase in CTNS mRNA levels was observed after inhibition of GSH synthesis and after induction of an oxidative stress, both of which caused an increase in cell ROS production. The role of ROS as broad activators of various biosynthesis and enzymatic pathways that regulate cell redox state has been well established [28].

Although we could not observe a clear correlation between CTNS mRNA and cell ROS content, activation of the CTNS gene could be expected in order to increase Cys availability for GSH synthesis in response to an oxidative stress. In support of this hypothesis, we have previously documented that cystinotic fibroblasts have relatively normal GSH/GSSG levels under normal cell culture conditions, but fail to increase substantially their GSH levels when exposed to hydrogen peroxide, in comparison to control cells [7]. Part of our data supports this concept.

In fact, lysosome CySS loading with CDME [29] induces a major increase in superoxide and hydrogen peroxide production and in glutathione peroxidase activity [30,31]. Accordingly, we have observed a very significant oxidation of GSH/GSSG and Cys/CySS redox couples after CDME exposure that was associated with enhanced CTNS mRNA expression, despite increased intracellular Cys levels. Taken together, these data indicate that cells increase CTNS mRNA levels in response to Cys depletion, but also that they can stimulate the same pathway when they need more Cys to respond to oxidation, suggesting different mechanisms of activation.

Cysteamine treatment had no significant effect on CTNS mRNA expression. Most tissues, in fact, constitutively generate cysteamine as a by-product of the degradation of coenzyme A. Its half-life is modulated by cysteamine dioxygenase activity, which is tightly regulated by means of ubiquitination and degradation in response to Cys levels [32,33]. These effects may explain in part the lack of changes observed in our experimental setting. It is likely that in cystinotic cells, in which CySS is accumulated inside lysosomes, the effects of cysteamine are dramatically different, as this compound allows CySS to reach the cytosol.

In our experimental setting, the effects of cell thiol depletion on CTNS mRNA levels could be successfully reverted by NAC and by GSHOEt. When the same treatments were applied to control cells, a suppression of CTNS mRNA levels was observed. Altogether, these results confirm that cells can sense thiol levels or their redox state to regulate the expression of cystinosin protein, but they do not add information on the specific stimuli that are sensed, because both of these compounds modify simultaneously thiol levels and their redox couples. In addition, NAC and GSHOEt also have other effects on cells, including inhibition of DNA synthesis and S-glutathionylation or S-cysteinylation of proteins and transcriptional regulation [34,35].

Unexpectedly, no significant changes in redox state were observed after overexpression or silencing of the CTNS gene. This may be related to the transient nature of these manipulations, but may also indicate that under normal circumstances, other CySS transporters such as the x_C, *4F2hc/LAT-2,* and *rBAT/*$b^{0,+}$*At* [36–39], or different metabolic pathways cooperate to regulate cell thiols.

Under stress conditions, on the other hand, CTNS may become essential for providing cells with additional CySS. Loss of the cystinosin contribution in cystinosis could result in the unregulated activation of other transporters and pathways, which may cause abnormal activation of redox-based signaling or protein cysteinylation. Cysteinylation of PKCδ has in fact been shown to occur in cystinotic cells, causing cell death by apoptosis [10].

Finally, the recent identification of cystinosin-LKG indicates that cystinosin is a complex CySS transport system that modulates CySS fluxes from different cell compartments [40]. Regulation of this pathway does not seem, at least in our experimental setting, to involve preferential expression of one isoform with respect to the other.

In conclusion, the present findings show for the first time that the CTNS gene is actively regulated and that cystinosin may represent a major player in the cell machinery that regulates cell thiol redox systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeadbiomed.2010.01.11.

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