Cystinosis Research Foundation Progress Report

Project Title: Role of nitric oxide in the kidney proximal tubular dysfunction associated with the Fanconi syndrome in cystinosis

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Overview

We intiated our investigations with using CTNS-targeting siRNA SMARTpool reagent prepared by Dharmacon, Inc., (Lafayette, CO, USA) to transiently silence the *CTNS* gene expression in HK-2 cell line as our proximal tubular cell culture system, thus, partially mimicking the genotypic defect in cystinosis. The presence of elevated cystine levels in CTNS knockdown HK-2 cells indicates that the storage defect in these cells is preserved, thus demonstrating a valid reproduction of the biochemical phenotype in cystinosis. Interestingly, these cells displayed elevated levels of intracellular superoxide and nitric oxide. As chronic elevation of reactive nitrogen species (RNS) is harmful, eventually the normal function of kidney proximal tubules is lost and apoptosis may occur. This is important as this will provide clues as to whether the production of NO in cystinotic cells is a physiological step or may be a response to stress.

Objective #1: To determine the intracellular nitric oxide and superoxide levels, 3-nitrotyrosines and 8-nitrogunanines, reduced and oxidized glutathione (GSH and GSSG, respectively) levels, general oxidative/nitrosative stress index, intracellular ATP levels, mitochondrial membrane potential and Na^+,K^+-ATPase activity in; (1) *Ctns* -/- and +/+ ciMPTEC lines and in (2) CTNS siRNA- and non-targeting siRNA-transfected HK-2 cells. The same cell models will be used in all cases described below. We will evaluate these parameters to determine the existence of nitrosative stress and assess the NO-mediated tubular epithelial cell injury.

Using FACS analysis, CTNS knockdown cells displayed elevated intracellular levels of superoxide (Fig. 1A) and nitric oxide (Fig. 1B). These alterations in ROS production were associated with a reduction in total GSH content in CTNS knockdown cells (Fig. 1C). Despite increased ROS load and reduced GSH content, CTNS knockdown cells showed a moderate, but not significant, increase in basal oxidative stress index. However, we have observed an increase in oxidative stress index in CTNS knockdown cells after exposure to oxidative stimuli suggesting decreased antioxidant capacity of these cells.

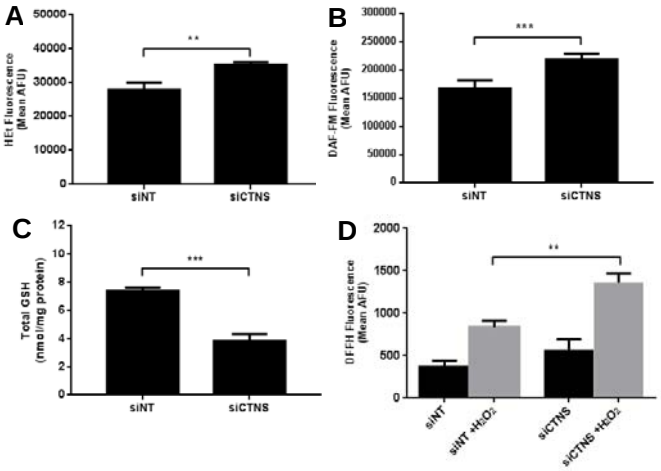

Fig. 1. Effect of CTNS gene silencing on intracellular ROS, total GSH, and general oxidative stress index. HK-2 cells were transfected with CTNS-targeting siRNA pool (50 pmol/µl) and levels of intracellular superoxide (A), nitric oxide (B), total GSH (C), oxidative stress index (D) were assessed 48-h post transfection. siNT, non-targeting siRNA; siCTNS, CTNS targeting-siRNA

Because unregulated production of intracellular superoxide and NO can lead to the formation of highly oxidizing peroxynitrite, we next assessed the nitrotyrosine levels in CTNS knockdown cells. Surprisingly, despite increased intracellular levels of superoxide and NO, the nitrotyrosine levels in CTNS knockdown cells remained unaltered (Fig. 2A). However, after exposure to an oxidative stimuli (i.e. 100 µM H_2O_2), CTNS knockdown cells displayed elevated levels of nitrotyrosines (Fig. 2A), implying increased sensitivity of these cells to nitrosative damage.

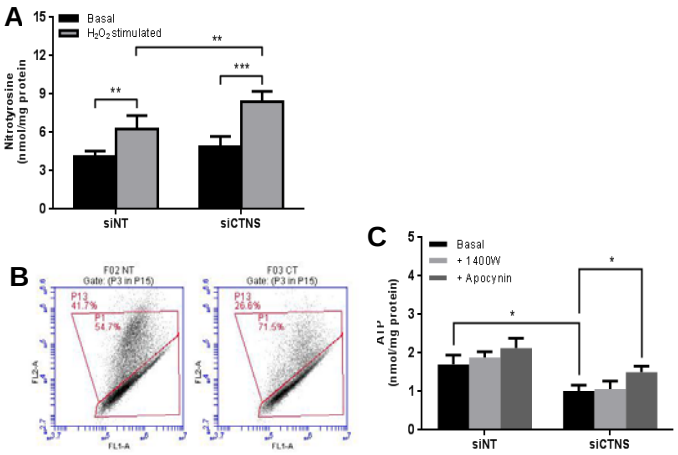

Fig. 2. Effect of CTNS gene silencing on intracellular 3-nitrotyrosine levels, mitochondrial membrane potential, and total ATP content. HK-2 cells were transfected with CTNS-targeting siRNA pool (50 pmol/µl) and 3-nitrotyrosine levels (A), mitochondrial membrane potential (B), and ATP content (C) were determined 48 h post transfection. siNT, non-targeting siRNA; siCTNS, CTNS targeting-siRNA

FACS analysis of CTNS knockdown cells revealed that these cells have compromised mitochondrial integrity (Fig. 2B), which possibly impacts on ATP synthesis. Indeed, concurrent with some studies performed on cystinotic cells, CTNS gene silencing in HK-2 cells reduced the intracellular ATP content (Fig. 2C). Interestingly, NOX inhibitor apocynin restored ATP content in CTNS knockdown cells to normal levels (Fig. 2C), suggesting that NOX may play a role in energy dysfunction associated with cystinosis. Apocynin treatment was also associated with an increase in total GSH content (data not shown).

Objective #2: To determine the inducible NO synthase (*i*NOS) and NADPH oxidase (NOX) expression and the expression of sodium transporters such as Na^+,K^+-ATPase and Type 3 Na/H exchanger (NHE3) in cell models described in Objective #1. These are some of the transporters affected in cystinosis and recent evidence suggested that endogenous NO plays an important role in the regulation of these membrane sodium transporters (3,4).

Using Western blot, we have observed an increased expression of iNOS and NOX proteins in CTNS knockdown cells (Fig. 3A and 3B, respectively), which probably contributes to the increased intracellular ROS load in these cells. Consistent with this observation, we have also documented an increased iNOS expression in both *Ctns* -/- ciMPTECs (CTP 188 and CTP 193) used in this study, suggesting enhanced nitrosative stress in these cells. Presently, we are investigating the effects of iNOS-specific inhibitor 1400W and NOX inhibitor apocynin on the expression of Na^+,K^+-ATPase and NHE-3 in ciMPTECs using Western blot and confocal microscopy. Our initial investigation showed that both Na^+,K^+-ATPase and NHE-3 expression is decreased in *Ctns* -/- ciMPTECs.

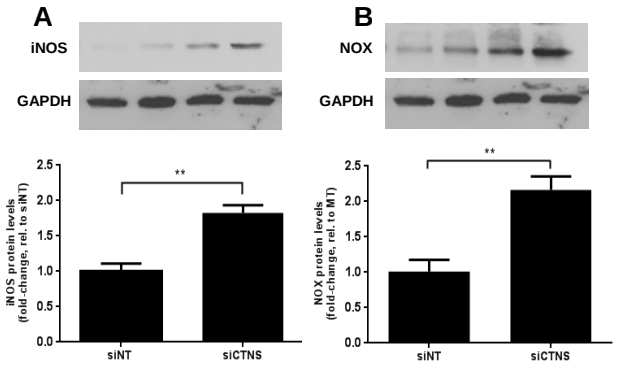

Fig. 3. Effect of CTNS gene silencing on the expression of iNOS and NOX. HK-2 cells were transfected with CTNS-targeting siRNA pool (50 pmol/µl) and levels of expression of iNOS (A) and NOX (B) were assessed by Western blot. siNT, non-targeting siRNA; siCTNS, CTNS targeting-siRNA

Objective #3: To compare the S-nitrosylated proteome patterns of cell models described in Objective #1. In view of this, it is important to understand the biological conditions under which S-nitrosylation occurs since this will provide clues as to whether this is a physiological step or may already be a response to stress. The identification of the full complement of S-nitrosylated proteins and the functional consequences of this modification is essential for the understanding the mechanisms of actions of NO and the signalling events that arise from its chronic release.

We are currently optimizing protocols for isolation and proteomic analysis of nitrosylated proteins in Ctns -/- ciMPTECs in collaboration with the UCD Mass Spectrometry Facility. Initial results will hopefully be available in the next couple of weeks.

Objective #4: If evidence of nitrosative stress is observed in *Ctns* -/- ciMPTEC lines and/or in CTNS siRNA-transfected HK-2 cells, we will investigate the effects NO synthase inhibitors, L-NAME and/or 1400W (in combination with cysteamine) on the same parameters mentioned above.

Our initial data showed that one of the *Ctns* -/- ciMPTEC clones provided to us (CTP 193) had a significantly increased intracellular NO level compared to its matched *Ctns* +/+ control (Fig. 4A). This was also associated with a decrease in total GSH levels (data not shown). CTP 193 clone also displayed increased nitrotyrosine levels as determined by ELISA (Fig. 4B). We are now investigating the effects of 1400W and apocynin on the GSH levels, nitrotyrosine content, and the expression of Na^+,K^+-ATPase and NHE-3 in *Ctns* -/- ciMPTECs.

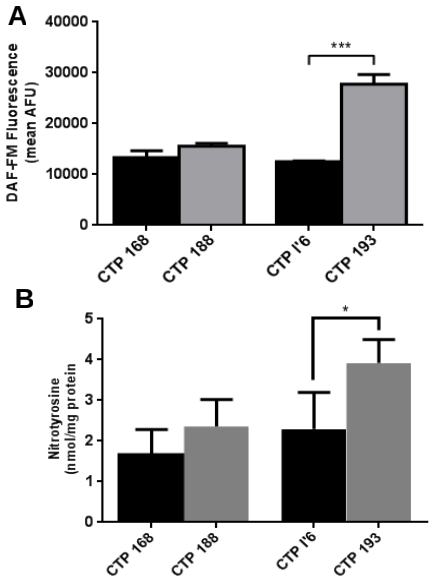

Fig. 4. Intracellular NO levels and nitrotyrosine content in *Ctns* -/- and +/+ ciMPTECs. ciMPTECs were cultured at 33°C until 40% confluence. Cells were then transferred to 37°C and intracellular NO levels (A) and nitrotyrosine content (B) were determined after 72 hours in culture. *Ctns* -/- (black bars); *Ctns* +/+ (gray bars).

Invited Oral Presentations 2012

- **CTNS gene inhibition alters redox status in a kidney proximal tubular epithelial cell line: implications for cell dysfunction associated with the Fanconi syndrome in cystinosis**

  Irish Nephrology Society and 25th Annual Conference of the European Diabetic Nephropathy Study Group (EDNSG)
  19th May 2012
  Crowne Plaza Hotel, Dublin-Northwood, Ireland

- **Role of nitric oxide in the kidney proximal tubular dysfunction associated with the Fanconi syndrome in cystinosis**

  7th International Cystinosis Congress
  28th June to 1st July 2012
  Marriott Charles de Gaulle, Paris, France

Publications 2012

Cystine dimethylester loading promotes oxidative stress independent of lysosomal cystine accumulation in a human proximal tubular epithelial cell line. 2012 Rodolfo Sumayao, Bernadette McEvoy, Natalia Martin-Martin, Tara McMorrow and Philip Newsholme. **Pediatric Research** *Provisionally accepted.*