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Activation of AMP kinase plays a role in the increased apoptosis in the renal proximal tubule in cystinosis

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ABSTRACT

In cystinosis, renal proximal tubule (RPT) function is compromised, due to mutations in *ctns*, which encodes for the transporter cystinosin, which removes cystine from lysosomes. Altered RPT function in cystinosis has been attributed to decreased ATP, as well as increased apoptosis. In this report, the role of AMPK was examined. AMPK was activated in primary rabbit RPT cells with a cystinosin knockdown, using cystinosin siRNA. The activation of AMPK was associated with a 50% decrease in ATP and a 1.7-fold increase in the ADP/ATP level. Cisplatin-induced apoptosis also increased in primary RPT cells with a cystinosin knockdown. The role of AMPK in the increased sensitivity to cisplatin was examined. The increased sensitivity to cisplatin was prevented in primary RPT cells with a cystinosin knockdown by the AMPK inhibitor Compound C. The effect of siRNAs against AMPKα1 and AMPKα2 was also studied. The siRNAs knocked down AMPKα, and prevented AMPKα activation by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR). The siRNAs against AMPKα1 and AMPKα2 also prevented the increased sensitivity to cisplatin in the primary RPT cells with a cystinosin knockdown. These results suggest that signaling through AMPK plays a role in the enhanced apoptosis in the RPT in cystinosis.

1. Introduction

Cystinosis is an autosomal recessive disease characterized by elevated levels of cystine in all tissues in the body [1]. Infantile cystinosis is characterized by renal problems. The reabsorption of solutes by the renal proximal tubule (RPT) is impaired, resulting in the Fanconi syndrome (the urinary loss of water, amino acids, glucose, bicarbonate, phosphate, and other ions) [1]. This disorder can be attributed to mutations in *ctns*, a gene that encodes for cystinosin, a cystine transporter responsible for cystine efflux from lysosomes [2]. A number of *ctns* mutations inactivate cystinosin, and result in renal disease. However, the manner by which these mutations result in the renal Fanconi syndrome are unknown.

A number of hypotheses have been proposed to explain the emergence of the Fanconi syndrome in cystinosis, including alterations in ATP metabolism, and apoptosis [3]. Evidence indicating that ATP metabolism was altered came from studies in which Cystine Dimethyl ester (CDME) was used to model cystinosis. CDME accumulates in lysosomes where it is hydrolyzed, generating cystine. The alterations in ATP metabolism which were observed following CDME treatment were proposed to be responsible for the reduced reabsorption of solutes by apical Na^+/solute cotransporters in cystinotic RPTs.

These conclusions were questioned recently, when investigators discovered that CDME inhibited mitochondrial ATP synthesis [3]. However, in other model systems, decreased intracellular ATP was observed, including conditionally immortalized human cystinotic RPT cell lines [4]. Although, the observed reductions in intracellular ATP could possibly explain the reduced activity of apical Na^+/solute cotransporters in cystinotic RPTs, our recent studies [5] do not support this hypothesis.

In our studies with primary RPT cells with a cystinosin knockdown, obtained using siRNA, the activity of the Na^+/phosphate (Pi) cotransport system and the Na^+/glucose cotransport system was reduced [5]. However, Na, K ATPase activity was not similarly reduced, and intracellular Na^+ levels were not altered, despite the observations of a 50% decrease in intracellular ATP [5]. Nevertheless, such a decrease in intracellular ATP could have other effects on cell function through the activation of AMPK.

AMPK is a heterotrimer, consisting of a catalytic α subunit, in addition to a regulatory β subunit and a regulatory γ subunit [6]. Increases in intracellular AMP and in ADP activate the enzyme allosterically, as well by the phosphorylation of the α subunit at Thr-172 [6,7]. In addition, AMPK can be activated independently of changes in energy charge [7]. Once activated, AMPK initiates phosphorylation events that cause processes that consume ATP to be inhibited, and processes that produce ATP to be activated.

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If conditions are particularly stressful, AMPK affects cell viability, activating such processes as apoptosis and/or autophagy [8,9].

This report examines the effects of a cystinosin knockdown in primary rabbit RPT cells on AMPK and apoptosis. AMPK was activated in primary RPT cells with a cystinosin knockdown. In addition, ADP was elevated, sensitivity to killing by cisplatin increased, and apoptosis occurred at a higher frequency. In order to determine whether AMPK was responsible, siRNA against AMPKα was employed. The results are reported here.

2. Materials and methods

2.1. Materials

Hormones, human transferrin, the ATP assay kit, X-ray film and chemicals were from Sigma Aldrich Chemical Corp. (St. Louis, MO). The Bioluminescent ADP/ATP Ratio Assay Kit was from Abcam, Inc. (Cambridge, MA). Collagenase was from Worthington (Freehold, NJ). Dulbecco's Modified Eagle's Medium (DME), Ham's F12 Medium (F12), soybean trypsin inhibitor, the Image-iT LIVE Red Caspase Detection Kit, lipofectamine and double stranded stealth siRNA were from Life Technologies Corp (Carlsbad, CA). The sequence of the cystinosin siRNA and the scrambled control was GAC AAU ACG UCU UGC UGC CCA GUU A (and the scrambled control GAC GCA UUU CUG UCG ACC CGA AUU A, while the sequence of AMPK α1 siRNA and AMPK α2 siRNA were, respectively CAGGGAACAUGAAUGGUUUAAACAA (scrambled control, CAGAAACGUAAGUUGUUAAAGGCAA), and AGGCAGAUUGUAUGCAGGUCCUGAA (scrambled control, UUCGUACAGGCGUCCAUACUAACCU).

Rabbit antibodies against pAMPKα1/α2, AMPKα1/α2, and AMPKβ were from Cell Signaling Technology, Inc., (Danvers, MA), and the mouse monoclonal antibody against βactin was from Santa Cruz Biotech (Santa Cruz, CA). Goat anti-rabbit and goat anti-mouse Horse Radish Peroxidase (HRP) conjugates, nitrocellulose, electrophoresis reagents, the Transblot apparatus, the Scanning densitometer, and the Quantity One software were from Bio-Rad Corp. (Hercules, CA). WesternBright Sirius Western blotting detection reagents and LucentBlue X-ray film was obtained from Advansta (Menlo Park, CA). The Micro BCA Protein Assay Kit was from Thermo Scientific, and Prism 5 software was from GraphPad, Inc. (San Diego, CA).

2.2. Primary RPT cell cultures

Primary rabbit RPT cell cultures were initiated from the kidney of a male New Zealand white rabbit (2–2.5 kg). As previously described [10], the renal artery was perfused first with Phosphate Buffered Saline (PBS) and then sterile 0.5% (w/v) iron oxide. Renal cortical slices were disrupted using a sterile dounce homogenizer (loose pestle), and tubule fragments separated through 253 μ and 83 μ mesh sieves. The proximal tubules and glomeruli on the 83 μ sieve, were transferred to a 50 ml conical tube containing basal medium (a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 (DME/F12), pH 7.4, containing 15 mM HEPES, 20 mM Na^+ bicarbonate) supplemented with penicillin (92 units/ml) and streptomycin (2%). After the glomeruli (with iron oxide) were removed with a sterile stir bar, the tubules were treated with 0.05 mg/ml collagenase class IV and 0.5 mg/ml soybean trypsin inhibitor for 2 min at 23 °C, followed by centrifugation. The tubules were re-suspended in basal medium, and plated into 35 mm cultures dishes (or 12 well plates) containing medium RK-1 (basal medium supplemented with 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 5×10^{-8} M hydrocortisone, 92 units/ml penicillin and 0.01% kanamycin). The medium with factors was changed the day after plating and every other day thereafter.

2.3. Transfection of primary RPT cells with siRNA

After 4 days in culture, primary RPT cells were transfected with 200 nM cystinosin siRNA or scrambled (scr) control siRNA using lipofectamine. An 80% knockdown of cystinosin was observed during the following 4 days [5]. The AMPK α subunit (AMPKα) was knocked down using a combination of 100 nM AMPKα1 siRNA and 100 nM AMPKα2 siRNA, in parallel with cultures treated with 100 nM scr AMPKα1 and scr AMPKα2 siRNA in combination. For a double knockdown of cystinosin and AMPKα1/α2, the primary RPT cells were first transfected with 100 nM AMPKα1 siRNA and 100 nM AMPKα2 siRNA (in parallel with cultures with 100 nM scr AMPKα1 and scr AMPKα2 siRNA in combination). One day later, primary RPT cell cultures that had been transfected with AMPKα1/α2 siRNA (or scr control AMPKα1/α2) were transfected either with 200 nM cystinosin siRNA or scr cystinosin siRNA. Three days later, cultures were used for studies with cisplatin and/or Western Blotting.

2.4. Apoptosis and toxicity studies

Immediately prior to the study, the culture medium was changed to fresh medium RK-1. Two hours later, 20 μM cisplatin was added (using a freshly prepared 4 mM cisplatin stock solution in water). After 6 h, the cells were labeled with FLICA, Hoechst 33342 and SYTOX Green (the components of an Image-iT LIVE Red Caspase Detection Kit). FLICA (excitation/emission maximum of 550/595 nm) stains cells with activated Caspase-3 and -7. SYTOX Green (excitation/emission maxima of 504/523 nm) stains only dead cells, while Hoescht 33342 (excitation/emission maxima of 350/461 nm) stains all cells.

Cells stained with FLICA, Hoescht 33342 and/or SYTOX Green were detected using a Zeiss Axio observer microscope. In each experimental condition, images of at least 25 microscope fields/culture in duplicate cultures were acquired using Axiovision software. The number of cells stained with FLICA, Hoescht 33342 and SYTOX Green was determined in each microscope field using the NIH Image J Program. The proportion of apoptotic and dead cells relative to the total number of cells (stained with Hoescht 33342) was then calculated. In cisplatin treated cultures, not only were apoptotic and dead cells identified, but in addition the number of attached cells was determined in triplicate cultures following a 24 h incubation, employing a Coulter counter (vs untreated controls).

2.5. Determination of intracellular ATP and the ADP/ATP ratio

Intracellular ATP was released from cells, and measured by the light emitted by firefly luciferase, using a Packard TriCarb scintillation counter with the coincidence turned off. To determine the ADP/ATP ratio determined using Abcam's assay kit, ATP was first measured from the light emitted following a luciferase reaction. Then ATP was generated from ADP using an ADP converting enzyme, followed by luminescence readings. ADP/ATP ratios were calculated.

2.6. Electrophoresis and western analysis

Primary cultures were washed with PBS at 4 °C, solubilized in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na^+ deoxycholate, 1 mM EDTA containing 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na^+ orthovanadate and 1 mM NaF), and removed from the dish with rubber policemen. Protein determinations made using a micro BCA Protein Assay Kit. Samples were equalized with respect to protein, and separated in 7.5% SDS poly-

acrylamide gels with molecular weight markers. Proteins were transferred to nitrocellulose using a Trans-Blot apparatus.

Blots were first blocked (1 h in Tris-buffered saline containing 0.1% Tween 20 (TTBS)), incubated with primary antibody in TTBS (2 h), followed by washing (6×; TTBS). The blots were then incubated with secondary HRP conjugated goat secondary antibody in TTBS (45 min), and washed (7×; TTBS). Antibody dilutions were as recommended by the vendor. To visualize bands, blots were incubated with WesternBright Sirius HRP substrate, and placed on X-ray film. X-ray film was scanned with a scanning densitometer and band intensities compared using Quantity One software.

3. Results

3.1. Effect of cystinosin siRNA on AMPK

Previously, cystinosin was knocked down by 80% in primary rabbit RPT cells using siRNA against rabbit cystinosin [5]. The primary RPT cells exhibited reduced Pi uptake under these conditions, and had reduced ATP levels [5]. To determine whether AMPK was activated, the level of pAMPKα subunit was examined. Fig. 1A shows that in scr control cultures, the level of pAMPK increased 8 ± 2-fold in the presence of AICAR. In contrast, the level of pAMPK in primary RPT cells with a cystinosin knockdown was elevated 7 ± 0-fold in the absence of AICAR (vs untreated scr controls). No further increase in pAMPK levels was observed when primary RPT cells with a cystinosin knockdown were treated with AICAR. These results suggest that AMPK is maximally activated in primary RPT cells with a cystinosin knockdown.

ADP, like AMP, is an activator of AMPK [11]. In order to evaluate whether the activation of AMPK in primary RPT cells with a cystinosin knockdown could be explained by an increase in ADP, the ADP/ATP ratio was determined. Fig. 1B shows that the ADP/ATP ratio increased 1.7 ± 0.2-fold with a cystinosin knockdown. Thus, the 0.5 ± 1-fold decrease in ATP in cells with a cystinosin knockdown (also shown in Fig. 1B) is accompanied by a similar increase in ADP. Such an increase in ADP would likely be accompanied by an increase in AMP, due to metabolism by adenylate kinase [12], causing further activation of AMPK.

3.2. Effect of a cystinosin knockdown on the sensitivity to apoptotic stimuli

Previously, human cystinotic RPT cell cultures were observed to have an increased sensitivity to apoptotic stimuli [12]. In order to determine whether similar changes occur in primary RPT cells with a cystinosin knockdown, the effect of 20 μM cisplatin was examined. Fig. 2A shows that after 6 h, 13 ± 1% of control cells were apoptotic with cisplatin. The proportion of apoptotic cells in primary RPT cells with a cystinosin knockdown was significantly higher, 24 ± 2%, than scr controls (i.e. 1.9 ± 0.2-fold increase). In addition, the number of dead cells also increased 2.2 ± 0.8-fold in primary RPT cells with a cystinosin knockdown (vs scr control cells).

The role of AMPK was examined because AMPK activation has been observed to increase the sensitivity of animal cells to such toxicants [13,14]. Initially, the effect of an AMPK inhibitor, Compound C (Cmpd C) was examined. Fig. 2B shows that 1 μM Cmpd C prevented the increase in cisplatin-induced killing of primary RPT cells with a cystinosin knockdown, completely inhibiting the cisplatin-induced cell death.

3.3. Effect of an AMPKα knockdown on the sensitivity to apoptotic stimuli

In order to further examine the role of AMPK in the increased sensitivity of primary RPT cells with a cystinosin knockdown to apoptotic stimuli, a combination of AMPKα1 and α2 siRNA was employed. Primary RPT cells were transfected with both AMPKα1 and AMPKα2 siRNA, in parallel with cultures transfected with scr AMPKα1 siRNA as well as scr AMPKα2 siRNA. Fig. 3 shows that transfection with both AMPKα1 and AMPKα2 siRNA prevented the activation of AMPK, and reduced the level of AMPKα subunits.

Fig. 4A shows the effect of the AMPKα1 and α2 knockdown on the sensitivity of primary RPT cells with a cystinosin knockdown to cisplatin. Following a 24 h incubation with 20 μM cisplatin, a 46 ± 4% decrease was observed in the cell number in control cultures transfected with scr siRNA against cystinosin (as well as scr AMPKα1 and AMPKα2). The effect of cisplatin increased significantly (to 70 ± 2%) when cultures were transfected with both cystinosin siRNA (as well as scr AMPKα1 and AMPKα2 siRNA). However, when siRNA against AMPKα1 and AMPKα2 was employed, (1) cisplatin no longer reduced the number of cells to this extent in cultures transfected with cystinosin siRNA, and (2) significant differences in cell number were no longer observed between cultures transfected with cystinosin siRNA and scr control cystinosin siRNA.

Fig. 4B shows that AMPK was activated after transfection of primary RPT cells with cystinosin siRNA (in addition to AMPKα1 and α2 scr siRNA), similar to the results shown in Fig. 3. However, the activation of AMPK was no longer observed in primary RPT cells treated with cystinosin siRNA, when also knocking down AMPKα

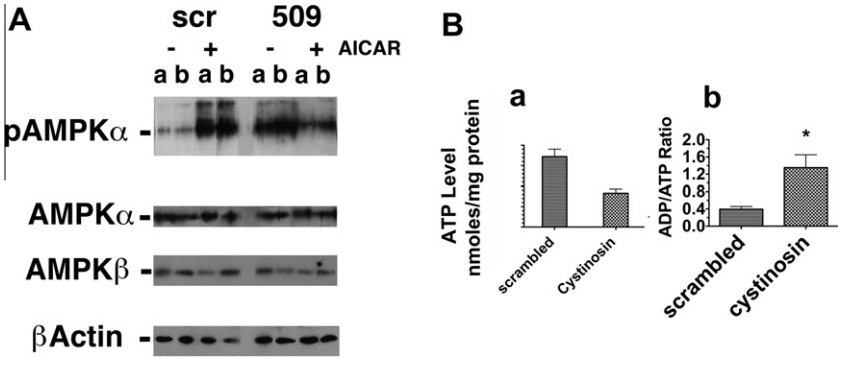

Fig. 1. Effect of cystinosin siRNA on AMPK, ATP and ADP levels. Primary RPT cells were transfected with either cystinosin siRNA or scr siRNA. (A) The level of pAMPKα, AMPKα, AMPKβ and βActin was determined in cultures treated for 4 h ±1 mM AICAR. Duplicate cultures from the same set are illustrated. (B) a. The level of ATP was the average of determinations made in triplicate in 5 independent culture sets treated either with scr siRNA or cystinosin siRNA. b. The ADP/ATP ratio was determined in triplicate cultures treated with either scr siRNA or cystinosin siRNA. Values are averages ± SEM. $^{*}p < 0.05$.

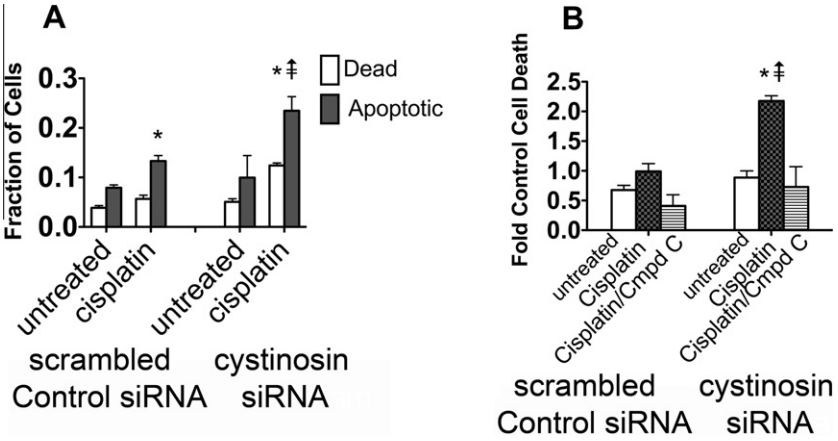

Fig. 2. Effect of cystinosin siRNA on apoptosis and cell death. (A) Effect of 20 μM cisplatin on the activation of caspase −3, −7 (apoptosis) and cell death in primary RPT cells treated with scr siRNA or cystinosin siRNA. (B) Effect of Compound C on cisplatin toxicity. The ability of 10 μM Compound C (Cmpd C) to prevent the death of primary RPT cells caused by 20 μM cisplatin was examined following a 6 h incubation. Comparisons are made to the fraction of cells that are dead in cultures treated with scr control siRNA (Control Cell Death) and cystinosin siRNA. Values are averages ± SEM of triplicate determinations. $^{*}p < 0.05$ relative to untreated; ‡ $p < 0.05$ relative to scr control cells treated with cisplatin.

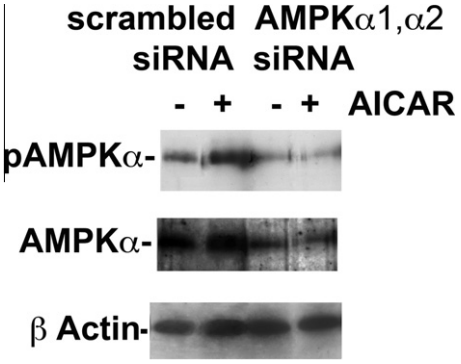

Fig. 3. Effect of AMPKα siRNA on activation of AMPK. Primary RPT cells were transfected with either AMPKα1 and AMPKα2 siRNA, or with AMPKα1 scr siRNA and AMPKα2 scr siRNA. Following the incubation of siRNA treated cultures ±1 mM AICAR for 1 h, the level of pAMPKα, AMPKα and β actin was determined by Western analysis.

using a combination of AMPKα1 and α2 siRNA. These results support the validity of the results in Fig 4A, and in addition, support the hypothesis that activation of AMPK is necessary for the increased sensitivity of RPT cells with a cystinosin knockdown to cisplatin.

4. Discussion

Previously, we observed that the level of cystinosin was reduced by 80% in primary rabbit RPT cells treated with cystinosin siRNA [5]. The reduction in the level of cystinosin was associated with a 70% reduction in Na^+ dependent Pi uptake and a 30% reduction in Na^+ dependent sugar uptake. Possibly, the reduced solute uptake could be explained by a decrease in the Na^+ gradient across the plasma membrane. However, intracellular Na^+ was not affected, and Na,K-ATPase activity was not reduced. Nevertheless, the cystinosin knockdown was associated with a 50% decrease in intracellular ATP, and a 1.7-fold increase in the intracellular ADP/ATP ratio. Consequences of increased intracellular ADP and AMP include the activation of AMPK [6,11]. Consistent with the hypothe-

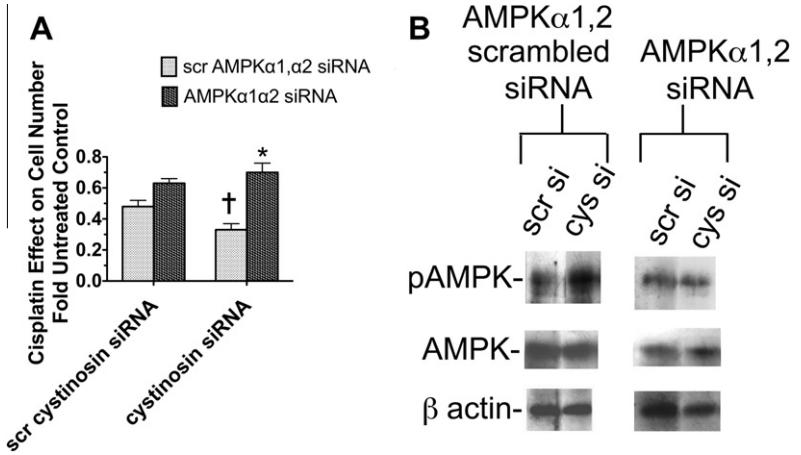

Fig. 4. Effect of AMPK siRNA on cisplatin toxicity. Primary RPT cells were transfected first with either AMPKα1 and AMPKα2 siRNA, or with AMPKα1 scr siRNA and AMPKα2 scr siRNA. Subsequently, the cultures were transfected with either cystinosin siRNA or scr control cystinosin siRNA. After 3 days the ability of the siRNAs to alter A. The effect of cisplatin, and B. The activation of AMPK was determined. In part A, the effect of a 24 h incubation with 20 μM cisplatin on the number of cells was determined in triplicate, relative to untreated controls. $^{*}p < 0.05$ relative to cystinosin siRNA cultures transfected with scr AMPKα1,α2 siRNA; $^{\dagger}p < 0.05$ relative to cystinosin scr siRNA cultures transfected with scr AMPKα1,α2 siRNA. In part B the level of pAMPK and AMPK was determined.

sis that AMPK is activated in primary RPT cells with a cystinosin knockdown, the level of the pAMPKα subunit was increased in RPT cells with a cystinosin knockdown to the level observed in control RPT cells treated with AICAR. The reduction in intracellular ATP is very likely responsible. However, AMPK activation may also occur via other mechanisms, including AMPK phosphorylation by Calmodulin-dependent protein kinase kinase (CaMKK) [6].

When AMPK is activated as a consequence of ATP depletion, a number of catabolic metabolic pathways are turned on, so as to generate ATP, while anabolic pathways that consume ATP are turned off [6].

Catabolic pathways that are activated include glycolysis and fatty acid oxidation, while anabolic pathways that are inhibited include lipogenesis and glycogen synthesis. Renal Na,K-ATPase is activated by AMPK [15], which can explain why Na,K-ATPase activity was maintained in primary RPT cells with a cystinosin knockdown, despite the 50% reduction in intracellular ATP. Activated AMPK may act either directly to phosphorylate target proteins, or indirectly on other signaling pathways. Included amongst the targets of AMPK is the mammalian target of rapamycin (mTOR), which is inhibited as a consequence of phosphorylation by AMPK [16]. Consequences include reduced protein synthesis and cell growth [16]. Other consequences of AMPK activation include induction of apoptosis and/or autophagy [16,17].

Previously, Park et al. [18] observed that apoptosis increased in cultured human cystinotic fibroblasts and RPT cells following treatment with either Tumor Necrosis Factor α (TNFα), or anti-Fas antibody. Park et al. [19] also observed that protein kinase C (PKC) δ was activated during the apoptosis of the cystinotic cells. Lysosomes are permeablized in early stages of apoptosis, releasing their contents (including lysosomal cystine). Presumably, the cystine that is released is responsible for the cysteinylation, and activation of PKCδ, which contributes to the increased apoptosis in cystinotic cells ([19]. Indeed, Ryer et al. [20] presented evidence that PKCδ does induce apoptosis in vascular smooth muscles through a process that involves p53, a tumor suppressor. Park et al. [19] proposed that increased apoptosis similarly occurs in cystinotic kidneys in vivo, resulting in a decreased in number of proximal tubules, contributing to the Fanconi syndrome.

The results presented here indicate that activation of AMPK contributes to the increased apoptosis in cystinotic RPT cells. Apoptosis occurred at a higher rate in primary rabbit RPT cells with a cystinosin knockdown following cisplatin treatment, relative to scr control cultures. The increased apoptosis in RPT cell cultures with a cystinosin knockdown was prevented by an AMPKα knockdown, as well as by the AMPK inhibitor Compound C. These observations are consistent with the results of several other investigators who studied cisplatin-treated animal cells, including Jin et al. [14], who observed that AMPK activation increased cisplatin-induced apoptosis in non-small cell lung cancer (NSCLC) cells. In addition, Yasmeen et al. [13] observed that activation of AMPK by metformin increased cisplatin-induced apoptosis in the ovarian epithelial cancer cell lines OVCAR-3 and OVCAR-4.

However, AMPK activation may have other consequences including autophagy, depending upon cell type and the cellular milieu [18]. Thus, the response of cisplatin-treated cells very likely depends upon differences in AMPK-mediated signaling through a number of pathways, including the mammalian Target of Rapamycin (mTOR). Activated mTOR phosphorylates a number of substrates, including S6 kinase and 4E-BP1, which results in an increase in protein synthesis and growth. However, when AMPK is activated, AMPK phosphorylates and inhibits mTOR, inhibiting mTOR mediated increases in protein synthesis and growth. Instead other cellular events may occur, including apoptosis (presumably via the dephosphorylation of S6 K and its pro-apoptotic substrate BAD) [18], or, in other cases, autophagy [18].

In cases where autophagy is induced, ULK1, an initiator of autophagy [21] is activated after AMPK forms a complex with ULK1 and mTOR (21). Normally, mTOR inhibits ULK1 activation. However, under appropriate conditions AMPK can lift the inhibitory effect of mTOR on ULK1 by phosphorylating raptor (another protein which complexes with mTOR). Thus, in cisplatin-treated U251 human glioma cells, AMPK activation induces autophagy, rather than apoptosis [22]. The cyclin inhibitor $p27^{kip1}$ is also phosphorylated by activated AMPK, and may contribute to the induction autophagy [8], as well as cell cycle arrest. In contrast, the induction and phosphorylation of the tumor suppressor p53 by AMPK induces apoptosis [9]. Further studies are in progress to determine whether p53 or other mediators such as $p27^{kip1}$ are activated by AMPK in primary RPT cells with a cystinosin knockdown, and whether they contribute to the increased sensitivity of cystinotic RPT cells to toxicants.

Acknowledgments

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