Arch Toxicol (2008) 82:607–614
DOI 10.1007/s00204-007-0275-5

ORGAN TOXICITY AND MECHANISMS

Mechanisms of the ifosfamide-induced inhibition of endocytosis in the rat proximal kidney tubule

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Received: 19 September 2007 / Accepted: 13 December 2007 / Published online: 23 January 2008

Abstract The Fanconi syndrome is a common side effect of the chemotherapeutic agent ifosfamide. Current evidences suggest that chloroacetaldehyde (CAA), one of the main metabolites of ifosfamide activation, contributes to its nephrotoxicity. However, the pathophysiology of CAA-induced Fanconi syndrome is not fully understood. The present work examined the adverse effects of CAA on precision-cut rat renal cortical slices, which allowed studying the toxic effect of CAA on proximal endocytosis. We demonstrated that clinically relevant concentrations of CAA ($\leq$200 μM) are able to inhibit the uptake of horseradish peroxidase, a marker of proximal tubular cell endocytosis in renal tubular proximal cells. CAA $\geq$75 μM has adverse effects, both on viability parameters and on energy metabolism, as shown by the great decrease in total glutathione and ATP levels. In addition, the V-ATPase, which plays a crucial role in intracellular vesicle trafficking, was inhibited by 100 μM of CAA. By contrast, the slight decrease in Na–K–ATPase activity observed for CAA$\geq$ 125 μM (maximum inhibition: 33%) could not totally explain the inhibition of the reabsorption processes. In conclusion, the addition of the two main adverse effects of CAA (decrease in ATP levels and inhibition of the V-ATPase) could explain the inhibition of endocytosis and the Fanconi syndrome observed during ifosfamide treatments.

Keywords Chloroacetaldehyde · Ifosfamide · V-ATPase · Nephrotoxicity · Proximal tubule

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Introduction

Ifosfamide (IFO) is an alkylating agent widely used in the treatment of various solid tumours in adult and pediatric patients (Carli et al. 2003). Although its early use was limited by severe urotoxic side effects (hemorrhagic cystitis), the introduction of the uroprotective thiol compound mesna (2-mercaptoethanesulfonate) has virtually eliminated urotoxicity. The introduction of mesna into IFO therapy has enabled higher and more frequent dosing of IFO but severe renal toxicity has been observed (Jurgens et al. 1989).

Interestingly, the antineoplastic medication cyclophosphamide (CP), a structural isomer of IFO, can cause hemorrhagic cystitis but is not nephrotoxic. Both IFO and CP are prodrugs that must be activated before they can cause both their therapeutic and toxic effects (Kaijser et al. 1994). The metabolism of the two drugs leads to the formation of alkylating mustard derivatives and acrolein, which probably causes cystitis. But unlike CP, a large part (about 50%) of IFO metabolism leads to the production not only of non-toxic 2- and 3-dechloroethyl IFO but also of chloroacetaldehyde (CAA), which is the metabolite responsible for renal toxicity.

Approximately 40% of IFO-treated children develop a permanent subclinical renal tubulopathy and 5% have a persistent Fanconi syndrome (Skinner et al. 1993,2000; Ho et al. 1995) characterized by a general proximal dysfunction

with the association of tubular proteinuria, amino-aciduria, hyperphosphaturia, loss of bicarbonates and glycosuria as main symptoms. Some authors (Caron et al. 1992; Foxall et al. 1997) showed that a constant acute toxicity characterized by a hyperaminoaciduria was observed during IFO treatment. Numerous in vitro studies have demonstrated that CAA induced alterations of cellular metabolism (Springate 1997; Benesic et al. 2006; Dubourg et al. 2001) and of transport processes (Mohrmann et al. 1992,1993, 1995; Springate and Taub 2007). However, the pathophysiology of CAA-induced Fanconi syndrome is not fully understood.

The aim of our study was to better understand the mechanism, which leads to the tubular dysfunction. Therefore, we studied the adverse effects of CAA on a long-term incubated precision-cut renal cortical slice model, which allowed reproducing the CAA-induced inhibition of proximal endocytosis.

Methods

Preparation and incubation of precision-cut renal cortical slices

Male Wistar rats were fed a standard rat chow ad libitum and water was provided continuously. All the studies were conducted according to a protocol approved by the local committee of the University Claude Bernard. Rats were anesthetized with sodium pentobarbital (35 mg/kg i.p.) and the kidneys were removed and placed in ice-cold Krebs–Henseleit medium. The kidneys were decapsulated and cylindrical cores of 5 mM diameter were prepared from cortical sections with a tissue-coring tool (Alabama R&D, Alabama, USA). The cores were maintained in oxygenated (95% O_2–5% CO_2) ice-cold Krebs–Henseleit buffer and transferred to a Krumdieck tissue slicer (Alabama R&D, Alabama, USA) filled with oxygenated ice-cold Krebs–Henseleit buffer. Preliminary experiments were used to determine settings that produced slices of approximately 250-µm thickness (Vittorelli et al. 2005). These slices were kept in ice-cold oxygenated Krebs–Henseleit buffer until incubation. The slices were floated on to Teflon®/Vitron rollers which were carefully blotted and loaded into scintillation vials containing 2 ml of L-glutamine—L-cystine- and D-glucose-free RPMI 1640 medium (EUROBIO, CXXRPM04-01) supplemented with 1 mM L-lactate and with various CAA concentrations. Vials were closed with a cap that had a central hole, and placed horizontally on a vial rotator in a humidified incubator set at 37°C and gassed with 40% O_2 and 5% CO_2. Then, the slices were incubated during a 6-h-incubation period.

Horseradish peroxidase uptake study

Horseradish peroxidase (HRP) is commonly used as a marker of kidney proximal cell endocytosis (Piwon et al. 2000; Christensen et al. 2003; Devuyst et al. 2005). Therefore, precision-cut rat kidney slices were incubated with various CAA concentrations during a 6-hour-incubation period in order to evaluate the adverse effects of CAA on tubular proximal endocytosis. HRP (final concentration 0.5 mg/ml) was added to the medium 60 min before the end of incubation. To prevent contamination by peroxidase activity contained in the extracellular spaces, the slices were then treated with collagenase as follows: six slices were incubated for 45 min in 4 ml of Krebs–Henseleit medium containing collagenase (2 mg/ml) in a 25-ml stoppered Erlenmeyer flask in an atmosphere of 95% O_2–5% CO_2 at 37°C in a shaking water bath. To remove collagenase and extracellular peroxidase, the suspension was rinsed three times with ice-cold Krebs–Henseleit medium and centrifuged at 1,000*g*. for 1 min. The final pellets were taken in 200 µl of Krebs–Henseleit and contained intact tubular proximal cells. Total protein content was determined in each sample according to the method of Lowry et al. (1951). Peroxidase activity of the samples diluted in a phosphate buffer (40 mM, pH 6.8) containing 0.5% Triton X100 and 0.25% bovine serum albumin was measured as described by Pütter and Becker (1983). For this, peroxidase activity was spectrophotometrically determined at 405 nm by the formation rate of the oxidized ABTS (2,2′-Azino-bis (3-Ethylbenzothiazoline-6-sulfonic acid)) in the presence of H_2O_2. Endogenous peroxidase activity (blank) was measured in incubated slices without HRP and was removed from the total peroxidase activity.

Metabolite assays

For the viability parameters, i.e. cellular ATP and total glutathione levels, two incubated slices were placed in 250 µl of cold 7% $HClO_4$ (v/v). After homogenization and centrifugation for 5 min at 3,000*g*, the supernatant was neutralized with 20% KOH (w/v) plus 1% H_3PO_4 (v/v) permitting ATP and glutathione level measurements. Pellets were solubilised in 100 mM NaOH for protein determination.

ATP concentration was quantified by using the method of Lamprecht and Trautschold (1974). The glutathione levels (the sum of the reduced and oxidized forms) were assessed as described by Griffith (1985). Total protein was determined in all flasks according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Measurement of V-ATPase activity

For ATPase activity measurement, four incubated slices were placed in 500 µl of cold homogenization's buffer

(Katz and Epstein 1967) containing saccharose (250 mM), EDTA (5 mM) and histidine (30 mM) to which 10 µl of 5% deoxycholate were added. Then, slices were ground using an Ultraturax homogenizer at 9,500 rpm. The activity measurement, adapted from Sheikh and Moller (1987), is based on ATPase activity measurement in the absence and in the presence of specific inhibitors. In order to minimize the interference of F_0-F_1-ATPase mitochondrial activity, mitochondria were eliminated by successive centrifugations (600*g*. for 10 min followed by 9,000*g*. 10 min); the resulting supernatant contained mainly the plasma membranes and the cellular content. Despite the elimination of most of the mitochondria, oligomycin was added in order to eliminate any F_0-F_1-ATPase residual activity. The incubation mixture, with a total volume of 0.3 ml, contained 30 mM histidine, 125 mM NaCl, 2 mM KCl, 6 mM $MgCl_2$. ATPase activity was measured by the assay of phosphate (Pi) liberation after addition of ATP (final concentration 3.3 mM) to the medium throughout a precise period of time at 37°C (5, 10 and 15 min for samples without inhibitors, with ouabain and oligomycin and with ouabain, oligomycin and concanamycin, respectively). In the preliminary experiments, we found that the ATPase reaction was linear for up to 15 min of incubation. The liberated Pi was measured by spectrophotometry at 850 nm after formation of a reduced phosphomolybdate complex. Total protein determination was measured in each sample after the homogenisation procedure according to the method of Lowry et al. (1951). A blank of each sample was measured by adding ATP after the ascorbate-molybdate solution preventing any ATP hydrolysis by ATPases. Total ATPase activity was measured without any inhibitor. The Na–K–ATPase and the residual F0-F1 ATPase activity were measured as the difference in ATPase activity in the absence and presence of 0.8 mM ouabain and 5 µg/ml oligomycin. To improve specificity, the activity measurement in the presence of ouabain was realised in a medium without potassium. The concanamycin-sensitive ATPase, i.e. the V-ATPase activity, was measured as the difference of ATPase activities (in the presence of 0.8 mM ouabain and 5 µg/ml oligomycin) with and without 100 nM concanamycin.

Reagents

Enzymes, coenzymes, and L-lactate were supplied by Roche (Meylan, France). CAA, concanamycin, oligomycin, ouabain and ABTS were from Sigma Chemical Co. (St. Louis, MO). The other chemicals used were of analytical grade.

Statistical analysis

The data presented in the Figures or in the Table represented four or six independent experiments. ATP and glutathione levels were expressed as micromoles per gram protein. ATPase activities were expressed in nmol Pi formed/min/mg protein, whereas peroxidase activity was expressed in mOD/min/g protein. The data are means ± SEM. The results were analysed by ANOVA for repeated measurements, followed by the Scheffé's test for comparison of the values obtained in the presence and the absence of CAA. Probabilities of less than 0.05 were considered to be significant.

Results

Effects of CAA on viability parameters of incubated cortical slices

In order to check their metabolic viability, rat renal cortical slices were incubated for 6 h with various CAA concentrations (0 to 150 µM). As shown in Fig. 1, the incubation of these slices with various concentrations of CAA led to a significant dose-dependent decrease in total glutathione levels and this effect was observed with the lowest concentration (25 µM) of CAA used. The cellular ATP level was also diminished in a dose-dependent manner but this diminution was observed only in the presence of 75, 100, 125 and 150 µM CAA (Fig. 2). Note that, with the 75-µM and 100-µM CAA concentrations, the cellular ATP level fell by 52 and 67%, respectively.

Effects of CAA on endocytic uptake of HRP in incubated cortical slices

Since the use of mesna, nephrotoxicity and especially the Fanconi syndrome, i.e. generalized proximal tubular dysfunction,

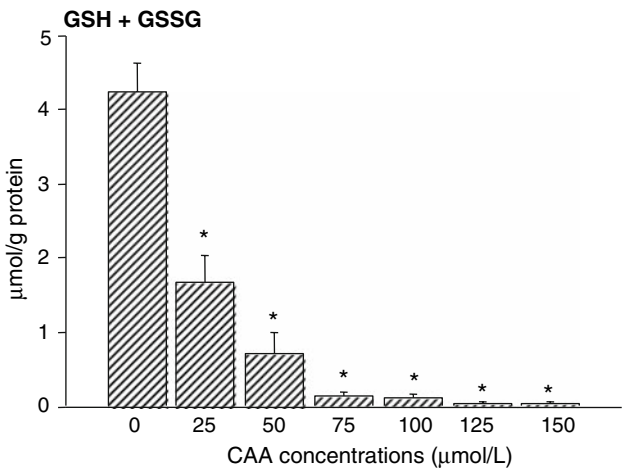

Fig. 1 Effects of various concentrations of chloroacetaldehyde (CAA) on total glutathione levels (the sum of the reduced and oxidized forms) of rat kidney-cortex slices incubated for 6 h. *$p < 0.05$ versus control (i.e., 0 mM chloroacetaldehyde). Bars represent means ± SEM for six experiments. *GSH*-reduced glutathione; *GSSG*-oxidized glutathione

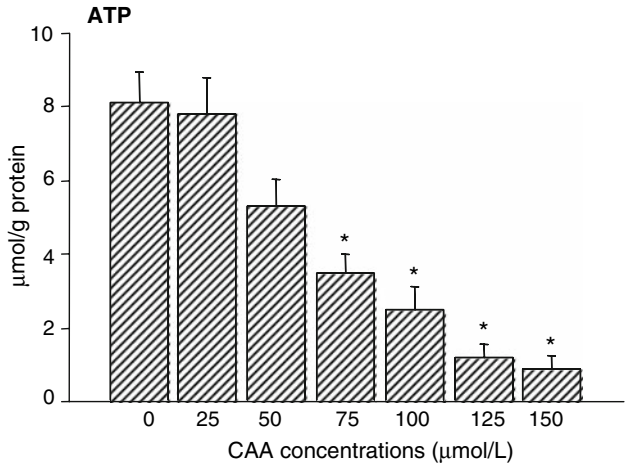

Fig. 2 Effects of various concentrations of chloroacetaldehyde (CAA) on ATP levels of rat kidney-cortex slices incubated for six hours. *$p < 0.05$ versus control (i.e., 0 mM chloroacetaldehyde). Bars represent means ± SEM for six experiments

is the main side effect of IFO treatment in humans or in experimental animals. Therefore, we examined the endocytosis capacity of proximal tubular cells by measuring HRP uptake by precision-cut cortical slices incubated for 6 h with various clinically relevant CAA concentrations (0–150 µM). As shown in Fig. 3, peroxidase uptake was greatly depressed in a statistically significant manner by the addition of 50, 75, 100, 125 and 150 µM CAA to the incubation medium.

Effects of CAA on the activity of ATPases

Total ATPase, Na–K–ATPase, and V-ATPase activities were measured in renal cortical slices incubated for 6 h without and with various CAA concentrations. As shown in Table 1, total ATPase activity of rat cortical slices fell by 27 and 33% in the presence of 125 and 150 µM CAA, respectively. This decrease was mostly explained by the fall of Na–K–ATPase activity which was diminished by 26 and 33% when renal cortical slices were incubated for 6 h with 125 and 150 µM CAA, respectively. Note that the blank did not change when CAA concentration was increased (Table 1).

The activity of V-ATPase was rather low compared with that of the other ATPases; indeed, it represented about 10% of the total ATPase activity. In contrast with what has been observed with total ATPase activity, CAA concentration ≥100 µM caused a great decrease in V-ATPase activity (Fig. 4): mean V-ATPase activity fell by 47, 59 and 67% in the presence of 100, 125, 150 µM CAA, respectively.

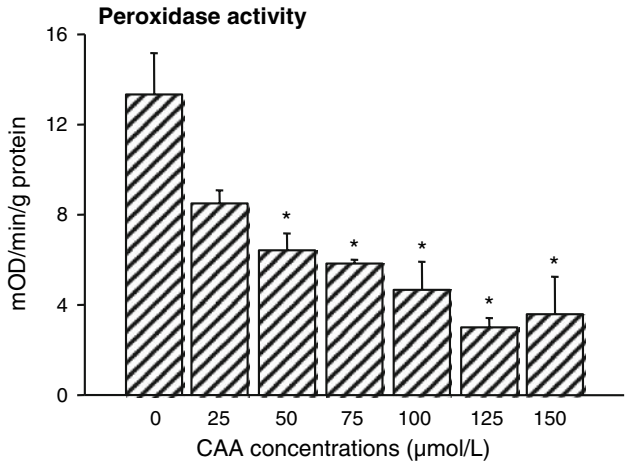

Fig. 3 Effects of various concentrations of chloroacetaldehyde (CAA) on horseradish peroxidase (HRP) uptake by rat kidney-cortex slices incubated for 6 h. Bars represent the peroxidase activity resulting from HRP endocytosis by the proximal tubular cells. *$p < 0.05$ versus control (i.e., 0 mM chloroacetaldehyde). Bars represent means ± SEM for four experiments

Discussion

Inhibition of protein endocytosis by CAA

Since approximately 40% of IFO-treated children develop a permanent subclinical renal tubulopathy and 5% have a persistent De-Toni-Debre-Fanconi syndrome (Skinner et al. 1993,2000; Ho et al. 1995), it is of the utmost importance to understand the pathophysiology of the renal proximal dysfunction leading to impaired reabsorption of low molecular weight proteins, amino-acids, glucose, bicarbonate and salts that can result in growth failure, rickets and progressive renal failure.

In the present study, we focused our attention on the defect of protein reabsorption, which is constantly observed in IFO-induced renal proximal dysfunction. For this, we studied in vitro the effects of CAA, a nephrotoxic metabolite of IFO, on the endocytosis of HRP, which can be easily detected thanks to its enzymatic activity. As shown in several studies (Brown 1989), renal proximal endocytosis is a very important process; indeed, it is not only responsible for reabsorption of filtered proteins but also is a part of the major recycling mechanism that is essential for trafficking of membrane proteins between the brush border and intracellular membranes. Any inhibition of this vesicle recycling process could impair protein uptake and could selectively diminish the abundance of various transporters in the brush membrane explaining the various reabsorption defects of the Fanconi syndrome.

Our results clearly demonstrate for the first time that clinically relevant concentrations of CAA inhibit the uptake of HRP, a marker of endocytosis in renal tubular cells (Piwon et al. 2000; Christensen et al. 2003; Devuyst et al. 2005), by rat renal cortical slices.

Table 1 Effect of various concentration of chloroacetaldehyde (CAA) on total ATPase and Na–K–ATPase activities of rat kidney-cortex slices incubated for 6 h

CAA concentration (µmol/l)	Total ATPase	Na–K ATPase	Blank
0	795.7 ± 43.4	562.9 ± 53.1	69.5 ± 2.0
25	774.4 ± 34.1	554.9 ± 35.0	74.0 ± 1.7
50	754.5 ± 49.8	536.6 ± 48.7	74.3 ± 2.3
75	736.4 ± 49.4	528.8 ± 52.9	77.3 ± 2.0
100	639.9 ± 47.2	449.8 ± 38.0	78.3 ± 2.2
125	577.6 ± 38.4*	410.0 ± 37.2*	79.0 ± 3.8
150	537.4 ± 55.5*	380.9 ± 50.1*	81.2 ± 2.4

ATPase activities are expressed in nmol of phosphate (Pi) formed / min/mg protein. Blank represents the endogenous Pi content of the samples. Results are reported as means ± SEM for six experiments. *$p < 0.05$ versus control (i.e., 0 mM chloroacetaldehyde)

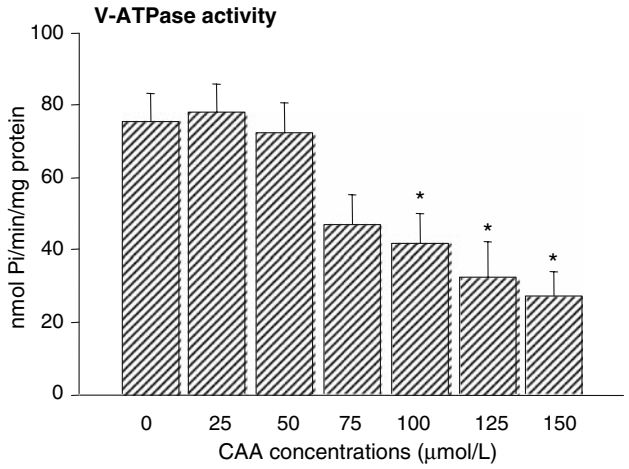

Fig. 4 Effects of various concentrations of chloroacetaldehyde (CAA) on V-ATPase activity of rat kidney-cortex slices incubated for 6 h. V-ATPase activity is expressed in nmol of phosphate (Pi) formed /min/mg protein. *$p < 0.05$ versus control (i.e., 0 mM chloroacetaldehyde). Bars represent means ± SEM for six experiments

Since the cellular endocytosis is an ATP-dependent process, which is inhibited by various metabolic inhibitors such as sodium azide and sodium fluoride (Clarke and Weigel 1985), we examined the effects of various concentrations of CAA on the level of ATP in renal cortical slices. Interestingly, except with the 50-µM CAA, there was a relatively good coincidence between the fall of the cellular ATP level and peroxidase endocytosis (see Figs. 2 and 3). Thus, our data clearly establish a link between the change in energy metabolism of renal tubular cells and protein reabsorption defects.

In a previous study conducted on a model of freshly isolated human kidney cortex tubules (Dubourg et al. 2001), we have shown that 0.5 mM CAA has adverse effects both on viability parameters and on energy metabolism of renal proximal cells. The observed fall of cellular ATP level caused by CAA could be attributed to the reduction of lactate oxidation secondary to the inhibition of flux through pyruvate dehydrogenase and through the subsequent reactions of the tricarboxylic acid cycle responsible for the release of lactate carbons as CO_2. Although we have not measured lactate removal, pyruvate accumulation and glucose synthesis in the present study, we assume that, in our rat renal cortical slices, CAA induced the same changes in lactate metabolism as those observed in isolated human proximal tubules. A similarity between the effects of CAA in the latter and those in rat renal cortical slices is strongly suggested by the similar fall of the cellular levels not only of ATP but also of total glutathione (see Figs. 2,3 for data obtained with slices, and Dubourg et al. 2001 for data obtained in tubules). With respect to the CAA-induced changes in energy metabolism, it should be underlined that Nissim et al. (2006) recently demonstrated that CAA inhibits complex I of the respiratory chain in isolated rat renal mitochondria; this effect may also explain the inhibition of lactate removal and oxidation leading to a fall of cellular ATP levels observed not only previously in human renal proximal tubules (Dubourg et al. 2001) but also in the present study in rat renal cortical slices.

It is important to emphasize that the effects observed in isolated human renal proximal tubules, which were incubated with CAA only for 1 hour (Dubourg et al. 2001), occurred at concentrations (at least 0.5 mM) of CAA that were higher than the circulating concentrations observed in patients during IFO treatment. Indeed plasma CAA levels of 10–35 µM (Pendyala et al. 2000) to 200 µM (Cerny and Küpfer 1989) have been measured among patients receiving IFO infusion. Aleksa et al. (2004) have also demonstrated that CAA can be produced in tubule cells with an estimated intra-cellular CAA concentration ranging from 35 to 320 µM. That such concentrations of CAA were toxic was strongly suggested by the fall of plasma glutathione in 60% of the patients treated (Pendyala et al. 2000). This is why, in the present study, the long-term toxicity of low concentrations of CAA was studied in precision-cut cortical slices incubated for 6 h.

Inhibition of V-ATPase by CAA

As documented by numerous recent studies, the disturbance of endosomal acidification in proximal tubule epithelial cells leads to a generalized dysfunction of the proximal tubule and to a Fanconi syndrome. The chloride channel 5 of the CLC family (ClC-5) and the vacuolar H^+-ATPase (V-ATPase) play a crucial role in the intracellular vesicle trafficking by maintaining an acidic environnement within the lumen of endo- and exocytic organelles in various

mammalian cells (Wall and Maack 1985; Mellman et al. 1986). As indicated in numerous studies (Mellman et al. 1986; Palokangas et al. 1994; Clague et al. 1994; Manabe et al. 1993; Desbuquois et al. 1990; Gekle et al. 1995), the inhibition of endosomal acidification by V-ATPase inhibitors or by acidotropic agents leads to a significant reduction in endocytosis and intracellular processing of various proteins and viruses. A previous study conducted in our laboratory demonstrated that the renal uptake of ^{125}I-lysosyme was strongly depressed by chloroquine, an alkalinizing agent of endosome (Simonnet et al. 1994). Several types of Fanconi syndrome have been linked to a defect in the endocytic acidification processes either by inhibiting the V-ATPase such as in cadmium and cisplatin nephrotoxicity (Herak-Kramberger et al. 1998; Takano et al. 2002; Marshansky et al. 2002) or by a mutation in ClC-5 in the Dent's syndrome (Fisher et al. 1994,1995; Lloyd et al. 1996; Steinmeyer et al. 1995) or the ClC-5 knockout mice (Piwon et al. 2000; Wang et al. 2000; Jentsch et al. 2002). Based on the fact that IFO treatment induces Fanconi syndrome and that its main metabolite CAA is responsible for such a renal toxic effect, we tested the hypothesis that CAA may affect acidification of intracellular organelles by inhibiting the proximal V-ATPase. In the present study, we demonstrated that CAA inhibits the V-ATPase activity (Fig. 4).

In the mammalian proximal tubule, V-ATPase is located both on the brush border membrane (Turrini et al. 1989; Brown et al. 1988) where it participates in the H^+ secretion and on various intracellular vesicles leading to the vesicle acidification (Brown 1989; Mellman et al. 1986), with a permanent recycling process between the brush border membrane and endosomes. The V-ATPase is a multisubunit complex composed of two functional domains: the peripherical V1 domain responsible for ATP hydrolysis and the V0 domain responsible for proton translocation (Wagner et al. 2004). It must be noted that the V1 domain is characterized by two conserved cysteine residues (cyst 254 and Cyst 532) located at the catalytic site of one of its subunits (Forgac 1999). Feng and Forgac (1994) have demonstrated that the V-ATPase can be reversibly inhibited by disulfide bond formation between two conserved cysteine residues (Cyst 254 and Cyst 532) located at the catalytic site on the A subunit. In addition, V-ATPase is sensitive to a number of oxidizing agents including nitrate (Dschida and Bowman 1995) S-Nitrosoglutathione (Forgac 1999) and nitric oxide (Tojo et al. 1994). CAA is a very reactive compound that can bind very easily to thiol groups such as cysteine or glutathione molecules (Dubourg et al. 2001; Benesic et al. 2006). The fact that CAA can bind to cellular thiols was demonstrated by the dramatic decrease in glutathione levels of our renal cells. The binding of CAA to free cysteine of one the catalytic site of the V-ATPase could explain the observed inhibition of the V-ATPase.

Some authors (Mohrmann et al. 1992,1993,1995; Springate and Taub 2007) have suggested that CAA caused alterations of sodium-dependent transports in renal tubular cells, which could explain the observed Fanconi syndrome. In our study, whereas the V-ATPase activity was greatly decreased with 100 μM CAA, the Na–K–ATPase activity remained unchanged at this CAA concentration. Therefore, our data strongly suggest that the V-ATPase inhibition per se may be, at least partially, responsible for the diminution of protein reabsorption observed in the CAA-induced Fanconi syndrome. However, this does not rule out a role for the inhibition of Na–K–ATPase in the pathophysiology of other symptoms encountered in the Fanconi syndrome.

In conclusion, we demonstrated that clinically relevant concentrations of CAA are able to both decrease ATP levels of the proximal tubular cells and to inhibit the cellular V-ATPase. The addition of these two adverse effects of CAA could lead to the inhibition of endocytosis as shown by the inhibition of HRP endocytosis. The fact that HRP uptake was decreased by CAA concentrations (50 μM) that did not reduce in a statistically significant manner the cellular ATP levels and V-ATPase activity, is a strong argument in favour of the addition of the two factors in the pathophysiology of proximal tubular dysfunction observed during IFO treatment.

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