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Original Paper

The Nephrotoxic Ifosfamide-Metabolite Chloroacetaldehyde Interferes with Renal Extracellular Matrix Homeostasis

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Key Words
Renal fibrosis • Extracellular matrix • Chloroacetaldehyde • Ifosfamide • Nephrotoxicity

Abstract
Background/Aims: Chronic renal proximal tubule dysfunction after therapy with the antineoplastic agent ifosfamide (IFO) is often attributed to the metabolite chloroacetaldehyde (CAA). Chronic IFO-nephropathy is reported to result in tubulointerstitial fibrosis and inflammation. ***Methods:*** To elucidate possible effects of CAA on extracellular matrix homeostasis, we investigated the action of CAA on markers of extracellular matrix (ECM) homeostasis in human proximal tubule cells (RPTEC) by use of direct ELISA for extracellular collagens and gelatin zymography. ***Results:*** An increase in type III collagen and a decrease in type IV collagen abundance in the media of RPTEC could be observed after exposure to CAA in clinically relevant concentrations. CAA increased intracellular type III and decreased intracellular type IV collagen. MMP-2 activity was decreased but MMP-9 activity unchanged. The enhanced CAA-induced collagen III formation could be attenuated by the intracellular Ca^{2+}-chelator BAPTA-AM, the PKA-antagonist H-89 and by extracellular acidification. CAA-induced collagen III abundance was enhanced by db-cAMP and IBMX and by protein overload. ***Conclusions:*** CAA exerts profibrotic effects on RPTEC dependent on Ca^{2+} and cAMP/PKA-signaling. These effects are enhanced by additional protein burden and attenuated by acidification.

Introduction

Chloroacetaldehyde (CAA) is a metabolite of the cancer therapeutic ifosfamide (IFO) generated mainly in liver by cytochrome P450-dependent mechanisms [1]. IFO is included in therapy protocols for the treatment of malignant solid tumors, especially in pediatric

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oncology, with response rates up to 80 % [2]. Yet, there is a significant number of patients developing chronic renal dysfunction, sometimes even years after the end of treatment [3, 4]. The predominant target for IFO-induced renal disease is the proximal tubule [5, 6]. The severity of renal impairment ranges from mild renal tubular acidosis (in up to 40% of all patients) to full blown Fanconi-syndrome (in up to 5 % of all patients) [3, 7, 8]. In pediatric patients treated with IFO wasting of phosphate by damaged proximal tubules often requires phosphate substitution to prevent hypophosphatemic rickets [3]. Additionally, the toxic effects of IFO treatment can persist over a long time period in children [9]. Thus, renal toxicity of IFO is of concern in oncology patients and especially in children. Until now, there are few prognostic parameters to identify patients who will develop chronic nephrotoxicty, since acute and chronic damage are not well correlated [10]. Furthermore, the mechanisms by which IFO leads to proximal tubular damage are poorly understood. Among IFO and its metabolites, CAA is most probably responsible for chronic renal disease [11-14]. Cell culture studies could show impairment of several transport mechanisms by CAA in renal proximal tubule cells and a concentration-dependent necrotic rather than apoptotic cell death [15-19]. Recently we could provide evidence for cAMP-dependent alterations in Ca^{2+}-signaling by CAA in human proximal tubule cells in primary culture [20]. CAA acts as a sulfhydryl reagent and thereby impairs the proper function of proteins necessary for the balance of proliferation and apoptosis by inhibition of caspases. We could also show that CAA inhibits cathepsin B, a lysosomal cysteine protease of importance for digestion of endocytosed protein [21]. Depletion of non-protein-sulfhydryl groups such as reduced glutathione (GSH) impairs the capacity of the cells to scavenge free radicals, thereby increasing the susceptibility to oxidative stress. The latter mechanism should account for acute toxicity that is frequently observed during IFO-infusion. It may also be a mechanism of antitumor action of ifosfamide [22]. Consequently, providing alternative free sulfhydryl group-carrying molecules protects renal cells in culture (GSH, mesna or cysteine [19, 23]) and probably also in vivo (by N-acteylcysteine in combination with its antioxidative action [24]). Inhibition of cysteine proteases and disturbed Ca^{2+}-handling do not necessarily lead to acute renal cell death, but are capable to induce chronic functional modifications leading to proximal tubule cell dysfunction, finally resulting in organ damage.

There are several case reports of pediatric and adult patients who developed chronic IFO-nephrotoxicity presenting as tubulointerstitial nephritis and fibrosis [25-27]. The hallmarks of chronic tubulointerstitial nephritis are chronic inflammatory events and altered extracellular matrix composition, which reinforce each other [28]. This vicious circle leads to epithelial mesenchymal transdifferentiation of proximal tubule cells, resulting in renal fibrosis in combination with impaired organ function. The major players in tubulointerstitial fibrosis are the fibrillar collagens type I and type III, which are often found to be increased in fibrotic disease [29]. Increases in collagen type IV as a constituent of the basal membrane are considered to be responsible for glomerular basement thickening, as it is found in diabetic nephropathy [30].

The purpose of this study was to identify possible profibrotic effects in human renal cells provoked by CAA and the signaling mechanisms involved. To work with a cell culture model in proximity to the clinical situation, human renal proximal tubule cells in primary culture (RPTEC) were used.

Materials and Methods

Cell culture

Human renal proximal tubule epithelial cells in primary culture (RPTEC) were purchased from Clonetics® (Cambrex Bioproducts) via CellSystems®, 53562 St. Katharinen, Germany. Cells were cultivated in plastic culture dishes (growth area 75 cm^2) in Ham F-12/Dulbecco's modified Eagle's medium (DMEM), supplemented with 1.1 g/l $NaHCO_3$, 3.57 g/l 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 5 mg/l human apo-transferrin, 5 mg/l (bovine)insulin, 500 µg/l hydrocortisone, 10 µg/l mouse EGF, 5 µg/l

Na^+-selenite, 0.5% fetal calf serum (Biochrom, Berlin, Germany), 6.5 µg/l thyroxin and 500 µg/l epinephrine under standard cell culture conditions (37 °C, 5% CO_2). The medium was changed 3 times a week and the cells were subcultivated every 14 days. In order to work with well differentiated cells, RPTEC were used for experiments from passage 5 to 9 [20]. These cells express the mRNA of organic anion transporters OCT1 and OCT2 but not of OCT3 (SLC22A1, SLC22A2 and SLC22A3, resp.) as confirmed by PCR and qRT-PCR.

Determination of collagen secretion by direct ELISA

Collagen I, III and IV secretion was determined by enzyme-linked immunosorbent assay (ELISA). According to [31], media and collagen standards (Sigma) were incubated overnight at 4 °C in 96-well Nunc-Immuno Maxisorb plates (Nalge Nunc International, Naperville, IL, USA). Following washing and blocking (2 h in 2 % bovine serum albumin in PBS/Tween 0.05 %), wells were incubated with rabbit antibody against collagen I, III or IV (1:1000, Rockland, Gilbertsville, PA) for 1 h at room temperature. After three washes, wells were incubated with HRP-conjugated secondary antibody (1:20000; Rockland, Gilbertsville, PA) for 1 h at room temperature and thereafter, after washing, incubated with *o*-phenylenediamine solution (0.5 mM *o*-phenylenediamine, 150 mM Na_2HPO_4, 3.5 mM citric acid, 0.015% H_2O_2) and the reaction was stopped after 15 min with 1 N H_2SO_4. The absorbance at 490 nm was determined using a multiwell-multilabel reader (Viktor^2, Wallac, Turku, Finland). Collagen secretion was normalized to total cellular protein content determined by the BCA-Assay [32].

Determination of intracellular collagen by western blotting

Cells were lysed in ice-cold buffer (1 mM Na-orthovanadate, 1% Nonidet P-40, 0.1% SDS, 0.1% Triton X-100, protease inhibitor cocktail, in PBS), protein therein determined and equal protein amounts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Subsequently, membranes were blotted with either rabbit anti-collagen type III or IV antibody (1:1000, Rockland, Gilbertsville, PA via Biotrend, Cologne, Germany). The primary antibody was detected using horseradish-peroxidase-conjugated secondary IgG (1:20 000) visualized by ECL. Protein bands were quantified using SigmaGel software (Jandel Scientific).

Determination of gelatinase activity

Gelatinase activity in cell culture media was determined as described in [33]. Shortly, the media were incubated with reaction Buffer (0.05 M Tris-Base, 150 mM NaCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.2 mM NaN_3, pH 7.6) and 1 µg FITC-labeled-gelatin (DQ-gelatin). Increase in fluorescence over time was determined at 37 °C at 490/535 nm excitation/emission. 1-10 ng collagenase I (*Clostridium histolyticum*, Sigma) served as positive controls. The measured increase in fluorescence/time was normalized for protein content. Possible direct effects of CAA on collagenase I were also tested, since CAA is capable of direct modification of proteins [21]. We found no alterations in collagenase I activity after 4 h incubation of the enzyme with CAA up to 150 µM.

Determination of CA-074 sensitive gelatinase activity

Contribution of cathepsin B to total gelatinase activity was determined by use of the cathepsin B inhibitor CA-074 (10 µM; Sigma). As described in the previous paragraph, two aliquots from cell culture supernatants were incubated with gelatinase assay reaction buffer containing either CA-074 or DMSO. After addition of 1 µg DQ-gelatine the effect of CA-074 on fluorescence increase over time was assessed. Fluorescence counts were normalized for control/DMSO and protein content. CA-074 did not affect the collagenase I controls.

Gelatin zymography

Gelatin zymography of cell culture media was performed using PAGE in 10 % polyacrylamide gels containing 0.5 % gelatin. Media (50 µl) were incubated for 30 min at 37 °C in Laemmli Buffer devoid of mercaptoethanol and subsequently underwent electrophoresis. Afterwards gels were washed in 2.5 % Triton X-100 in H_2O for 1 h, followed by overnight incubation at 37 °C in enzyme buffer (50 mM TRIS; 10 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.1% Triton X-100, 3 mM NaN_3, pH 7.5). After Comassie staining and destaining of the gels, areas of gelatinase activity appeared as nonstained, light bands. Afterwards, gels were dried, scanned and analyzed by SigmaGel-software. Intensity values were normalized to total cellular protein content.

Determination of Cathepsin B activity

Cathepsin B activity was measured by means of the specific fluorescent substrate z-Arg-Arg-AMC (AMC: 7-amino-4-methyl-coumarin) [34]. After incubation for up to 3 h at 37°C, fluorescence of the cleavage product AMC was measured at 355 nm excitation and 460 nm emission. Activity was normalized to protein content.

PCR experiments

The mRNA of organic anion transporters SLC22A1, SLC22A2 and SLC22A3 (OCT1, OCT2 and OCT3) was detected by PCR and qRT-PCR using the following conditions and primers: 10 min 95°C followed by 45 cycles with 10 seconds 95°C, 10 seconds 60°C and 30 seconds 72°C, in case of qRT-PCR followed by melting point determination. The quality of the primers (5´- 3´) has been validated by sequencing the unique amplificate found in cells overexpressing the resp. mRNAs.

SLC22A1 (OCT1), forward: ACATTCGTCAGGAACCTCGG
reverse: CGCCGCAAAACATCTCTCTC
SLC22A2 (OCT2), forward: GCTACGAGGTGGACTGGAAC
reverse: CTCCGATATCTCCGCCCAAC
SLC22A3 (OCT3), forward: GTTTTTGCTGCTGTGCCTGAC
reverse: TCCAGCATCCACGCATTGAC

Statistics

All data are presented as mean values ± S.E.M. For statistical testing prophet 5.0 software was used. The Shapiro-Wilks-test was performed to test data for normality (H_0 was rejected when $p < 0.05$). When none of the tested samples failed the normality test, samples were compared by one-way ANOVA, otherwise by Kruskal-Wallis-test. Statistical significance was assumed when $p < 0.05$.

Results

Chloroacetaldehyde causes time- and dose-dependent alterations in extracellular matrix composition of renal cells

Following 24 h of serum starvation, human proximal tubule cells in primary culture were incubated for 24–48 h with CAA in concentrations from 15 up to 150 µM. According to literature, 15 µM resemble CAA levels detected in patient plasma during ifosfamide therapy [35]. Figures 1A and B show the effect of 15 µM and 150 µM CAA on extracellular matrix composition in RPTEC after 24 h and 48 h incubation. As shown in Figure 1A, 15 µM CAA induced a progredient increase in type III collagen (182.4 ± 13.8 % of control after 24 h and 266.4 ± 46.2 % of control after 48 h), whereas collagen I was not affected (123.1 ± 9.1 % of control after 24 h and 99.7 ± 6.5 % of control after 48 h). Type IV collagen was reduced during the incubation period (80.2 ± 4.8 % of control after 24 h and 82.3 ± 5.4 % of control after 48 h). When RPTEC were challenged with 150 µM CAA (Fig. 1B), a massive and progredient increase in secreted collagen III was observed (703.6 ± 108.2 % of control after 24 h and 2286.2 ± 834.1 % of control after 48 h). Collagen I secretion was elevated but failed to be statistically significant (314.9 ± 44.1 % of control after 24 h and 302.1 ± 107.8 % of control after 48 h). Also, collagen type IV secretion was elevated (135.0 ± 14.9 % of control after 24 h and 214.3 ± 82.6 % of control after 48 h). Yet, the effect of 150 µM CAA on type IV collagen failed to reach statistical significance. The parent compound ifosfamide (150 µM) and its metabolite acrolein (15 µM) failed to interfere significantly with RPTEC collagen secretion (not shown). These data show that CAA at clinical relevant concentrations enhances collagen III secretion of human renal proximal tubule cells in primary culture.

Western blot analysis of lysates from RPTEC showed slightly increased intracellular levels of collagen type III (Fig. 1C: 148.0 ± 27.2 % of control after 24 h and 256.3 ± 62.0 % of control after 48 h), whereas intracellular collagen IV was decreased after challenge with 15 µM CAA (29.2 ± 7.1 % of control after 24 h and 40.2 ± 16.1 % of control after 48 h). Thus, the results indicate that altered intracellular collagen metabolism is involved in the changes of secreted collagen pattern caused by CAA.

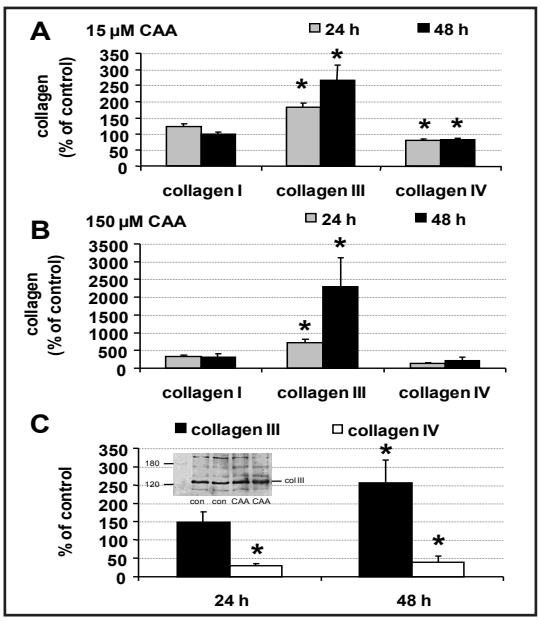

Fig. 1. Effects of CAA on collagen secretion and intracellular collagen III and collagen IV content of RPTEC. 15 µM (A) or 150 µM (B) CAA were applied and collagen content was determined in media after 24 h and 48 h of exposure (n = 16 - 48; * p < 0.05 vs. control). (C) Intracellular collagen content after exposure to 15 µM CAA. Insert shows a representative western blot against collagen III after 24 h 15 µM CAA (n = 4 – 7; * p < 0.05 vs. control).

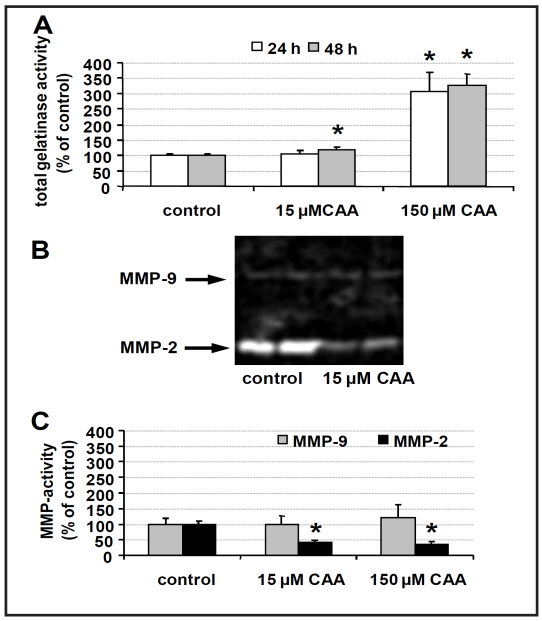

Fig. 2. (A) Total gelatinase activity in cell culture supernatants of RPTEC after 24 h and 48 h exposure to 15 or 150 µM CAA (n = 8 – 26; * p < 0.05 vs. control). (B) Gelatin zymography of RPTEC media. Typical result with a faint gelatinolytic band representing MMP-9 and a solid band representing MMP-2. (C) Quantification of MMP-2 and MMP-9 activity (n = 6 – 9; * p < 0.05 vs. control).

Chloroacetaldehyde alters activities of secreted collagenolytic and gelatinolytic enzymes

To test a possible interference of CAA with enzymes involved in matrix degradation, gelatinase activity was determined in media collected after CAA exposure. Furthermore, gelatin zymography was used to specify MMP´s possibly involved. Figure 2A shows that incubation with CAA caused an increase in total gelatinase activity 24 h after exposure of RPTEC to 150 µM CAA. After exposure to 15 µM CAA total gelatinase activity was slightly elevated after 48 h incubation (Fig. 2A; total gelatinase activity: 106.6 ± 7.9 % of control after 24 h and 118.8 ± 8.4 % of control after 48 h). 150 µM CAA led to an elevation of total gelatinase activity to 307.2 ± 60.0 % of control after 24 h and 327.1 ± 36.7 % of control after 48 h, respectively. In the additionally performed gelatin zymography experiments (Fig. 2B and C) MMP-2 and MMP-9 were found to be predominantly secreted by RPTECs, with much higher MMP-2 activity rates than MMP-9 activity. The faint gelatinolytic band representing weak MMP-9 activity was unaltered by CAA treatment. To our surprise, MMP-2 activity was significantly reduced after CAA exposure for 24 h or 48 h (15 µM CAA reduced relative MMP-2 activity to 41.1 ± 6.6 % of control after 24 h and to 33.8 ± 10.0 % of control after 48 h). In order to explain this discrepancy between the induction of total gelatinase activity and reduction of MMP-2 activity, we investigated the contribution of the lysosomal protease cathepsin B in CAA-treated RPTECs. Since CAA induces lysosomal protein overload and leakage of lysosomes [21], we further investigated the sensitivity of total gelatinase activity against the specific cathepsin B inhibitor CA-074. As shown in Figure 3A, CA-074 (10 µM) led to a significant reduction of total gelatinase activity in media of CAA-treated cells (15 µM CAA: 111.9 ± 14.6 vs. 77.5 ± 11.4 % of control; 150 µM CAA: 182.0 ± 19.8 vs. 101.4 ± 18.8 % of control), whereas no significant effect of CA-074 in media of control cells was observable (92.7 ± 6.4 % of control). To support this hypothesis, cathepsin B activity in cell culture

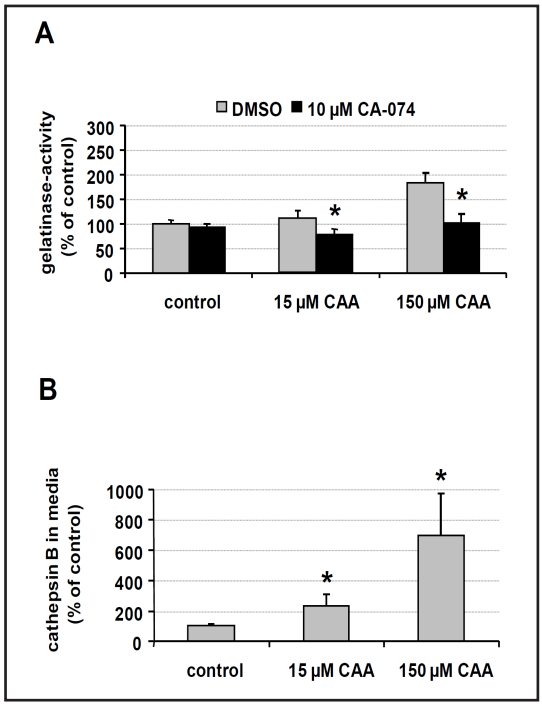

Fig. 3. (A) Total gelatinase activity after 48 hours in cell culture supernatants of RPTEC in presence and absence of the cathepsin B inhibitor CA-074 (n = 16 – 20; * p < 0.05 vs. DMSO; n.s. = not significant). (B) Cathepsin B activity in RPTEC media after 48 hours (n = 12 – 16; * p < 0.05 vs. control).

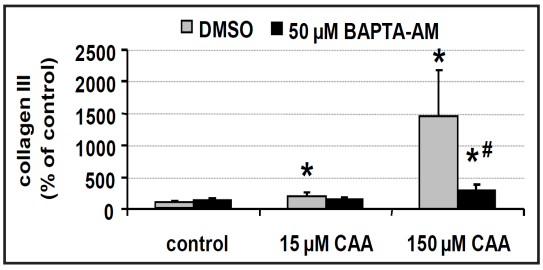

Fig. 4. Effect of calcium chelation by BAPTA-AM (50 µM) on CAA-induced collagen III secretion after 48 hours (n = 8; * p < 0.05 vs. control; # p < 0.05 vs. 150 µM CAA).

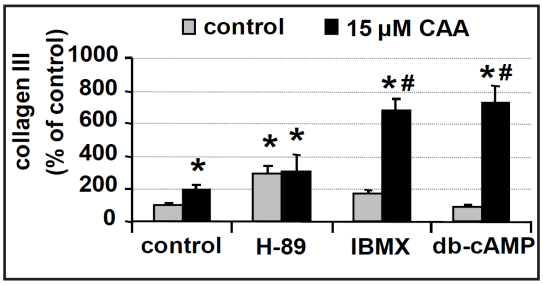

Fig. 5. Influence of PKA-cAMP-signaling pathway on CAA-induced collagen III secretion. Cells were incubated for 48 hours with or without 15 µM CAA and 1 µM H-89, 500 µM IBMX or 1 mM cAMP (n = 6 - 8; * p < 0.05 vs. control; # p < 0.05 vs. 15 µM CAA).

media was determined. As shown in Figure 3B, a significant increase in media cathepsin B activity induced by CAA occurred (15 µM: 230.9 ± 77.7 % of control; 150 µM: 702.1 ± 273.9 % of control). Under control conditions, media cathepsin B release was 0.44 ± 0.05 % of total cathepsin B activity. Thus, CAA inhibits MMP-2-activity, but due to concomitant cathepsin B leakage from the cells, total gelatinase activity is unchanged (15 µM CAA) or even enhanced (150 µM CAA). These data reveal a severe interaction of CAA with ECM turnover, since not only collagen secretion is disturbed by CAA, but also the balance of secreted matrix-remodelling enzyme activities is heavily disordered.

Contribution of signaling pathways in CAA induced matrix derangement

Calcium and PKC

To identify mechanisms by which CAA affects ECM homeostasis of RPTEC, pharmacological approaches were used. Collagen III secretion by RPTEC was used as surrogate marker, because it was the most sensitive parameter after CAA treatment. Since CAA induces sustained increases of intracellular Ca^{2+} in RPTECs [20], the effect of intracellular Ca^{2+} chelation with BAPTA-AM on CAA induced collagen III secretion was examined. Incubation of RPTEC with BAPTA-AM (50 µM) reduced CAA induced collagen III secretion after 48 h incubation (Fig. 4). The use of the PKC inhibitors BIM I (500 nM) and rottlerin (10 µM) had no effect (data not shown). Thus, the effect of CAA on collagen type III secretion seems to be dependent on intracellular Ca^{2+} but not on PKC activation.

Role of PKA and cAMP signaling pathways

Earlier studies from our laboratory could show a dependence of CAA-induced Ca^{2+}-elevations in RPTEC on cAMP/PKA-signaling [20]. Therefore, the impact of the membrane

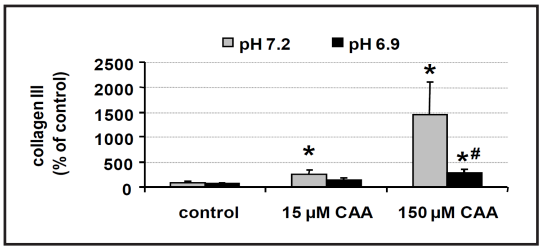

Fig. 6. Extracellular pH-dependent CAA-evoked collagen III secretion after 48 hours. (n = 6 - 14; * p < 0.05 vs. control; # p < 0.05 vs. 150 µM CAA).

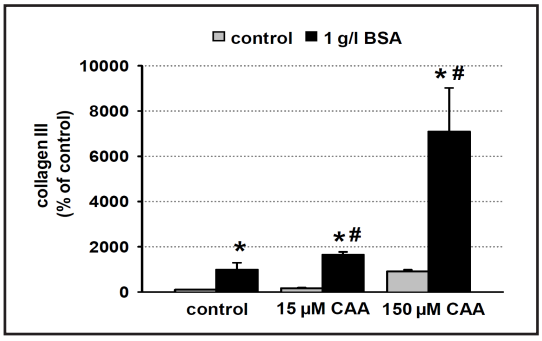

Fig. 7. Effect of protein load on CAA-induced collagen III formation. RPTEC were incubated with 15 or 150 µM CAA for 24 h and afterwards challenged with 1 g/l bovine serum albumin (BSA / BSA+CAA) for another 24 h (n = 8; * p < 0.05 vs. control; # p < 0.05 vs. CAA).

permeable cAMP-analogue dibutyryl-cAMP (db-cAMP; 1 mM), the phosphodiesterase-inhibitor IBMX (isobutylmethylxanthine 0.5 mM) and the PKA-inhibitor H-89 (1 µM) were tested on collagen III secretion by RPTEC. Figure 5 shows the effects of H-89, IBMX and db-cAMP on collagen III secretion in the presence or absence of 15 µM CAA. 48 h incubation with H-89 alone led to a significant increase in collagen type III production (295.7 ± 50.5 % of control), that was not further increased by CAA (306.8 ± 96.8 % of control). IBMX (168.9 ± 26.2 % of control) and db-cAMP (88.7 ± 12.2 % of control) did not significantly alter the amount of secreted collagen III, whereas both compounds potentiated the effect of CAA: IBMX + CAA increased type III collagen to 679.1 ± 66.0 % of control whereas CAA alone led to an increase to 194.5 ± 28.7 % of control. Coincubation of CAA and dibutyryl-cAMP elevated collagen III secretion to 730.8 ± 99.4 % of control. Thus, there is evidence for a contribution of the cAMP/PKA-signaling pathway in CAA-induced collagen type III secretion.

Extracellular pH and sulfhydryl-ractivity

Since acute CAA toxicity can be partly reduced by extracellular acidification due to reduced sulfhydryl reactivity of the compound in acidic environment [21], the effects of lowering extracellular pH on collagen III secretion provoked by CAA was investigated. Figure 6 shows the amount of extracellular collagen III in acidified media (pH_o 7.2 or 6.9) of RPTEC after 48 h CAA exposure. The extracellular acidification per se had no significant effect on collagen type III secretion. At pH_o 7.2, 15 µM CAA led to an increase of collagen III to 245 ± 100 % of control and to 1445 ± 666 % of control when 150 µM CAA were used. At pH_o 6.9, collagen III secretion was 150 ± 50 % of control in presence of 15 µM CAA and 278 ± 89 % of control after challenge with 150 µM CAA. These results indicate a reduced effect of CAA on collagen type III secretion in an acidic extracellular environment.

Protein challenge aggravates CAA-induced collagen III increase

The effects of CAA with regards to changes in lysosomal morphology and permeability could be enhanced by additional protein challenge [21]. Therefore, a possible contribution of an elevated protein load on collagen III secretion in response to CAA was investigated. After 24 h exposure to 15 or 150 µM CAA, RPTEC were challenged by 1 g/l bovine serum albumin for additional 24 h. The results are summarized in Figure 7. BSA alone led to an increase in type III collagen to 986 ± 325 % of control according to known effects of protein challenge on proximal tubule cells [36]. Addition of BSA to cells exposed to CAA induced an increase of collagen III already at 15 µM CAA and up to 7093 ± 2058 % of control (150 µM CAA). Thus, CAA-induced collagen III secretion can be potentiated by protein overload.

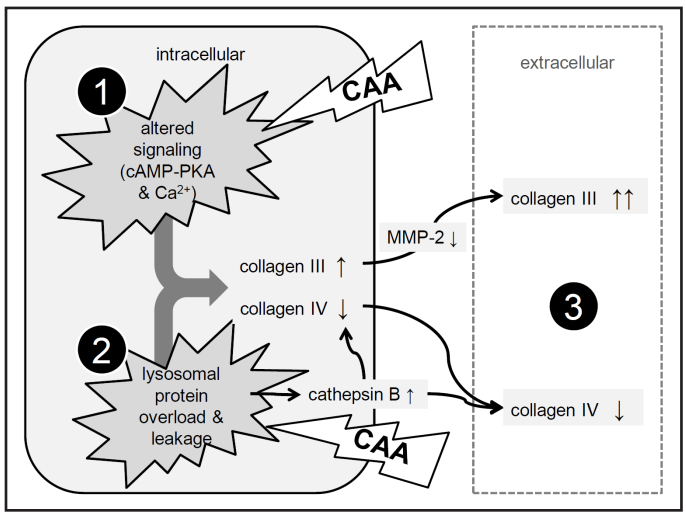

Fig. 8. Scheme of the putative profibrotic action mechanism of CAA. 1: CAA disturbs signaling via cAMP-PKA and intracellular Ca^{2+} leading to an increase of intracellular collagen III. 2. CAA induces lysosomal protein overload and leakage, further promoting collagen synthesis. Cathepsin B released from lysosomes cleaves intra- and extracellular collagen IV. 3. Reduced MMP-2 activity further aggravates extracellular collagen III accumulation. Extracellular cathepsin B exacerbates ECM disturbance by further collagen IV depletion.

Table 1. Summary of CAA effects on the parameters tested in this study

	effect
Collagen III (intracellular)	↑
Collagen III (extracellular)	↑
Collagen IV (intracellular)	↓
Collagen IV (extracellular)	↓
MMP2 activity	↓
MMP9 activity	±
Cathepsin B activity (intracellular)	↓
Cathepsin B activity (extracellular)	↑

Discussion

The data presented here show that the ifosfamide metabolite chloroacetaldehyde is a potent profibrotic agent in renal proximal tubule cells in primary culture. We tested also its parent compound, ifosfamide, which turned out to have no profibrotic action although we were able to demonstrate the mRNA expression of IFO-transporting organic cation transporters [37] SLC22A1 and SLC22A2 (OCT1 and 2) but not SLC22A3 (OCT3) and although the presence of certain IFO-metabolizing cytochrome P450 enzymes at least in tubular cells of the developing kidney has been shown [13]. Obviously, either ifosfamide was not taken up sufficiently or not metabolized to a sufficient amount to exert the effects which has CAA on extracellular matrix homeostasis described here.

The effect of CAA is predominantly characterized by a marked increase in type III collagen synthesis and a decrease in type IV collagen in cells of proximal tubule origin. These data point towards extracellular matrix disarrangement provoked by CAA, which on the one hand leads to deposition of fibrillar collagen type III, associated with fibrosis, inflammatory events and wound healing. The decrease in type IV collagen could reflect reduction of epithelial tightness by impaired composition of basal membranes, negatively influencing tubular transport mechanisms. These results are in good agreement with case reports from patients who developed chronic ifosfamide nephrotoxicity presenting as tubulointerstitial fibrosis [25-27].

To distinguish between enhanced synthesis of type III collagen and reduction in matrix degradation induced by CAA, western blot analysis of intracellular collagen was performed and gelatinase/matrix metalloproteinase activity in media of CAA treated cells was determined. The data show that CAA exposure led to an increase of intracellular collagen III as well as a reduction in intracellular collagen IV immunoreactivity, in accordance to extracellular matrix alteration evoked by CAA. Total gelatinolytic capacity, determined by gelatinase-assay, was

not reduced by CAA. Nevertheless MMP-2 activity, determined by gelatin zymography was reduced. These apparently conflicting results can be explained by increased cathepsin B activity in RPTEC supernatants, most probably due to release of cathepsins from ruptured lysosomes as well as other collagenolytic proteases that can access the extracellular space due to CAA-induced membrane disruption. The increased cathepsin B activity measured in RPTEC media after CAA exposure coincided with an increasing fraction of gelatinase sensitive to the specific cathepsin B-inhibitor CA-074. In a previous study, we could show lysosomal leakage after CAA exposure in coincidence with increased cell membrane permeability [21]. It is known, that amongst lysosomal proteases especially cathepsin B can be stable and active in the extracellular space under non-acidic conditions [38].

As shown previously [20], CAA is capable to interfere with Ca^{2+}- and cAMP-PKA-signaling. Thus, the possible contribution of these signaling pathways on CAA-induced collagen type III synthesis was investigated. Chelation of intracellular free Ca^{2+} by BAPTA-AM reduced collagen III accumulation in response to CAA. These results point towards an involvement of Ca^{2+} in the fibrotic action of CAA probably including calcium-dependent kinases or calcium-calmodulin complexes. Furthermore, chelation of Ca^{2+} by BAPTA-AM is able to reduce the CAA-invoked collagen III synthesis even when CAA is used in a concentration of 150 µM. Since Ca^{2+}-chelation prevents protein loss induced by 15 µM CAA, but not by 150 µM CAA [20], it can be speculated that Ca^{2+} is a signal transducer for chronic CAA effects, such as fibrosis, but not for the toxic effects of high CAA concentrations that are most probably a direct effect of thiol-group depletion [23]. Additionally, involvement of the cAMP-PKA-pathway in enhanced collagen III formation was investigated. Inhibition of PKA by H-89 abrogated the effect of CAA on collagen type III, whereas stimulation of adenylyl cylclase by db-cAMP and inhibition of phosphodiesterases by IBMX potentiated CAA-induced collagen III synthesis. These data implicate that the cAMP-PKA pathway is involved in the increased extracellular type III collagen secretion in response to CAA.

In order to investigate the possibility that extracellular acidification could be a prevention method for the profibrotic effects of CAA, the effect of CAA on collagen III was determined after lowering extracellular pH. As previously reported, the ability of CAA to react with sulfhydryl groups is reduced by acidification in vitro [21]. Reducing extracellular pH from 7.2 to 6.9 diminishes the profibrotic action of CAA. This is most probably due to impaired SH-reactivity by CAA. In addition, inhibition of cathepsin B activity by CAA is lessened when cells are exposed to CAA in acidic media. Taken together, these results point towards SH-reactivity of CAA as a cause for the fibrotic effects, especially inhibition of lysosomal protein degradation which leads to cellular protein overload.

Additionally, protein overload can contribute to chronic renal damage by CAA. When human proximal tubule cells are challenged with BSA after CAA exposure, the profibrotic effects of CAA are potentiated, indicating that inhibition of lysosomal cysteine proteases by CAA worsens the effect of protein challenge. Thus, there is evidence for disturbed protein handling by proximal tubular cells after CAA exposure. In proteinuria-induced proximal tubular damage, free radicals play a major role in progression of fibrosis. In order to test the contribution of free radicals to CAA-induced type III collagen synthesis, the radical scavenger tiron was used. In these experiments, no positive effect of tiron could be observed on collagen III accumulation as well on LDH-release (data not shown). These results are in accordance with data published on CAA-toxicity in osteosarcoma cells [22].

In conclusion, this study shows that the ifosfamide metabolite CAA is able to induce extracellular matrix alterations in human proximal tubule cells in primary culture. The predominant feature is an increase in collagen III, which is due to enhanced synthesis of type III collagen accompanied by a decrease in MMP-2 activity. The effect of CAA on collagen III depends on intracellular free Ca^{2+} and cAMP-PKA signaling. Protein challenge aggravates the fibrotic effect of CAA. Reducing sulfhydryl reactivity of CAA by acidification ameliorates CAA-induced fibrosis. Thus, there is evidence for chronic alterations in proximal tubule cell homeostasis by CAA that could translate into the clinically observed chronic and progredient course of ifosfamide nephropathy.

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Disclosure Statement

The authors have no conflict of interests.

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