biomedicines

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Simulating the physiological environment for kidney tubular crystallopathy *in vitro* utilizing uric acid.

Klein Chaja [1], Barnes Devon [1], Janssen Manoe [1]*

[1] Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, 3584 CG Utrecht, The Netherlands; c.n.klein@students.uu.nl (C.K.)

* Correspondence: m.j.janssen1@uu.nl

Citation: Klein, C.; Barnes, D.; Janssen, M. *Stimulating kidney tubular crystallopathy in vitro utilizing uric acid*. *Biomedicines* **2022**, *10*, x. https://doi.org/10.3390/xxxxx

Academic Editor: x

Received: 01-07-2022
Accepted: x
Published: x

Abstract: Aberrant crystallization of uric acid (UA) in the kidney can cause an inflammatory response that is associated with the identification and progression of chronic kidney disease and end-stage renal failure. It had previously been reported that UA could trigger inflammatory responses by activating some transcription factors, such as Nuclear Factor-κB (NF-κB), and induce the expression of proinflammatory cytokines of the interleukin-1 family, such as IL-1β and IL-18. Although the NLRP3-dependent inflammation by UA is studied in multiple cell types, simulation of the physiological environment for UA crystal formation in conditionally immortalized primary tubular epithelial cells (ciPTECs) and the down-stream inflammatory processes has not been well defined. Here, we evaluated the effect of UA on the activation of the NLRP3 inflammasome considering different pH levels and timepoints. Exposure of ciPTECS to UA in reduced pH levels resulted in more significant changes in cell viability across UA concentrations compared to physiological pH. Also, exposure to UA increased expression of IL-1β on protein level, particularly in lower pH levels. Furthermore, UA induced expression of Caspase-1, TNFα and ASC in a concentration-, and pH-dependent way. However, it was observed that expression of IL-1β and NLRP3 did not follow a concentration-dependent trend across some pH-levels. In this case, other mechanisms might be involved in UA induced IL-1β and NLRP3 mRNA expression. In conclusion, we showed that UA reduces viability in ciPTECs and inducing expression of inflammasome-mediated markers of caspase-1, ASC, TNFα on mRNA and IL-1β on protein level, particularly when UA concentrations were elevated and pH levels were lowered.

Keywords: crystallopathy; chronic kidney disease; renal inflammation; uric acid; pH; NLRP3 inflammasome; IL-1 β; conditionally immortalized proximal tubule cells

1. Introduction

Crystals of intrinsic or extrinsic origin may induce inflammation and tissue injury when deposited inside the body triggering diverse medical disorders termed as "crystallopathies" [1]. Although numerous examples exist in which physiological crystals do not trigger the immune system, aberrant crystallization of organic materials within the body, or exposure to external crystalline material, can cause an inflammatory response that is associated with pathogenesis in various diseases [2].

Hyperuricemia has been confirmed as an independent predictor for the development, progression and prognosis of chronic kidney disease (CKD) [3–6]. The incidence of elevated levels of uric acid (UA) increased markedly in the last decade [6]. It had previously been reported that UA could trigger inflammatory responses by activating some

transcription factors, such as Nuclear Factor-κB (NF-κB), and induce the expression of proinflammatory cytokines of the interleukin-1 family (IL-1β, IL-18). IL-1 cytokines are seen as the main drivers of crystal induced pathology [2]. Studies found that both soluble UA and UA crystals are able to trigger inflammation through activation of the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome, either in a crystal-dependent or independent manner [5–7].

The NLRP3 inflammasome is a multiprotein oligomer complex formed by the cytosolic proteins NLRP3, ASC and pro-caspase 1 [8]. The NACHT, LRR, and PYD domain of NLRP3 interacts with the adapter molecule, ASC, which in turn activates pro-caspase 1 to its active form Active caspase-1 subsequently cleaves pro-IL1β and pro-IL-18 mature pro-IL1β and pro-IL-18 [9].

The pathological mechanism of UA-induced renal-inflammation is complex. When UA reaches abnormally high concentrations, UA crystals form. These crystals can aggregate in the kidney or urinary tract leading to impaired kidney functioning by acting as a damage-associated molecular pattern (DAMP) that alerts the immune system to cell injury and helps trigger both innate and adaptive immune responses [2,7,10].

In human circulation, under physiological conditions UA is soluble inside cells as a weak acid. With a pKa of 5.8, 98% of UA released in the extracellular space at a physiological pH of 7.4 is in its ionized form as monosodium urate (MSU). However, when serum urate level exceeds 6.8 mg/dl, ionized MSU begins to form MSU crystals [3].

An acidic environment appears to facilitate UA crystallization and is an identifiable risk factor in the majority of UA crystal formers [11]. Whereas serum pH are maintained within a narrow range, urine pH can vary more widely. At a urinary pH of less than 5.5, urinary urate exists in the more hydrophobic and less soluble form, allowing crystals to precipitate [11]. Therefore, acidic urine pH is now recognized as a risk factor for CKD [4].

The pathway in which UA is able to trigger NLRP3 inflammasome activation has been studied in multiple cell types [1,3,6,7,10,12–15]. Yet, simulation of the physiological environment for UA crystal formation in conditionally immortalized primary tubular epithelial cells (ciPTECs) has not been explored. Our aim was to determine interchangeable effects of prolonged exposure to UA and medium pH on ciPTECs and evaluate the inflammasome activation and down-stream inflammatory process in ciPTECs. This project focuses on disease modelling and initially aims to investigate the pathophysiological mechanisms associated with UA-induced crystal formation. Also, since urinary pH plays a key role in the formation of UA crystal formation, it is of interest to explore whether it is possible to simulate an environment *in vitro* were UA crystals form with subsequent inflammation as a consequence. To our knowledge, there are limited studies assessing the effect of pH on UA crystal development *in vitro*.

2. Materials and Methods

2.1. Cell culture

Human conditionally immortalized renal PTECs were purchased from Cell4Pharma (Nijmegen, The Netherlands). The cells ranging from 40 to 50 passages were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1:1 DMEM/F-12) (Gibco, Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS) (Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 5 μg/mL insulin, 5 μg/mL transferrin, 5 μg/mL selenium, 35 ng/mL hydrocortisone, 10 ng/ml epidermal growth factor and 40 pg/mL tri-iodothyronine, without addition of antibiotics, as reported previously [16]. Cell culture plates were obtained from Greiner Bio-One (Monroe, NC, USA). ciPTEC 14.4s were kept in culture at 33 °C and 5% (v/v) CO_2. Prior to the experiments, cells were seeded at a density of 48,400 cells/cm^2, grown for one day at 33 °C, 5% (v/v) CO_2, and cultured for 7 days at 37 °C, 5% (v/v) CO_2 to allow maturation, refreshing the medium every other day. Prior to experiments medium was changed to FCS-free DMEM/F12.

2.2 Preparation of soluble UA in pH adjusted DMEM/F12 medium

UA was purchased from Sigma (St. Louis, MO, USA). Soluble UA was prepared by dissolving UA in 1 M NaOH at a concentration of 50 mg/ml, as previously described [17]. The 50 mg/ml UA solution was heated in a water bath for 6-8 hours at 80 °C until fully dissolved. In total, three different pH levels were prepared by adding 1 M HCl to FCS-free DMEM/12. A pH probe was used to adjust the pH of the culture mediums to either 6, 5.5 or 5.The stock concentration of 50 mg/ml was further diluted in FCS-free DMEM/F12 in order to prepare the concentration of 800 μg/ml, required for the study. After this, the pH was adjusted again to initial pH by adding 1 M HCl. Again, a pH probe was used to obtain the desired pH levels. All solution were filtered (22 μm pore size) before use to ensure sterility.

2.3 Cell treatments

CiPTEC 14.4s were seeded in 96-well plates at a density of 48,400 cells/cm2. After aspiration and washing with HBSS buffer (pH 7.4), the cells were divided in different pH level groups and exposed to soluble UA (0-800 μg/ml) for 24, 48 and 72 h, respectively.

2.4 Cell viability analysis

PrestoBlue™ Cell Viability Reagent was purchased from Invitrogen (Carlsbad, CA, USA) to measure cell viability. Following treatment as described above, the reagent was diluted 1:10 in FCS-free DMEM/F12 and added into each well after the supernatant was aspirated. The plate was further incubated for 1 h at 37 °C in the dark. Fluorescence was measured at an emission of 580nm- 640nm and excitation of 520nm using the GloMax Explorer Multimode Miroplate Reader (Promega, Leiden, The Netherlands).

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Cell culture supernatants were collected after 24, 48 and 72 h of treatment and stored at -20°C until analyzed. Protein levels of IL-1β were determined using Human Uncoated ELISA Kit Enzyme immunoassay kits for the detection of IL-1β according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

2.6 Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR)

RNA extraction, cDNA synthesis and qPCR were performed on multiple treated samples, and negative controls. For RNA extraction, total RNA from ciPTEC 14.4s treated as described above for 24h was collected using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. Thereafter, the samples were quantified using the NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Primers for qPCR were synthesized by Thermo Fisher Scientific (Waltham, MA, USA). The primer sequence is listed in Table 1. To synthesize cDNA, 600 ng of total mRNA per sample was mixed with iScriptTM Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and nuclease free water. After cDNA synthesis, the cDNA was diluted 10 times in MilliQ water. The qPCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) with the corresponding primers listed in Table 1. Gene expression were analyzed using the $2^{-\Delta\Delta Ct}$ formula and expression levels were reported as ratio relative to gene expression, using untreated cells as the reference sample. Ribosomal protein S13 was used as a housekeeping gene for normalization.

Table 1. qPCR primer sequences.

Gene	Forward sequence	Reverse sequence
NLRP3	GATCTTCGCTGCGATCAACA	GGGATTCGAAACACGTGCATTA
PYCARD (ASC)	CAGCAACACTCCGGTCAG	AGCTGGCTTTTCGTATATTGTG
CASP-1	GCCTGTTCCTGTGATGTGGAG	TGCCCACAGACATTCATACAGTTTC
IL-1B	TCGCCAGTGAAATGATGGCT	TGGAAGGAGCACTTCATCTGTT
TNFA	TGTTGTAGCAAACCCTCAAGC	TATCTCTCAGCTCCACGCCA
RPS13	GCTCTCCTTTCGTTGCCTGA	ACTTCAACCAAGTGGGGACG

2.7 Data Analysis

All data were expressed as mean ± standard error of the mean (SEM). Intergroup differences for continuous variables were assessed by two-way ANOVA; Dunnett's test was used for multiple comparisons. A p-value <0.05 was considered statistically significant. Software used for statistical analysis was GraphPad Prism (version 8.43; GraphPad software, La Jolla, CA, USA).

3. Results.

3.1. Viability of ciPTECs Decreases with Increasing Concentrations of UA and Reduced pH Levels

CiPTECs were treated with a variety of UA concentrations and pH conditions for either 24, 48 or 72h, ranging from 0-800 μg/ml and from physiological pH (7.4) to pH 5 respectively. Subsequently, cell-mediated cytotoxicity was measured. As demonstrated in Figure 1, at all timepoints cell viability decreased with an increasing concentration of UA. This decrease in viability seems to occur at lower UA concentrations in lower pH levels compared to higher pH levels. Also, the initial viability in untreated cells declined remarkably in pH 5, while there wasn't a distinct difference between the initial cell viability between pH 6 and 5.5.

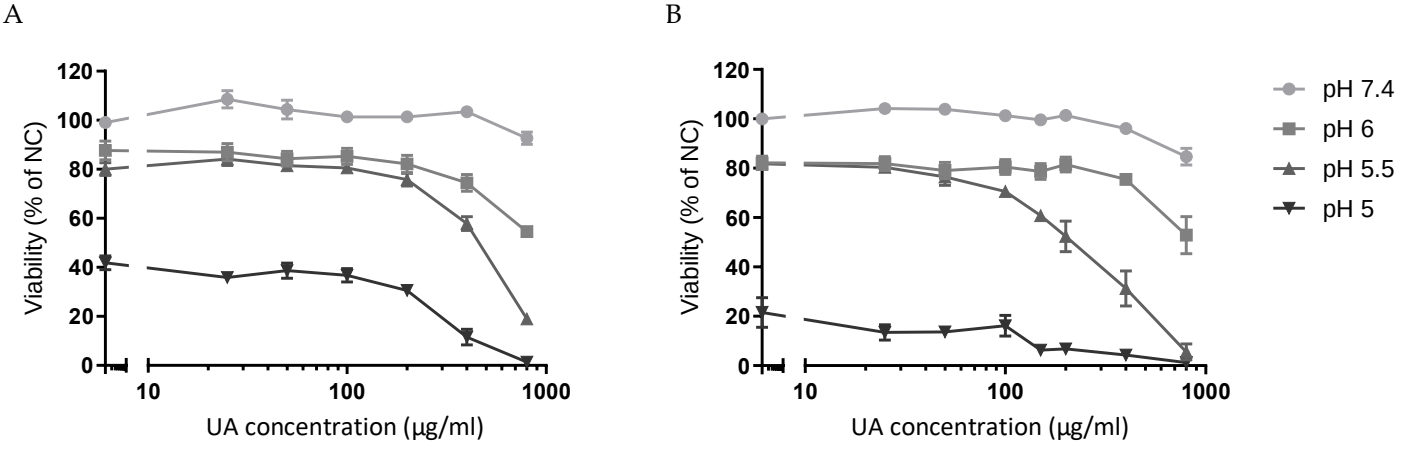

Figure 1. *Cont*.

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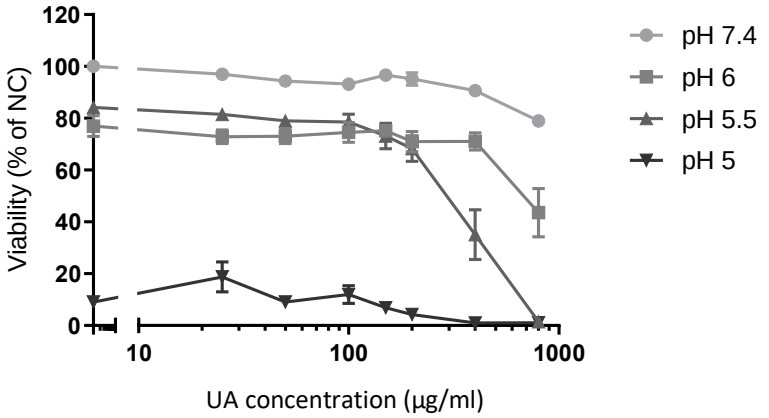


Figure 1. Viability of cultured immortalized human primary renal proximal tubule epithelial cells (ciPTECs) following incubation with dissolved uric acid (UA). ciPTECs were cultured with various concentrations of UA (0-800 µg/ml) across different pH levels (7.4, 6, 5.5, 5), with viability measured after **A)** 24 h **B)** 48 h **C)** 72 h. Results are expressed as percentage difference in fluorescence relative to untreated cells that serve as the negative control (NC). All results are represented as means ± SEM obtained from 3 independent experiments, each of which was performed in triplicates.

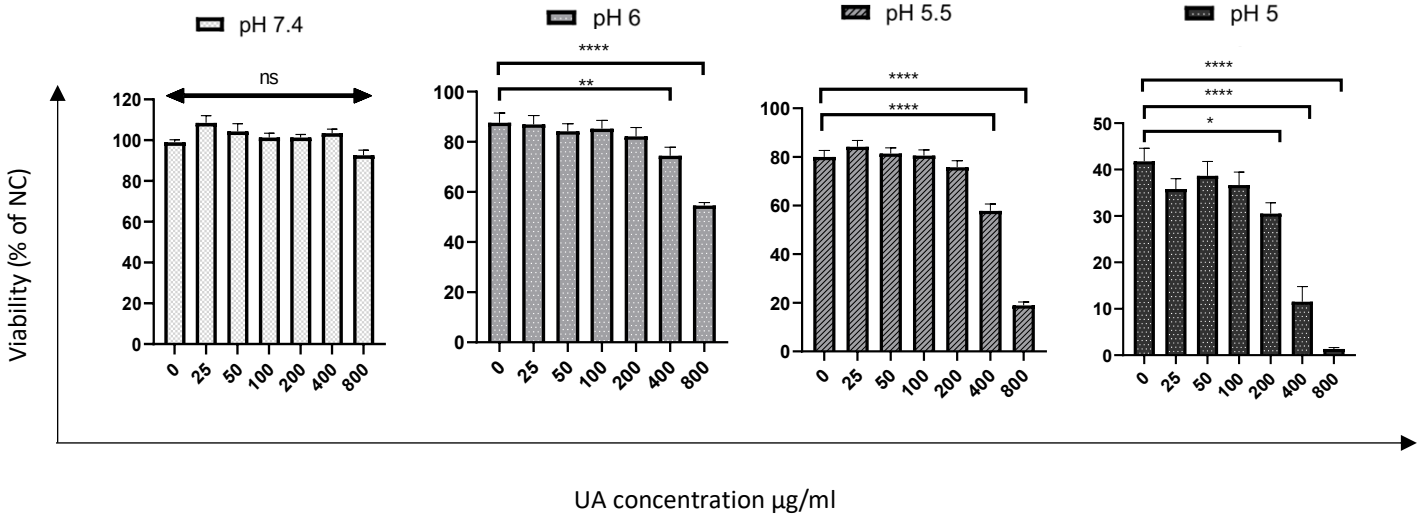
3.2. UA has a more detrimental effect on cell viability in lower pH levels

Reduction in viability as a percentage change relative to the untreated samples within each pH is shown in Figure 2.

After 24 h treatment (Fig 2A) no significant difference in viability was measured across UA concentrations ranging from 0-800 µg/ml within physiological pH. However, in pH 6 and pH 5.5, significant reductions in viability were seen in the UA concentrations 400 µg/ml and 800 µg/ml compared to their own untreated controls. In pH 5, significant changes in viability were seen in the concentrations 200, 400 and 800 µg/ml UA compared to its own untreated control.

After 48h treatment (Fig 2B), a significant change in viability was measured at a UA concentration of 800 µg/ml in pH 7.4 compared to the untreated cells. In pH 6, 800 µg/ml UA changed significantly from its own untreated control. In pH 5.5 and pH 5, the viability in the concentrations 200, 400 and 800 µg/ml differed significantly from their own controls

After 72h treatment (Fig 2C), a significant change in viability was again measured at a concentration of UA 800 µg/ml compared to the untreated cells. In pH 6, the viability in 800 µg/ml UA also again changed significantly from its own control. In pH 5.5 the viability in the concentrations 200, 400 and 800 µg/ml differed significantly from their own controls. Nevertheless, in pH 5 no significant differences across UA concentrations were visible, due to the low viability.



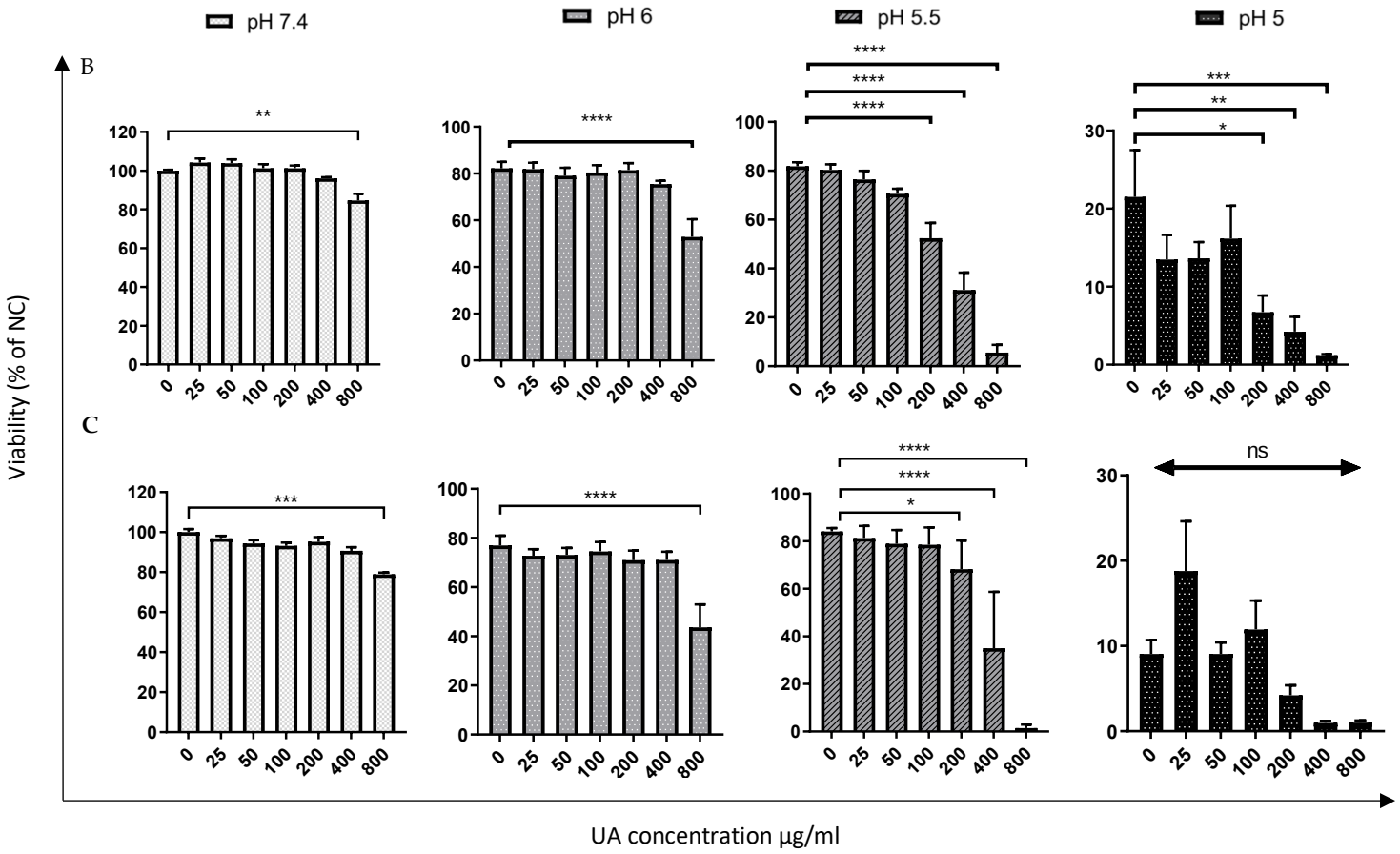


Figure 2. Viability in percentage change relative to the negative control (NC) (UA 0 µg/ml, pH 7.4) . Statistical significance was measured as viability in percentage change relative to the untreated samples within each pH level after A) 24h, B) 48 and C) 72h *P<0,05, **P<0,01, ***P<0,001, ****<0,0001 *versus* control (0 µg/ml UA, relative to each pH). All results represent mean ± SEM obtained from three independent experiments performed in triplicates.

3.2 UA Increases IL-1β Secretion by ciPTECs after Exposure in an Acidic Environment

Following treatment, secretion of IL-1β in the supernatant by ciPTECS increased in all conditions compared with that of the untreated control group (UA 0, pH 7.4). As represented in Figure 2, IL-1β reached a maximum level of 182,26 pg/ml (UA 800 µg/ml, pH 5, n=1). The maximum level of IL-1β secreted in the supernatant decreased considerably after 48h exposure to a level of 12,35 pg/ml (UA 800 µg/ml, pH 5, n=1) and to 2,55 pg/ml after 72 h exposure (UA 800 µg/ml, pH 5).

A

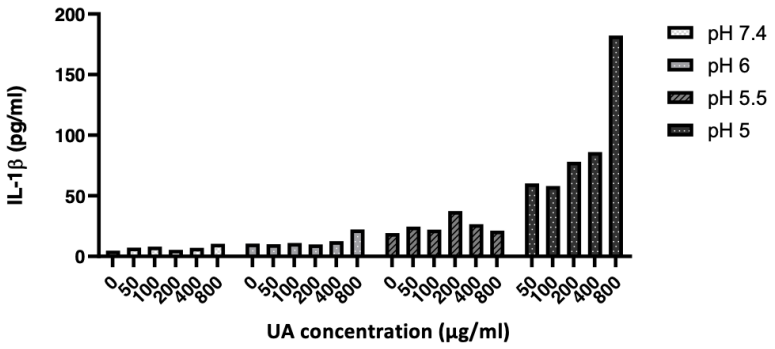


Fig 2. *Cont.*

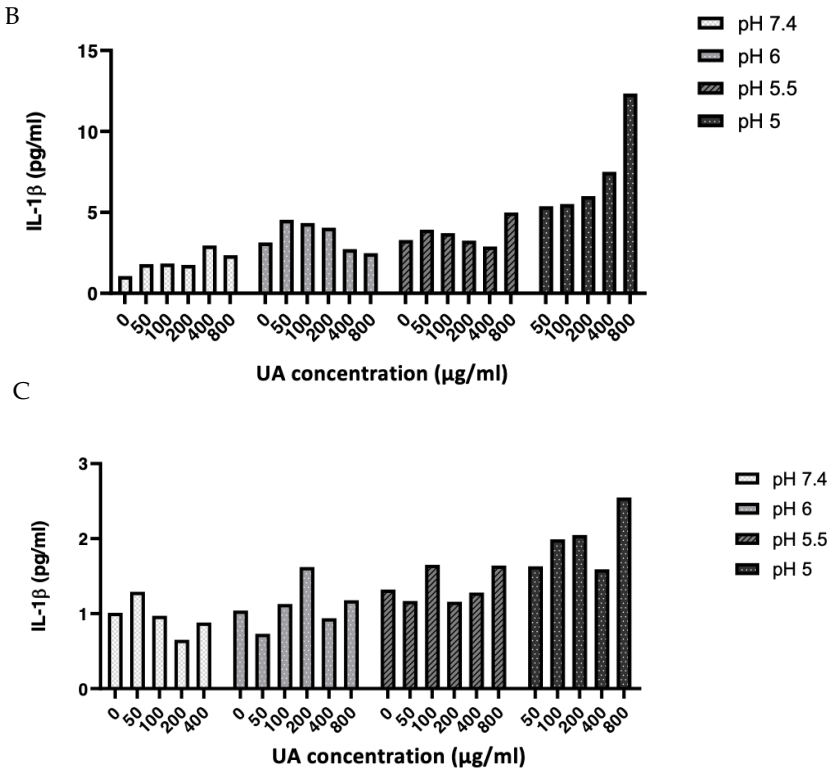


Figure 3 . IL-1β production by ciPTECs after exposure to increasing concentrations of UA ranging from 0-800 µg/ml and different pH levels ranging from physiological pH to 5 after A) 24, B) 48 or B) 72h. Levels of IL-1β were determined using ELISA. These results are derived from one experiment and protein levels are expressed as a relative expression compared to untreated cells in physiological pH (NC).

3.3. UA Induced Expression of Caspase-1, TNFα and ASC in a Concentration-, and pH-Dependent Way

A qPCR assay was performed after 24h of treatment. Caspase-1 and TNF*α* showed relatively concentration-dependent trends in activity (Fig 4A, Fig.4C). These trends became more distinct when the pH decreased. Also, the expression of ASC was increased in more acidic environments and somewhat higher in higher UA concentrations. TNF*α* was the most highly expressed (mean: 53,9 fold higher than NC), followed by Caspase-1 (mean: 14,4 fold higher than NC) and ASC (mean: 4,0 fold higher than NC).

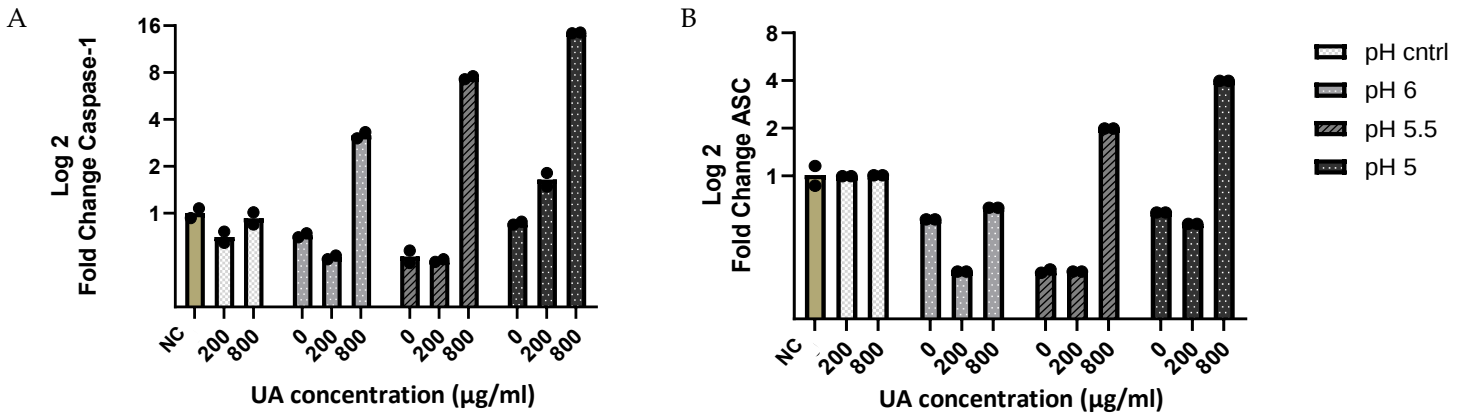


Figure 4. *Cont.*

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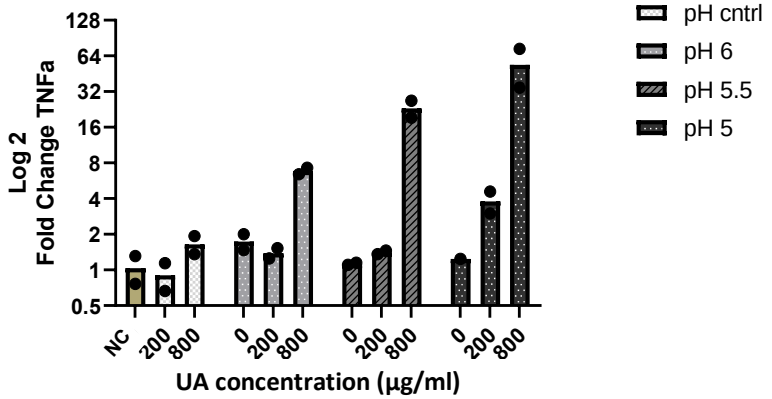

Figure 4. Expression levels of A) Caspase-1 B) ASC and C) TNFα by ciPTECs after 24h exposure to increasing concentrations of UA ranging from 0-800 µg/ml and different pH levels ranging from physiological pH to pH 5. Data is derived from one experiments performed in duplicate and expressed as mean Log 2 of the expression ratio compared to the negative control.

3.4 Other mechanisms besides NLRP3 activation might be influencing IL-1β and NLRP3 expression

Concerning IL-1β a concentration dependent-trend was seen in physiological pH and pH 5. 800 µg/ml. However, low expression levels of IL-1β close to the detected mRNA level in untreated control samples were found in pH 6 800 µg/ml, pH 5.5 800 µg/ml and pH 5 0 µg/ml. (Fig 5A). Apparently, IL-1β mRNA has very low expression in these conditions.

Regarding NLRP3 expression, a concentration dependent-trend was only seen in pH 5 800 µg/ml (Fig 5C). In this condition UA increased the relative NLRP3 mRNA expression level about 16 times compared to the NC (physiological pH, 0 µg/ml UA).

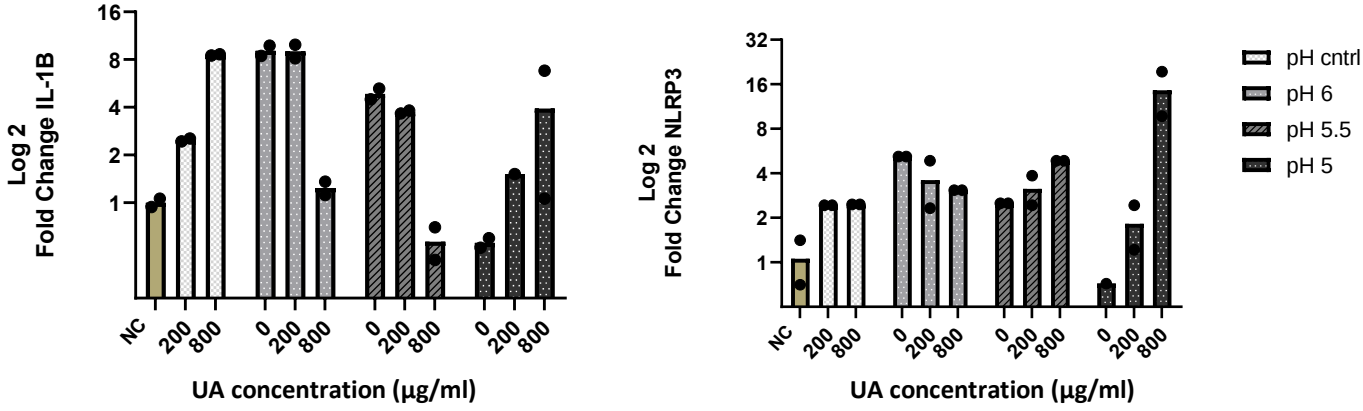

Figure 5. Expression levels of A) IL-1β and B) NLRP3 by ciPTECs after 24h exposure to increasing concentrations of UA ranging from 0- 800 µg/ml and different pH levels ranging from physiological pH to pH 5. Data is derived from one experiments performed in duplicate and expressed as mean Log 2 of the expression ratio compared to the negative control.

3.5 UA crystals developed during treatment

Although not consistent throughout all experiments, crystal formation showed after 72 h of incubation with soluble UA [Image 1]. Further elaboration on this is warranted to obtain more insight into naturally occurring crystal formation and growth in this *in vitro* model since most studies primarily focused on exposure to either soluble UA or with pre-formed crystals [3,13].

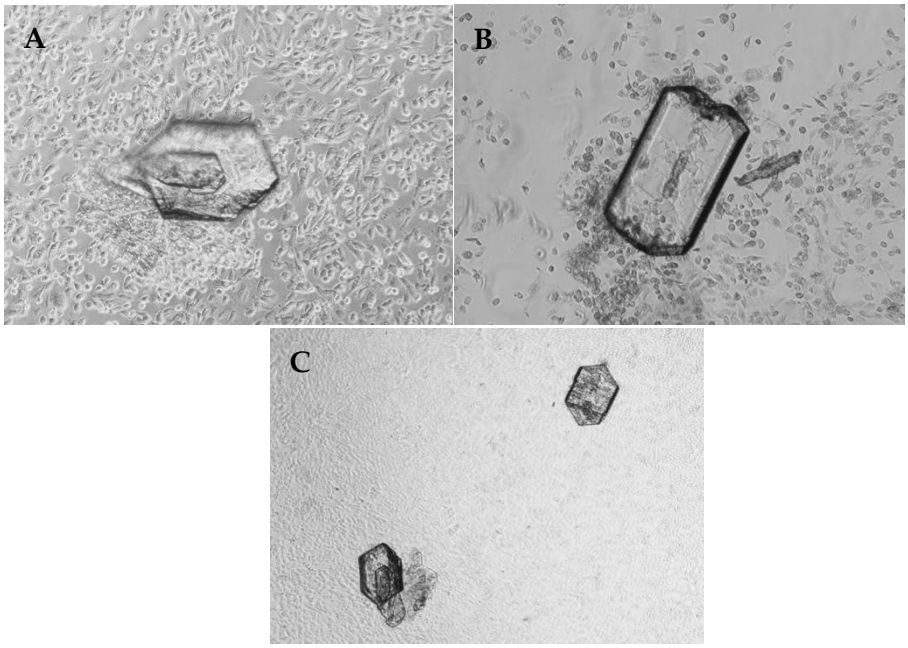

Image 1. A) six-sided shaped UA crystal formed after 72h incubation with 800 μg/ml UA at pH 5 in a 24 well plate, 100x magnification. B) Barrel shaped UA formed after 72h incubation with 600 μg/ml UA at pH 5 in a 24 well plate,, 100x magnification. C) six-sided shaped UA crystal formed after 72h incubation with 800 μg/ml UA at pH 5 in a 24 well plate,, 40x magnification

4. Discussion

Elevated serum UA levels are closely related to CKD and the progression of renal disease [3–6] . When UA crystallizes, their crystals act as DAMPs and may eventually lead to downstream activation of the NLRP3 inflammasome, which in turn leads to secretion of IL-1B and IL-18 from the cells. The pathway in which UA, either in soluble or crystalline form, is able to trigger the NLRP3 inflammasome was previously studied in intestinal epithelial cells [12], mesangial cells [3], innate immune cells [10,13], Human Proximal Tubule Epithelial Cells (HK-2) [1,14,15] and Primary human Tubular Epithelial Cells [6,7]. Our results are of interest since 1) to our knowledge NLRP3 inflammasome activation by UA in ciPTECS has not been previously described and 2) simulating the physiological environment of crystal induced inflammation investigating the effects of changes in pH levels has not been done so far. Since urinary pH plays a key role in the formation of UA crystals, it is of interest to investigate the system's ability to simulate a physiological environment *in vitro* in which UA crystals can form, with crystal-induced inflammation as a consequence.

In this study, we demonstrated that after 24 hours of treatment as described above, increasing concentrations of UA had no significant effect on cell viability compared with non-treated cells within physiological medium pH (7.4). These results are consistent with data obtained by Yang et al and Xiao et al [6,7] in PTECS. However, in these studies pH values were all within range of 7.1 to 7.4. This present study includes multiple pH levels ranging from 7.4 – 5 since low urinary pH has also been identified is a risk factor for crystallopathy. Our results showed that the combination of low pH and high UA reduce cell

viability more so than when compared to these effects in isolation. The effect is observed even more over time, with exception of pH 5 after 72 hours where a majority of the cells had died.

Furthermore, IL-1β, a pro-inflammatory interleukin known to have systemic consequences, is an indicator of NLRP3 inflammasome activation and is able to promote kidney inflammation [16]. This study provides further evidence that UA increased IL-1β protein expression compared to non-treated cells in most conditions on both the protein and mRNA level. Elevated IL-1β levels after exposure to sUA is in line with the previous studies of Xiao et al [3] and Chunling et al [12], where renal mesangial cells and intestinal cells were utilized respectively. Contradictory results are found in the study of Alberts et al [13] where addition of UA did not increase IL-1β secretion in either THP-1 cells or primary human monocytes.

NLRP3 had elevated expression compared to the NC as well, although levels were expressed lower than the NC at 0 μg/ml UA at pH 5.

Besides this, we noticed that relative mRNA expression of IL-1β and NLRP3 did not follow a particular concentration-dependent trend in pH 6 and 5.5. NLRP3 and IL-1β both play a critical role in UA-mediated inflammation. We expected to see induced transcription of these genes with increasing concentrations of UA and lower pH levels. In this case, other mechanisms might be involved in UA induced IL-1β and NLRP3 mRNA expression. Also, we only recorded one timepoint in the process of mRNA production. Remarkable is, that in the lowest pH (pH 5), we did measure a concentration-dependent trend in expression levels of IL-1β and NLRP3.

Caspase-1 and ASC expression levels were elevated in almost all the cells treated with 800 μg/ml UA, the highest concentration used. However, surprisingly low levels were detected in almost all 200μg/ml and 0 μg/ml UA concentrations. Therefore, a high concentration of UA seems to be required to trigger the expression of these components needed for NLRP3 induced inflammation. Interestingly, within these high concentrations (800 μg/ml UA) an upward trend in mRNA expression was visible when the pH level decreased. This indicates that UA in an acidic environment induces NLRP3 inflammation to a higher extend than in pH levels closer to physiological pH. This may have to do with the UA's pKa of 5.8, since the balance will shift to the less soluble form of UA. TNFα , which indicates general inflammation, was highly expressed across multiple conditions and showed a concentration and pH- dependent trend towards higher UA concentrations and lower pH levels.

Certain limitations were associated within the present study. Ideally, it would have been interesting to include more extreme conditions e.g. higher concentrations or lower pH levels. However, one practical shortcoming throughout the investigation was the fast crystallization of UA in vials during solution preparation. Once sings of crystal formation appeared, vials had to be disposed since we wanted to investigate the ability of sUA to form these crystals *in vitro.* Moreover, repeats of the ELISA and qPCR assays are warranted to obtain increased certainty regarding the results and to be able to perform improved statistical analysis on this. Also, we did not manage to include a positive control in ELISA assays. Lipopolysaccharide (LPS) may serve in as a positive control since it is able to provoke an extreme inflammatory condition in ciPTECs [18]. Repeats of prolonged incubation with high UA concentrations at low pH are required to test the system's ability to form UA crystals.

5. Conclusions

In conclusion, our present findings suggest that UA is capable of reducing viability in ciPTECs and inducing expression of NLRP3 inflammasome-mediated markers of caspase-1, ASC and TNFα on mRNA level and IL-1β on protein level, especially when UA concentrations are elevated and pH levels are lowered. Naturally occurring crystal formation in the system was demonstrated, but further research is warranted.

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