Published in final edited form as:

J Urol. 2015 May ; 193(5): 1684–1691. doi:10.1016/j.juro.2014.11.093.

Activation of the NLRP3 Inflammasome in association with Calcium Oxalate crystal-induced Reactive Oxygen Species in kidneys

Sunil Joshi¹, Wei Wang¹, Ammon B. Peck², and Saeed R. Khan^{1,3}

¹Department of Pathology, Immunology & Laboratory Medicine, University of Florida College of Medicine, Gainesville, Florida 32610 USA

²Department of Infectious Diseases and Pathology, University of Florida College of Veterinary Medicine, Gainesville, Florida 32610 USA

³Department of Urology, University of Florida College of Medicine, Gainesville, Florida 32610 USA

Abstract

Purpose—To investigate association between hyperoxaluria and calcium oxalate (CaOx) crystal induced production of reactive oxygen species (ROS) and activation of NLRP3 inflammasome.

Materials and Methods—Eight week old male rats were given hydroxy-L-proline (HLP) to induce hyperoxaluria. A group of rats on HLP diet also received apocynin, antioxidant and non-specific inhibitor of NADPH oxidase. After 28 days rats were euthanized and kidneys were extracted. Microarray analysis was conducted using Illumina bead array readerTM. Gene ontology (GO) analysis and the pathway analysis of the genes was done using DAVID (Database for Annotation, Visualization of Integrated Discovery) enrichment analysis tool. Quantitative RT PCR and immunohistochemical staining were performed to confirm microarray results.

Results—Analysis of 22,226 genes revealed 20 and 24 pathways were highly significant in cortex and medulla respectively. In cortex, ECM-receptor interaction, complement and coagulation cascades, focal adhesion and hypertrophic cardiomyopathy were most significant pathways, whereas in medulla, complement and coagulation cascades, ECM-receptor interaction and dilated cardiomyopathy were major pathways. Genes encoding for PYCARD (ASC: apoptosis-associated spec-like protein containing a CARD), TXNIP (Thioredoxin-interacting protein), NLRP3, Caspase-1, IL-1 β and IL-18 were significantly up regulated in HLP-fed rats, but the group receiving apocynin had these genes down regulated in the cortex and medulla respectively. These results were verified using quantitative RT PCR with Syber Green assays and Immunohistochemical staining.

Conclusions—Results indicate a role for ROS in activation of NLRP3 inflammasome via TXNIP leading to a robust inflammatory response in kidneys of rats with hyperoxaluria and CaOx nephrolithiasis.

Keywords

Reactive Oxygen Species; Oxidative Stress; Inflammasome; Calcium Oxalate; Nephrolithiasis

Introduction

Hyperoxaluria, primary, enteric or idiopathic, is a major risk factor for calcium oxalate (CaOx) crystal formation and deposition within the kidneys and CaOx nephrolithiasis, which is a chronic disease, with greater than 50% recurrence within a decade.¹ Previous studies from our laboratory have demonstrated that cellular exposure to oxalate and CaOx crystals causes the activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), leading to the excessive production of reactive oxygen species (ROS).²⁻⁴ Results of recent studies also show that CaOx crystals activate innate immunity through Nucleotide binding oligomerization domain-like receptor family, pyrin domain containing -3 (NLRP3) inflammasome.^{5, 6}

The ROS under normal conditions are involved as mediators in various regulatory and signaling pathways, controlling growth and proliferation, regulation of different transcriptional activities, and activation and inhibition of different molecules. ROS are in a balanced state with the antioxidants present and are only produced when they are needed and the excess are removed by various scavenging enzymes and antioxidants.³ ROS, when produced in excess cause oxidative stress, leading to change in the redox state, causing permanent damage to macromolecules and disruption of crucial redox-dependant signaling processes. Excessive ROS production leads to conformational changes caused by oxidation of proteins, such as kinases and phosphatases and NF κ B activation, which plays a crucial role in the regulation of immune responses.⁷ ROS also activate different cytosolic molecular complexes known as inflammasomes that have enzymatic activity mediated by the activation of caspase-1. Inflammasomes are involved in the proteolytic activation of proinflammatory cytokines interleukin-1 beta (IL-1 β) and interleukin-18 (IL-18).⁸

The most characterized and understood inflammasome is the nucleotide binding oligomerization domain-like receptor family, pyrin domain containing-3 (NLRP3) also known as NALP3. NLRP3 constitutes the NLRP3 scaffold, the apoptosis associated speck like protein containing a CARD (ASC) also known as PYCARD adaptor, and caspase-1.⁹ Caspase-1 dependent activation of NLRP3 leads to processing of cytokines like IL-1 β .⁹ The NLRP3 inflammasome is known to be activated by pathogens, pathogen associated molecular patterns (PAMPs), damage associated molecular patterns (DAMPs), and different environmental agents. Inflammatory pathologies and tissue restructuring in various disease such as gout (due to monosodium urate crystals), pseudo gout (due to calcium pyrophosphate crystals),¹⁰ silicosis (due to silica), asbestosis (due to asbestos),¹¹ and atherosclerosis (due to cholesterol),¹² all follow the IL-1 β secretory pathway. Recent studies have shown CaOx crystals induced inflammation in the kidneys is also due to the NLRP3 mediated IL-1 β secretory pathway.^{5, 6} In addition oxalate up regulates expression of IL-2RB and activates IL2R signaling in HK2 cells, a line of human renal epithelial cells.¹³

In the present study, we hypothesized that hyperoxaluria and CaOx crystal deposition in the kidneys leads to the production of ROS triggering NLRP3 inflammasome via TXNIP leading to an inflammatory response in the kidneys. To test this hypothesis, we examined changes in the global transcriptome of the rat kidneys following hyperoxaluria in the rats fed hydroxy-l-proline (HLP) over a 4 week period, and whether changes could be specifically reversed by drug intervention using apocynin, an antioxidant and a non-specific small molecule NADPH oxidase inhibitor.¹⁴

Materials and Methods

Details of animal experimentation, RNA extraction, and microarray analyses are provided in our earlier publications and will only be briefly described here.⁴ Methods employed to study the expression of inflammasome related genes and proteins are however described in detail.

Animal Studies

Eight weeks old, male Sprague- Dawley rats (n =18), with average weight of about 150 grams, were bought from Harlan Laboratories and divided into three groups as previously described. Group 1 rats were fed normal rat chow, Group 2 rats received chow supplemented with 5% hydroxy-l-proline (HLP) while the Group 3 rats got diet similar to group 2 rats, except their water was supplemented with 4 mM Apocynin. Rats were sacrificed after 28 days and their kidneys explanted.¹⁵ Data was previously collected but not analyzed for the genes reported in this study. All the experimental procedures were approved by the University of Florida's IACUC Protocol # 201101850 and were in accord with the recommendations of the NIH Guide for the Care and Use of Laboratory Animals.

RNA Extraction And Differential Expression Of Genes By Microarray Analysis

The total RNA from each of the 36 different specimens was isolated as described in our previous studies. Single hybridizations were carried out with each of the RNA specimens using the IlluminaTM RatRef-12 Expression Bead Chip. So, there was a replicate of n=6 for each group and mean value and standard deviation were calculated. The RatRef-12 Expression Bead Chip contained >22,000 genes expressed in the Rat genome. All microarray data have been deposited with the Gene Expression Omnibus (GSE36446).

The microarray analysis was done in the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida, using the IlluminaTM bead array reader, while all the gene expression data analysis was done using the Genome Studio Gene Expression Module V1.0. as described previously. We studied the gene expression between the cortex and medulla tissues from control versus HLP-treated rats and between control and HLP-Apocynin treated rats. Database for Annotation, Visualization of Integrated Discovery (DAVID) enrichment analysis tool from the Bioinformatics resources, National Institute of Allergy and Infectious Diseases, NIAD, NIH was used for GO: TERM and KEGG pathway analysis based on differentially expressed genes.

RT-PCR

The quantitative real time PCR was done to determine the mRNA expression of inflammasome related genes such as TXNIP, NLRP3, IL-1 β , IL-18, ASC/PYCARD and Caspase-1. The mRNA of these genes was PCR amplified and detected using the Roche's FastStart High Fidelity PCR System (Indianapolis, IN, USA; Cat. # 03553426001).¹⁶

The forward and reverse primers are as follows: TXNIP: Forward 5' CTGAAGTTACCCGAGTCAAAGC 3' Reverse 5' CTCACCTGTAGCCTGGTCTTCT 3'; NLRP3: Forward 5' CAGACCTC CAAGACCACGACTG 3' Reverse 5' CATCCGCAGCCAATGAACAGAG 3'; IL-1 β : Forward 5' CACCTCTCAAGCAGAGCACAG 3' Reverse 5' GGGTTCCATGGTGAAGTCAAC 3'; IL-18: Forward 5' GACTGGCTGTGACCTATCTGTGA 3'; Reverse 5' TTGTGTCCTGGCACACGTTTC 3'; caspase-1: Forward 5' TGCCTGGTCTTGTGACTTGGAG 3' Reverse 5' ATGTCCTG GGAAGAGGTAGAAACG3'; ASC/PYCARD: Forward 5' TTATGGAAGAGTCTGG AGCTGTGG 3' Reverse 5' AATGAGTGCTTGCCTGTGTTGG 3'.

The Statistical analysis was performed using Graph Pad Prism version 5.0 for Windows (Graph Pad, Software, La Jolla, CA, USA). *P* values were calculated by one-way ANOVA for nonparametric data. *P* < 0.05 is considered statistically significant.

Histological Examinations

The formalin fixed kidney tissues were embedded in paraffin and sectioned to a thickness of 5- μ m. Deparaffinization of paraffin-embedded slides was performed by xylene immersion followed by dehydration in ethanol.¹⁵ Slides were incubated overnight at 4°C with primary antibodies reactive to TXNIP (sc-33099, Santa Cruz Biotechnology, Inc.), ASC/TMS1 (NB100-92442, Novus Biologicals, LLC, Littleton, CO), NLRP3 (NBP2-12446, Novus Biologicals, LLC, Littleton, CO), Caspase-1 (ab-1872, Abcam, Cambridge, MA), IL-18 (sc-7954, Santa Cruz Biotechnology, Inc.), and IL-1 β (ab-9722, Abcam, Cambridge, MA). Isotype controls were performed using rabbit IgG.

Results

Administration of HLP resulted in increased urinary excretion of oxalate by the rats and CaOx crystal deposition in their kidneys, as previously described (Table 1).¹⁵ Urinary oxalate excretion increased in rats consuming HLP or HLP+ apocynin but treatment with apocynin reduced CaOx crystal deposition in HLP fed rats. Urinary excretion of kidney injury molecule (KIM) and hydrogen peroxide (H₂O₂) also increased in HLP fed rats. Apocynin treatment produced a significant reduction in both KIM and H₂O₂. As described previously, there was crystal deposition in all sections of the kidney, including the cortex, medulla and renal papilla, the maximum crystals were found in the tubular lumens of the distal tubules and the collecting ducts.

Of the 22226 rat genes represented on the array, 3302 and 2894 were found to be significantly differentially-expressed at a 2-fold change in the cortex and medulla, respectively, in a comparison between control and HLP-fed rats, while 3000 and 2505

genes, respectively, were found to be significantly expressed in the cortex and medulla in the comparison between control and HLP+Apocynin-fed rats. Using Kyoto Encyclopedia of Genes and Genome (KEGG) to curate the differentially-expressed genes, several gene clusters were identified that defined specific biological processes for the HLP-fed and HLP +Apocynin-fed rats, as presented in Table 2 (URL address provided for Journal's website) for cortex and medulla, respectively. These data strongly indicate that the effects of both HLP and Apocynin on renal tissues are complex and involve distinct activities on cellular functions. KEGG pathway analysis of some 3000 genes per run (using a p-value < 0.1 cut-off) was carried out for two different sets of genes, one cortex tissue-derived and the other medulla tissue-derived. . It should be mentioned, however, that dissection of kidney into cortex and medulla is not precise most likely resulting in cross contamination of cells derived from each tissue section. Using the pathway analysis of these 3000 gene sets by DAVID, we identified several pathways which were highly significant and unique within the experimental groups. We were able to show that genes encoding ASC/PYCARD and TXNIP were significantly up regulated in the HLP-fed rats and down regulated in the HLP + Apocynin treated rats (Figure 1A and B), similarly the gene encoding NLRP3 and Caspase -1 were shown to be significantly up regulated in the HLP-fed rats and down regulated in the HLP + Apocynin treated (Figure 1C and D). We also found that gene encoding IL-1b and IL-18 were significantly up regulated in the HLP-fed rats and down regulated in the HLP + Apocynin treated rats (Figure 1E and F). These genes act as key mediators of inflammation.

Quantitative Real Time PCR was done to verify the micro array results. As expected, there was significant difference between the control vs. HLP group and the HLP vs. HLP+APO group in both cortex and medulla for the TXNIP expression ($p < 0.05$)(Figure 2A). For ASC/PYCARD, there was significant difference between the control vs. HLP expression ($p < 0.05$) and between the control vs. HLP+APO group ($p < 0.05$), in the cortex and in the medulla, there was significant difference between the expression levels of the control vs. HLP group ($p < 0.05$), and HLP vs. HLP+APO group ($p < 0.05$) (Figure 2B). Similarly, the expression levels of control vs. HLP and HLP vs. HLP+APO were significantly different for NLRP3 ($p < 0.05$) in both cortex and medulla (Figure 2C). For both ASC/PYCARD and NLRP3, the expression level was calculated by normalization to the internal standard GAPDH. Similar results were obtained for the Caspase-1 and IL-1 β expression levels in the cortex and medulla with control vs. HLP ($p < 0.05$), and HLP vs. HLP+APO ($p < 0.05$) being significantly different (Figure 2D & E). For the proinflammatory cytokine, IL-18, the expression levels of the control group were significantly different from the HLP group ($p < 0.05$), and the HLP group was significantly different from HLP+ APO group with HLP group having higher expression, in the cortex. In the medulla, there was significant difference between all the groups when compared such as control vs. HLP ($p < 0.05$), control vs. HLP+APO ($p < 0.05$), and HLP vs. HLP+APO ($p < 0.05$) (Figure 2F).

The immunohistochemical staining of the kidney sections of the control, HLP-fed and HLP +Apocynin-fed fed rat's revealed results as anticipated. There was no staining in the kidney sections of the control for TXNIP, ASC/PYCARD, NLRP3, Caspase-1, IL-1 β , and IL-18. However, there was strong staining of the renal tubular epithelial cells in the tubules of HLP treated rat kidney sections (Figure 3). The kidney sections of the HLP+Apocynin-fed group

showed reduced staining as compared to the HLP group. These results were in accord with the quantitative real time PCR and the microarray results obtained for these genes involved in the activation of NLRP3 inflammasome, leading to the production of pro inflammatory cytokines such as IL-1 β and IL-18.

Discussion

In this study, we report the activation of NLRP3 inflammasome and related proinflammatory genes encoding cytokines in the rat model of hyperoxaluria. In our previous study, we have shown that Ox and CaOx crystals lead to the production of ROS concomitantly with the activation of NADPH oxidase complex. Results of a number of studies have shown that ROS play a crucial role in the activation of NLRP3.^{17, 18} There are different mechanisms and stimuli for the activation of the NLRP3 inflammasome but it is still unclear how it gets activated. Many studies suggest K⁺ efflux is needed for NLRP3 activation and when it is inhibited by drugs or by artificial conditions, such as high concentration of K⁺ in the cell culture media, the NLRP3 inflammasome is not activated.¹⁹ Alternatively, as suggested in our study, the production of ROS is considered crucial for its activation. Previous research has shown that blocking or reducing excessive production of ROS by chemical scavengers of ROS, inhibitors of NADPH oxidase, or siRNA mediated knockdown of the p22phox subunit of the NADPH oxidase hindered the activation of NLRP3, in response to a vast variety of stimuli including ATP,²⁰ *C. albicans*,²¹ and different crystals such as MSU, asbestos, silica and hemozoin. It has also been shown that TXNIP can directly bind to NLRP3 in a ROS sensitive manner, further strengthening the relation between ROS production and inflammasome activation.²² We hypothesized as shown in Figure 4, that Ox and CaOx crystals in the kidneys, via the renin angiotensin system, activate the NADPH oxidase complex, which produces ROS in excess leading to the dissociation of TXNIP from Thioredoxin (Trx1). Thioredoxin is a crucial redox protein that regulates the levels of ROS in cells. High levels of TXNIP in the cells inhibit the redox activity of Thioredoxin leading to increased levels of ROS²³ ultimately causing the activation of the NLRP3 inflammasome via TXNIP resulting in a robust inflammatory response in the kidneys of rats with hyperoxaluria and CaOx nephrolithiasis. Previous studies have shown that ROS causes high levels of TXNIP in the cell to associate with NLRP3,²⁴ thus causing its activation. The current study supports the concept that under oxidative stress, excessive ROS caused Trx1-TXNIP dissociation, leading to increased levels of TXNIP in the cell that bind to NLRP3, increasing a NLRP3-TXNIP association that ultimately leads to the activation of NLRP3 inflammasomes. Our data clearly shows an increase in TXNIP expression under HLP-fed rats.

Published studies have shown that the subunits of NLRP3 inflammasome, when assembled, induce the activation of caspase-1 that subsequently leads to the processing and activation of IL-1 β .⁹ Our microarray results, along with quantitative real time PCR analyses, revealed that the HLP-fed rats exhibited high expression of caspase-1 and IL-1 β , while interestingly the HLP+Apocynin fed rats had decreased expression of both caspase-1 and IL-1 β . Apocynin is an inhibitor of NADPH oxidase complex by blocking the translocation of p47 subunit of NADPH oxidase to the cytosolic subunits, thus inhibiting the assembly of the complex that leads to reduction in production of ROS and oxidative stress.²⁵ We also showed that the

proinflammatory cytokine IL-18 was also highly expressed in our results in the HLP-fed rats and the expression levels decreased in the HLP+Apocynin group. A recent study has shown that CaOx crystals induce renal inflammation, leading to activation of NLRP3 inflammasome, which ultimately lead to IL-1 β production.⁵ This study also revealed that crystal deposition in the kidneys led to tubular damage, cytokine expression, neutrophil recruitment and renal failure. In addition, it demonstrated that CaOx crystals trigger IL-1 β dependant innate immunity by the NLRP3 assembly activation. In our rat model of hyperoxaluria and nephrolithiasis, our immunohistochemical staining results in the HLP treated rats showed destruction of the lining of epithelium and tubular damage where crystals were present and strong epithelial staining for IL-1 β . Whereas in the HLP +Apocynin-fed group, there was reduced staining and crystal deposition as compared to the HLP group.

It has been recently shown that high oxalate diet results in increased NALP3/NLRP3 expression in the kidneys, and progressive renal failure associated with oxalate nephropathy in wild type mice is not seen in NALP3/NLRP3 deficient mice.⁶ Our results are supportive of these findings, showing an increased expression of NLRP3 in the HLP-fed rats, an expression that was reduced in the HLP+Apocynin-fed rats. Furthermore, our studies also showed an increased expression of ASC (Apoptosis associated spec like protein containing a CARD)/PYCARD, and the proinflammatory IL-18 in the HLP fed rats group and their expression was decreased in the apocynin treated group. In summary, our study indicates that expressions of NLRP3, ASC, TXNIP, Caspase-1, IL-1 β , and IL-18 in the HLP fed rats were up-regulated, whereas these expressions were reduced in the apocynin treated group. A concomitant activation of NLRP3 inflammasomes was observed with production of ROS, presumed to be induced by Ox and CaOx crystal. Our results show the role of ROS in the activation of NLRP3 inflammasome via TXNIP leading to a robust inflammatory response in the kidneys of rats with hyperoxaluria and nephrolithiasis.

Numerous studies have been performed investigating experimentally induced CaOx nephrolithiasis in rats to better our understanding of the role of hyperoxaluria in the formation of CaOx stones.²⁶ Most of the previous studies including our own have examined involvement of a limited number of molecules and pathways.² We decided to perform genome wide analyses of the kidneys of hyperoxaluric rats and investigate genes and molecules involved in specific pathways and steps during stone formation.⁴ We showed production of an oxidative burst leading to injury and inflammation in kidneys of hyperoxaluric rats, through an increase in various subunits and activation of the NADPH system via angiotensin-II pathway.⁴ We also showed that NADPH oxidase induced reactive oxygen species play significant role in the regulation of macromolecules involved in modulating crystals formation and retention within the kidneys.²⁷ Recent studies have shown activation of NLRP3 inflammasomes in hyperoxaluric mice.^{5, 6} Results of our examination described here show involvement of NADPH oxidase induced reactive oxygen species in the activation of NLRP3 inflammasomes. We have previously suggested that markers of oxidative stress should be increased in stone patients particularly those with high levels of urinary oxalate such as primary hyperoxaluria.³ Circulating cytokines should also be increased in such patients. Interestingly multiple markers of inflammation have been detected in idiopathic stone formers.²⁸ Anti-inflammatory therapies including blockade of

NADPH oxidase activation may not only reduce renal inflammation but also crystal deposition in patients with hyperoxaluria. Statins have been shown to even reduce stone recurrence.²⁹

Acknowledgments

The authors appreciate Dr. Jin Yao, Ms. Ginger Clark, and Dr. Yijun Sun efforts from the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR) for running the microarrays and providing expert assistance in data analyses. The funding for the research was supported by National Institute of Health (NIH) grant number RO1-DK078602.

Abbreviations Used

CaOx	Calcium oxalate
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
ROS	reactive oxygen species
NLRP3	Nucleotide binding oligomerization domain-like receptor family, pyrin domain containing -3
NO	nitric oxide
eNOS	endothelial nitric oxide synthase
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
HLP	hydroxyl-L-proline
KEGG	Kyoto Encyclopedia of Genes and Genome
DAVID	Database for Annotation, Visualization of Integrated Discovery
APO	apocynin
CARD	C-terminal caspase-recruitment domain
PYCARD	apoptosis –associated spec-like protein containing a CARD
PAMPs	Pathogen associated molecular patterns
DAMPs	Damage associated molecular patterns
IL-1β	Interleukin-1 beta
IL-18	interleukin-18

REFERENCES

1. Pearle MS, Calhoun EA, Curhan GC. Urologic diseases in America project: urolithiasis. *J Urol.* 2005; 173:848. [PubMed: 15711292]
2. Khan SR. Crystal-induced inflammation of the kidneys: results from human studies, animal models, and tissue-culture studies. *Clin Exp Nephrol.* 2004; 8:75. [PubMed: 15235923]
3. Khan SR. Hyperoxaluria-induced oxidative stress and antioxidants for renal protection. *Urol Res.* 2005; 33:349. [PubMed: 16292585]
4. Joshi S, Saylor BT, Wang W, et al. Apocynin-treatment reverses hyperoxaluria induced changes in NADPH oxidase system expression in rat kidneys: a transcriptional study. *PLoS One.* 2012; 7:e47738. [PubMed: 23091645]

5. Mulay SR, Kulkarni OP, Rupanagudi KV, et al. Calcium oxalate crystals induce renal inflammation by NLRP3-mediated IL-1 β secretion. *J Clin Invest*. 2013; 123:236. [PubMed: 23221343]
6. Knauf F, Asplin JR, Granja I, et al. NALP3-mediated inflammation is a principal cause of progressive renal failure in oxalate nephropathy. *Kidney Int*. 2013; 84:895. [PubMed: 23739234]
7. Anrather J, Racchumi G, Iadecola C. NF-kappaB regulates phagocytic NADPH oxidase by inducing the expression of gp91phox. *J Biol Chem*. 2006; 281:5657. [PubMed: 16407283]
8. Martinon F. Signaling by ROS drives inflammasome activation. *Eur J Immunol*. 40:616. [PubMed: 20201014]
9. Schroder K, Tschopp J. The inflammasomes. *Cell*. 140:821. [PubMed: 20303873]
10. Martinon F, Petrilli V, Mayor A, et al. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*. 2006; 440:237. [PubMed: 16407889]
11. Dostert C, Petrilli V, Van Bruggen R, et al. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science*. 2008; 320:674. [PubMed: 18403674]
12. Duewell P, Kono H, Rayner KJ, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*. 464:1357. [PubMed: 20428172]
13. Koul S, Khandrika L, Pshak TJ, et al. Oxalate upregulates expression of IL-2R β and activates IL-2R signaling in HK-2 cells, a line of human renal epithelial cells. *Am J Physiol Renal Physiol*. 2014; 306:F1039. [PubMed: 24523387]
14. Heumuller S, Wind S, Barbosa-Sicard E, et al. Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. *Hypertension*. 2008; 51:211. [PubMed: 18086956]
15. Zuo J, Khan A, Glenton PA, et al. Effect of NADPH oxidase inhibition on the expression of kidney injury molecule and calcium oxalate crystal deposition in hydroxy-L-proline-induced hyperoxaluria in the male Sprague-Dawley rats. *Nephrol Dial Transplant*. 2011; 26:1785. [PubMed: 21378157]
16. Khan SR, Khan A, Byer KJ. Temporal changes in the expression of mRNA of NADPH oxidase subunits in renal epithelial cells exposed to oxalate or calcium oxalate crystals. *Nephrol Dial Transplant*. 26:1778. [PubMed: 21079197]
17. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol*. 2009; 27:229. [PubMed: 19302040]
18. Bryant C, Fitzgerald KA. Molecular mechanisms involved in inflammasome activation. *Trends Cell Biol*. 2009; 19:455. [PubMed: 19716304]
19. Petrilli V, Papin S, Dostert C, et al. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ*. 2007; 14:1583. [PubMed: 17599094]
20. Cruz CM, Rinna A, Forman HJ, et al. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem*. 2007; 282:2871. [PubMed: 17132626]
21. Gross O, Poeck H, Bscheider M, et al. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature*. 2009; 459:433. [PubMed: 19339971]
22. Zhou R, Tardivel A, Thorens B, et al. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol*. 2010; 11:136. [PubMed: 20023662]
23. Li X, Rong Y, Zhang M. Up-regulation of thioredoxin interacting protein (Txnip) by p38 MAPK and FOXO1 contributes to the impaired thioredoxin activity and increased ROS in glucose-treated endothelial cells. *Biochem Biophys Res Commun*. 2009; 381:660. [PubMed: 19254690]
24. Zhou R, Yazdi AS, Menu P, et al. A role for mitochondria in NLRP3 inflammasome activation. *Nature*. 2011; 469:221. [PubMed: 21124315]
25. Joshi S, Saylor BT, Wang W, et al. Apocynin-treatment reverses hyperoxaluria induced changes in NADPH oxidase system expression in rat kidneys: a transcriptional study. *PLoS One*. 7:e47738. [PubMed: 23091645]
26. Khan SR, Glenton PA, Byer KJ. Modeling of hyperoxaluric calcium oxalate nephrolithiasis: experimental induction of hyperoxaluria by hydroxy-L-proline. *Kidney Int*. 2006; 70:914. [PubMed: 16850024]

27. Khan SR, Joshi S, Wang W, et al. Regulation of Macromolecular Modulators of Urinary Stone Formation by Reactive Oxygen Species: Transcriptional Study in an Animal Model of Hyperoxaluria. *Am J Physiol Renal Physiol*. 2014
28. Tsao KC, Wu TL, Chang PY, et al. Multiple risk markers for atherogenesis associated with chronic inflammation are detectable in patients with renal stones. *J Clin Lab Anal*. 2007; 21:426. [PubMed: 18022927]
29. Sur RL, Masterson JH, Palazzi KL, et al. Impact of statins on nephrolithiasis in hyperlipidemic patients: a 10-year review of an equal access health care system. *Clin Nephrol*. 2013; 79:351. [PubMed: 23195830]
30. Schroder K, Zhou R, Tschopp J. The NLRP3 inflammasome: a sensor for metabolic danger? *Science*. 327:296. [PubMed: 20075245]

Figure 1.

Relative expression of genes in HLP-fed rats vs. control compared to HLP+Apocynin fed rats vs. control. Genes encoding (A) ASC/PYCARD and (B) TXNIP were significantly up regulated in the HLP-fed rats in both cortex and medulla and down regulated in HLP +Apocynin fed rats, genes encoding the inflammasome (C) NLRP3 and (D) Caspase-1 were significantly up regulated in the HLP-fed rats in both cortex and medulla and down regulated in HLP+Apocynin fed rats, and similarly genes encoding the proinflammatory cytokines, (E) IL-1 β and (F) IL-18 were significantly up regulated in the HLP-fed rats (red

bars) in both cortex and medulla and down regulated in HLP+Apocynin fed rats (green bars). Most of the genes shown had values > 0.3 (or > 2 fold ratio differential expression).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2.

Quantitative RT-PCR of control, HLP-fed, and HLP+APO fed rat's gene expression of (A) TXNIP, (B) ASC/PYCARD, (C) NLRP3, (D) Caspase-1, (E) IL-1 β and (F) IL-18.

Expression values of the gene were determined by quantitative PCR using Syber Green assays. The expression level was calculated by normalization to the internal standard β-actin for TXNIP, Caspase-1 and IL-18 using Ct method and normalization to the internal standard GAPDH for ASC/PYCARD, NLRP3, and IL-1β. Data is presented as Mean ± SD.

* $p < 0.05$ (control vs. HLP) and ** $p < 0.05$ (HLP vs. HLP+APO), *** $p < 0.05$ (control vs. HLP+APO).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

Immuno histochemical staining of paraffin embedded kidney sections of control, HLP treated, and HLP+Apocynin treated rats. The figures show the control, HLP-fed, and the HLP+Apocynin fed rats kidney sections stained for NLRP3, and IL-1 β . Similar results were found for other genes. The control showing no staining at all, while the HLP-fed rat kidney sections show strong staining in the tubular epithelial cells. The renal tubules that contained crystals were dilated and showed destruction of the lining epithelium, while HLP-Apocynin rat kidney sections were lightly stained as compared to the HLP-fed rat kidney sections.

Images were taken using the Zeiss Axiovert 200 M microscopes at 10X (Carl Zeiss Microimaging, Inc., Thornwood, NY).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4.

ROS are important contributor to renal injury and inflammation. These ROS, when produced in excess cause oxidative stress. This leads to dissociation of TXNIP (Thioredoxin interacting protein) from Trx1 (Thioredoxin), binding of TXNIP to NLRP3, and inflammasome activation³⁰. The activated inflammasome leads to production of caspase-1, IL-1 β , and IL-18 and associated inflammatory response. The NF κ B pathway also plays an important role in production of cytokines, chemokines and adhesion molecules. The pathways are interrelated with cross talks between them.

Urinary Chemistry of the rats at different time periods, under different treatment conditions showing urinary oxalate (mg/24hours), hydrogen peroxide (µM/24 hours), and kidney injury molecule (pg/ml).

Table 1

	Day	Control	Apocynin	HLP	HLP+APO
Oxalate (mg/24 hours)	0	0.460	-	-	-
	7	0.577	0.627	1.724 [*]	1.675 ^a
	14	0.546	0.546	2.441 [*]	2.443 ^a
	28	0.541	0.559	2.185 [*]	2.393 ^a
Hydrogen Peroxide (µM/24 hours)	0	2.240	-	-	-
	7	3.430	3.810	4.710 [*]	3.090 ^δ
	14	2.660	2.690	7.530 [*]	4.300 ^{aδ}
	28	2.650	2.630	4.740 [*]	3.620 ^{aδ}
Kidney Injury Molecule (pg/ml)	0	249.045	-	-	-
	7	286.724	380.189	652.829 [*]	574.744 ^a
	14	435.369	453.389	1030.551 [*]	543.467 ^{aδ}
	28	367.105	437	1113.393 [*]	751.949 ^{aδ}

* p < 0.05 Control vs. HLP treated,
^a p < 0.05 Control vs. HLP+Apocynin,
^δ p < 0.05 HLP vs. HLP + Apocynin