Activation of the NLRP3 Inflammasome in Association with Calcium Oxalate Crystal Induced Reactive Oxygen Species in Kidneys

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Abbreviations and Acronyms

APO = apocynin

CaOx = calcium oxalate

CARD = C-terminal caspase-recruitment domain

HLP = hydroxy-L-proline

IL = interleukin

KEGG = Kyoto Encyclopedia of Genes and Genome

KIM = kidney injury molecule

NADPH oxidase = nicotinamide adenine dinucleotide phosphate-oxidase

NFκB = nuclear factor κ-light-chain enhancer of activated B cells

NIH = National Institutes of Health

NLRP3 (NALP) = nucleotide binding oligomerization domain-like receptor family, pyrin domain containing-3

PCR = polymerase chain reaction

PYCARD (ASC) = apoptosis associated spec-like protein containing CARD

ROS = reactive oxygen species

Trx1 = thioredoxin

TXNIP = thioredoxin-interacting protein

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Purpose: We investigated the association of hyperoxaluria and calcium oxalate crystal induced production of reactive oxygen species with activation of the NLRP3 inflammasome.

Materials and Methods: Eight-week-old male rats were given hydroxy-L-proline to induce hyperoxaluria. A group of rats on the hydroxy-L-proline diet also received apocynin, an antioxidant and nonspecific inhibitor of NADPH oxidase. At 28 days the rats were sacrificed and the kidneys were extracted. Microarray analysis was done with the BeadArray™ Reader. Gene ontology and gene pathway analyses were done with the DAVID (Database for Annotation, Visualization of Integrated Discovery) enrichment analysis tool. Quantitative real-time polymerase chain reaction and immunohistochemical staining were performed to confirm microarray results.

Results: Analysis of 22,226 genes revealed that 20 and 24 pathways were highly significant in the cortex and medulla, respectively. In the cortex extracellular matrix-receptor interaction, complement and coagulation cascades, focal adhesion and hypertrophic cardiomyopathy were the most significant pathways. In the medulla complement and coagulation cascades, extracellular matrix-receptor interaction and dilated cardiomyopathy were the major pathways. Genes encoding for PYCARD (ASC), TXNIP, NLRP3, caspase-1, and IL-1β and 18 were significantly up-regulated in hydroxy-L-proline fed rats but in the group that received apocynin these genes were down-regulated in the cortex and medulla. Results were verified by quantitative real-time polymerase chain reaction with SYBR® Green assay and immunohistochemical staining.

Conclusions: Results indicate a role for reactive oxygen species in activation of the NLRP3 inflammasome via TXNIP. This led to a robust inflammatory response in the kidneys of rats with hyperoxaluria and calcium oxalate nephrolithiasis.

Key Words: kidney, nephrolithiasis, inflammasomes, reactive oxygen species, oxidative stress

PRIMARY, enteric or idiopathic hyperoxaluria is a major risk factor for CaOx crystal formation and deposition in the kidneys and for CaOx nephrolithiasis, a chronic disease with a greater than 50% recurrence rate in a decade.[1] Previous studies from our laboratory demonstrated that cellular exposure to oxalate and CaOx crystals causes NADPH oxidase activation, leading to excessive ROS production.[2-4] Results of recent

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studies also show that CaOx crystals activate innate immunity through the NLRP3 inflammasome.[5,6]

Under normal conditions ROS are involved as mediators in various regulatory and signaling pathways, controlling growth and proliferation, regulating different transcriptional activities, and activating and inhibiting different molecules. ROS, which are in a balanced state with the antioxidants present, are only produced when they are needed and the excess is removed by various scavenging enzymes and antioxidants.[3] When produced in excess, ROS cause oxidative stress, leading to a change in the redox state, causing permanent damage to macromolecules and disrupting crucial redox dependent signaling processes. Excessive ROS production leads to conformational changes caused by oxidation of proteins such as kinases and phosphatases, and activation of NFκB, which has a crucial role in immune response regulation.[7] ROS also activate different cytosolic molecular complexes known as inflammasomes, of which the enzymatic activity is mediated by caspase-1 activation. Inflammasomes are involved in proteolytic activation of the proinflammatory cytokines IL-1β and 18.[8]

The most characterized and understood inflammasome is NLRP3, also known as NALP3. NLRP3 represents the NLRP3 scaffold ASC, also known as PYCARD adaptor and caspase-1.[9] Caspase-1 dependent activation of NLRP3 leads to the processing of cytokines such as IL-1β.[9] The NLRP3 inflammasome is activated by pathogens, PAMPs (pathogen associated molecular patterns), DAMPs (damage associated molecular patterns) 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] follow the IL-1β secretory pathway. Recent studies showed that CaOx crystal induced inflammation in the kidneys is also due to the NLRP3 mediated IL-1β secretory pathway.[5,6] In addition, oxalate upregulates IL-2RB expression and activates IL2R signaling in human renal epithelial HK2 cells.[13]

We hypothesized that hyperoxaluria and CaOx crystal deposition in the kidneys lead to ROS production, triggering the NLRP3 inflammasome via TXNIP and leading to an inflammatory response in the kidneys. To test this hypothesis we examined changes in the global transcriptome of rat kidneys after hyperoxaluria was induced in rats that received HLP for a 4-week period. We also evaluated whether changes could be specifically reversed by drug intervention using APO, an antioxidant and a nonspecific small molecule NADPH oxidase inhibitor.[14]

MATERIALS AND METHODS

Details of animal experimentation, RNA extraction and microarray analyses were previously reported.[4] However, methods used to study the expression of inflammasome related genes and proteins are described in detail.

Animal Studies

A total of 18 male 8-week-old Sprague Dawley® rats with an average weight of about 150 gm were divided into 3 groups as previously described.[15] Group 1 rats received normal rat chow, group 2 rats received chow supplemented with 5% HLP and group 3 received a diet similar to that of group 2 but with water supplemented with 4 mM APO. Rats were sacrificed at 28 days and the kidneys were explanted. Data were collected previously but not analyzed on the genes reported in this study. All experimental procedures were approved by the University of Florida institutional animal care and use committee, and were in accord with the recommendations of the NIH Guide for the Care and Use of Laboratory Animals.

RNA Extraction and Differential Gene Expression Microarray Analysis

Total RNA from each of the 36 specimens was isolated as previously described.[4,15] Single hybridizations were performed in each RNA specimen using the RatRef-12 Expression BeadChip®. Thus, there was a replicate of 6 preparations per group and the mean ± SD was calculated. The BeadChip contained more than 22,000 genes expressed in the rat genome. All microarray data were deposited with GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/, GSE36446).

Microarray analysis was done at the Interdisciplinary Center for Biotechnology Research, University of Florida, using the BeadArray Reader. All gene expression data analysis was done with GenomeStudio™ Gene Expression Module, version 1.0 as described previously.[4] We studied gene expression between the cortex and medulla tissues of control vs HLP treated rats and control vs HLP-APO treated rats. The NIH DAVID (Database for Annotation, Visualization of Integrated Discovery, http://david.abcc.ncifcrf.gov/) enrichment analysis tool was used for GO (gene ontology) TERM and KEGG pathway analysis based on differentially expressed genes.

Real-Time PCR

Quantitative real-time PCR was done to determine mRNA expression of inflammasome related genes such as TXNIP, NLRP3, IL-1β and 18, ASC/PYCARD and Caspase-1. The mRNA of these genes was PCR amplified and detected using the FastStart High Fidelity PCR System (catalogue No. 03553426001, Roche™).[16]

Forward and reverse primers were TXNIP forward 5′ CTGAAGTTACCCGAGTCAAAGC 3′ and reverse 5′ CTCACCTGTAGCCTGGTCTTCT 3′, NLRP3 forward 5′ CAGACCTC CAAGACCACGACTG 3′ and reverse 5′ CATCCGCAGCCAATGAACAGAG 3′, IL-1β forward 5′ CACCTCTCAAGCAGAGCACAG 3′ and reverse 5′ GGGTTCCATGGTGAAGTCAAC 3′, IL-18 forward 5′ GACTGGCTGTGACCCTATCTGTGA 3′ and reverse 5′ TTGTGTCCTGGCACACGTTTC 3′, caspase-1 forward 5′ TGCCTGGTCTTGTGACTTGGAG 3′ and reverse 5′