Research Paper

Urate oxidase knockdown decreases oxidative stress in a murine hepatic cell line

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Humans, birds, and some primates do not express the uric acid degrading enzyme urate oxidase (UOX) and, as a result, have plasma uric acid concentrations higher than UOX expressing animals. Although high uric acid concentrations are suggested to increase the antioxidant defense system and provide a health advantage to animals without UOX, knockout mice lacking UOX develop pathological complications including gout and kidney failure. As an alternative to the knockout model, RNA interference was used to decrease UOX expression using stable transfection in a mouse hepatic cell line (ATCC, FL83B). Urate oxidase mRNA was reduced 66% (p < 0.05) compared to wild type, as measured by real time RT-PCR. To determine if UOX knockdown resulted in enhanced protection against oxidative stress, cells were challenged with hexavalent chromium (Cr(VI)) or 3-morpholinosydnonimine hydrochloride (SIN-1). Compared to wild type, cells with UOX knockdown exhibited a 37.2 ± 3.5% reduction (p < 0.05) in the electron spin resonance (ESR) signal after being exposed to Cr(VI) and displayed less DNA fragmentation (p < 0.05) following SIN-1 treatment. Cell viability decreased in wild type cells (p < 0.05), but not cells with UOX knockdown, after treatment with SIN-1. These results are consistent with an increased intracellular uric acid concentration and an increased defense against oxidative stress.

Introduction

In the genomes of both prokaryotes and eukaryotes lies the gene for the uric acid degrading enzyme, urate oxidase (UOX, EC 1.7.3.3.), however, not all organisms express this gene. Among those organisms in which its expression is silenced are humans, birds, some primates, terrestrial reptiles and most insects. In these organisms, uric acid is excreted as the terminal product of purine degradation. In UOX expressing animals, UOX further degrades uric acid to allantoin, the terminal product of purine degradation. When UOX is expressed, the plasma concentration of uric acid is much lower than in those animals that do not express the enzyme. For example, in humans the normal range of serum uric acid is 200 to 400 µM, which is similar to that found in birds[1] and 10 to 20 times that found in UOX expressing animals. Without UOX, humans have achieved a state of hyperuricemia that approaches the saturation point[2,3] and, as a result, gout is a prevalent disease in humans.

The association of hyperuricemia with hypertension[4] and cardiovascular disease (CVD)[5] in humans, as well as the pathological complications resulting from uric acid crystallization in the kidneys and joints makes the functional advantage of a lack of UOX expression unclear. One widely accepted theory is that increased serum uric acid may provide humans with an increased antioxidant capacity for free radical scavenging, which may reduce the accumulation of oxidative damage and extend the life span.[6] Supporting this theory is the positive correlation between plasma urate and life span among the primate species.[7] Also, numerous reports cite the ability of uric acid to protect cellular components from reactive oxygen and nitrogen species,[8,9] which may contribute to animal longevity. However, uric acid exhibits pro-oxidant[10,11] and pro-inflammatory[12] properties when concentrations are elevated, suggesting that hyperuricemia may be involved in the onset and/or exacerbation of diseases associated with high levels of oxidative stress, like CVD,[13,14] hypertension[15,16] and ischemic stroke.[17]

To further understand the potential role that uric acid has in longevity, disease development and disease treatment, a mouse model for hyperuricemia was created that lacks urate oxidase expression.[18,19] These mice were severely hyperuricemic, exhibiting serum uric acid concentrations ten times higher than that

found in the wild type mice and twice that found in humans. Uric acid crystals developed in the kidneys and led to urate nephropathy and nephrogenic diabetes insipidus, resulting in dehydration and death of most mice within several weeks of birth. The extreme hyperuricemia and resultant pathological complications obviate the use of these mice, therefore a more appropriate animal model of human hyperuricemia should be established. Using RNA interference to reduce UOX expression in mice may elevate serum uric acid without it reaching the lethal concentrations observed in the knockout model, thereby potentially providing an effective experimental model of human hyperuricemia.

The objective of this study was to utilize RNA interference to "knockdown" UOX expression in a mouse hepatic cell line and to determine the effects on the oxidative stress response. This will provide a direct link between reduced UOX expression and the resultant increased protection this affords from oxidative damage by reactive species. The advantage of using small interfering RNAs is that this approach affords an element of control over UOX gene expression and subsequently the degree to which serum uric acid concentrations are elevated.

Results

There was a 66% knockdown in UOX mRNA (Fig. 1) ($p < 0.05$) in the cell line expressing shRNA sequence 1. This was the numerically greatest level of UOX knockdown among the cell lines expressing shRNA sequences that target UOX. The reduction of UOX mRNA in the cell lines expressing shRNA sequences 2–4 ranged between 0% and 60% of wild type levels (data not shown). No difference in UOX mRNA abundance between the wild type cell line and the cell line serving as the negative control was detected ($p > 0.05$).

Representative ESR spectra are shown in Figure 2. This spectrum consists of a 1:2:2:1 quartet with hydrogen and nitrogen splitting constants of $a_H = a_N = 14.9$ G. Based on these splitting constants, the quartet was assigned to a DMPO/·OH adduct, which can also be generated by decomposed superoxide free radicals. There was no measurable signal in cells not exposed to Cr(VI). When exposed to Cr(VI), cells with UOX knockdown exhibit a 37% smaller ($p < 0.05$) free radical signal than wild type cells.

Data presented in Figure 3 were tested for fit to a linear regression line to determine if SIN-1 exposure had an effect on cell viability. In both the wild type and negative control cell lines, cell viability decreased with increasing concentrations of SIN-1 ($p < 0.01$). However, no significant decrease in cell viability occurred in the cell line with UOX knockdown ($p = 0.99$). Across all cells lines, after 24 hours of exposure to 1,000 μM SIN-1, there was no cell survival (data not shown).

Tail length, which was measured in arbitrary units and indicative of the extent of DNA fragmentation, from all cell line by treatment combinations was quantified and is presented in Figure 4. Analysis of variance indicated a main effect of cell line and SIN-1 concentration, as well as a cell line by SIN-1 concentration interaction, on tail length ($p < 0.05$). Pooled across all cell lines, comet
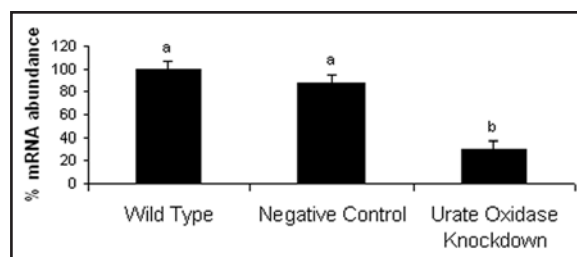

Figure 1. Urate oxidase mRNA abundance in wild type cells, negative control cells, or cells with urate oxidase (UOX) knockdown. Urate oxidase mRNA abundance is scaled to wild type levels, which is set at 100%. Bars represent means ± SEM, n = 2. Different letters indicate differences (p < 0.05) between cell lines.
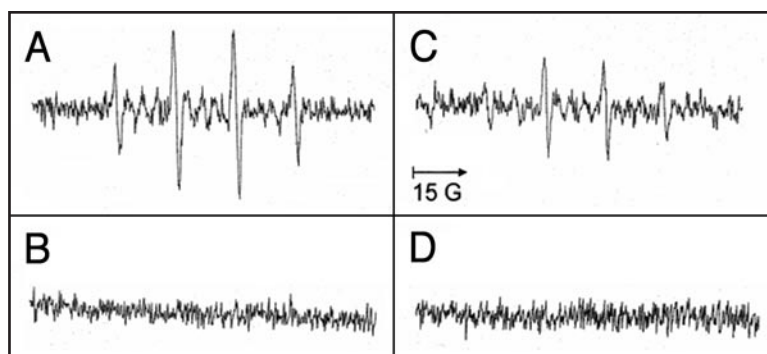

Figure 2. Electron spin resonance in wild type and cells with urate oxidase (UOX) silencing. Spectra (A and B) were generated in the presence of wild type cells and spectra (C and D) were generated in the presence of cells with urate oxidase knockdown. Only cells in spectra (A and C) were exposed to Cr(VI) at 200 μM.
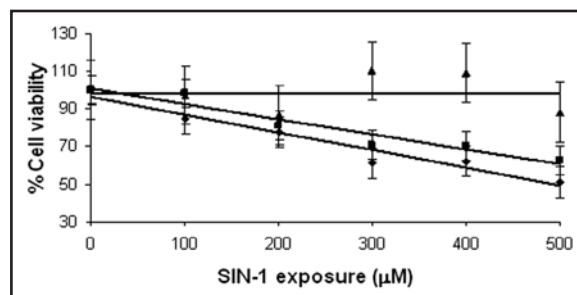

Figure 3. Cell viability of wild type (diamonds), negative control (squares) and urate oxidase (UOX) knockdown (triangles) cell lines exposed to various concentrations of 3-morpholinosydnonimine hydrochloride (SIN-1) for 24 hours. Data points represent means ± SEM, n = 2. Data within each cell line were scaled to the viability of cells exposed to 0 μM SIN-1, which was set at 100% viability. The regression line for each cell type is indicated. Wild type: p < 0.0001, R^2 = 0.78; Negative Control: p < 0.0001, R^2 = 0.51; UOX Knockdown: p > 0.9, R^2 < 0.1.

tail length increased with an increase in SIN-1 concentration ($p < 0.05$). Across SIN-1 concentrations, the overall tail length of wild type cells and the negative control cell line was 27.5 ± 0.9 and 27.0 ± 0.9, respectively, which were longer ($p < 0.05$) than the 22.0 ± 0.9 overall tail length in the cells with UOX silencing. There was no difference in tail length between cell lines not exposed to
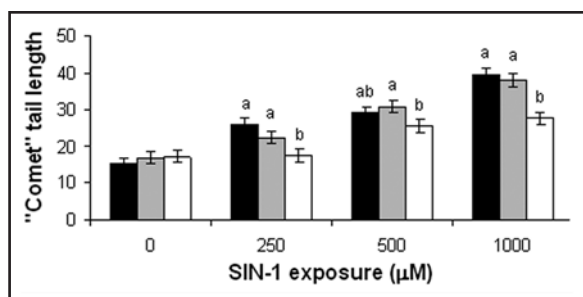
Figure 4. Comet tail length (arbitrary units) of the wild type (black bars), negative control (gray bars) and urate oxidase (UOX) knockdown (white bars) cell lines exposed to various concentrations of 3-morpholinosyd-nonimine hydrochloride (SIN-1). Bars represent means ± SEM. Letters represent significant differences (p < 0.05) between cell lines within the same concentration of SIN-1.

SIN-1. The UOX knockdown cells had a significantly shorter tail length (p < 0.05) than both wild type and negative control cells at 250 μM and 1,000 μM SIN-1. When exposed to 500 μM SIN-1, cells with UOX knockdown had shorter tails (p < 0.05) than only the negative control cell line.

Discussion

In the present experiment, short interfering RNAs were used to silence UOX mRNA approximately 66% compared to wild type. Exposure to Cr(VI) resulted in a less intense ESR signal in cells with UOX silencing, indicative of lower hydroxyl and/or super-oxide radical concentrations. In wild type and negative control cells, cell viability decreased with increasing concentrations of the peroxynitrite producing chemical, SIN-1, up to the 500 μM level. This relationship was not observed in cells with urate oxidase silencing. The comet assay, used to detect DNA fragmentation induced by SIN-1, indicated that cells with UOX silencing had less DNA denaturation when exposed to SIN-1. These aforementioned findings are consistent with increased protection from oxidative damage in UOX silenced cells.

The effect of reducing UOX mRNA on intracellular uric acid concentrations is unknown. Attempts were made to assess concentration differences but technical difficulties prevented accurate quantification. However, because of existing differences in the physiological responses to oxidative stimuli between the cell lines, it is likely that an increase in the intracellular concentration of uric acid occurred with UOX mRNA silencing. Additionally, neither the transfection conditions nor the expression of the shRNA affected the handling of oxidative stress, since the effects of oxidative stress in the negative control cell line remain similar to wild type cells. Reducing UOX activity levels with oxonic acid, a competitive inhibitor of the enzyme, is effective in increasing circulating uric acid concentrations.[20] Therefore, assuming the intracellular concentration of uric acid is increased in cells with UOX silencing, these results support the role of uric acid as an antioxidant.

Hexavalent chromium is a carcinogen in both human and animal models.[21] The most documented evidence of Cr(VI)

toxicity is from inhalation of the molecule, which is most apparent in the increased rate of lung,[22] kidney, prostrate and bladder cancers[23] and mental illness[22] in those exposed in the workplace. Once in the cell, Cr(VI) is reduced by intracellular antioxidants like glutathione, ascorbic acid and cysteine or through the intermediates Cr(V) and Cr(IV) to trivalent chromium (Cr(III)).[24] Trivalent chromium can interact with DNA to form Cr-DNA adducts,[25] which can induce apoptosis or mutagenesis. Interaction of the hexavalent chromium with cellular components can induce production of hydroxyl and superoxide free radicals detected by ESR, inducing oxidative stress and causing oxidative damage.[26,27] The present study indicates that cells with UOX silencing are exposed to less reactive oxygen species than wild type cells when treated with Cr(VI). This supports an existing theory that, in vitro, uric acid was effective at reducing DNA damage induced by Cr(III) exposure.[28] The probable increase in the intracellular concentration of uric acid either contributes to greater free radical scavenging or reduces the rate of free radical production, or a combination of both, thus protecting cellular components from Cr(VI)-induced reactive oxygen species damage. This raises the possibility that uric acid may be a therapeutic agent for Cr(VI) exposure, a concept that warrants further investigation.

In addition to Cr(VI), SIN-1 was used in the present experiment to induce oxidative stress. Decomposition of SIN-1 in solution at pH > 5 causes production of superoxide and nitric oxide. Together, these chemicals spontaneously form peroxynitrite, which is detected both in the media[29] and inside cells.[30] It has been well established in numerous cell types that treating cells with SIN-1 causes intracellular oxidative stress and accumulation of intracellular markers of oxidative damage in a dose-dependent manner, resulting in a reduction in cell viability.

Exposure of human lymphoid blastoma cells to SIN-1 concentrations between 1 mM and 5 mM caused a dose-dependent increase in cell death.[31] This is supported by data in human premonocytic cells where a reduction in viability was found after treatment of cells with 1 mM and 2 mM SIN-1.[32] Interestingly, rodent cell culture exhibits a lower tolerance to SIN-1-induced reduction in viability. In the present experiment, wild type cell viability began to decrease when the SIN-1 concentration in the media was 100 μM and decreased to approximately 50% viability when the concentration reached 500 μM, while a 1 mM concentration resulted in no viable cells after 24 hours. The 50% reduction in cell viability at 500 μM was also observed in rat adrenal gland cell culture.[33] Biological fluids have a quenching effect on reactive species produced by SIN-1,[34] so the origin of animal sera used to supplement growth media likely influences SIN-1-induced peroxynitrite exposure.

Incubating cells with antioxidants or antioxidant enzymes reduces the toxicity of SIN-1 in cell culture. Exposure to 1 mM or 5 mM uric acid[31,33] or a combination of catalase and superoxide dismutase[31] limited the reduction in cell viability or cell death caused by SIN-1. In the present experiment, the maintenance of cell viability between 100 μM and 500 μM SIN-1 that occured with UOX silencing supports the concept that intracellular uric acid is increased in these cells, protecting the cellular components from oxidative damage that leads to cell death.

One of the specific sites of SIN-1 induced oxidative damage is denaturation of genomic DNA. The data presented here agrees with numerous reports utilizing the comet assay as an indicator of DNA damage induced by SIN-1 in human lymph cells[29,31] and Chinese hamster fibroblasts[35] that was preventable by inclusion of catalase,[29] lycopene or β-carotene[35] in the cell culture media. The ability of antioxidants or antioxidant enzymes to limit the strand breaks implies that an increased antioxidant capacity is present in cells with UOX silencing and presumably this is an increased uric acid concentration.

As demonstrated in this study, RNA interference can be used in a mouse-derived cell culture system to reduce UOX expression, which resulted in increased protection against oxidative stress. Additionally, the results in the present study suggest that intracellular uric acid concentration was not increased to a level that became pro-oxidative. The next logical experiment would be to apply this concept in a living mouse. The advantage of retaining a certain level of UOX expression, as opposed to eliminating it completely as in the knockout models, is that the elevation of uric acid to lethal levels can be avoided. Therefore, moderate to dramatic increases in circulating uric acid concentrations would likely occur proportional to the degree of UOX knockdown which would allow for further investigation into the role that uric acid may play in disease treatment and prevention.

Several reports have established an association between serum uric acid and death due to cardiac mortality[36-38] and some evidence indicates that hyperuricemia can predict development of hypertension,[39,40] obesity,[41] kidney disease[42] and diabetes.[43,44] It has been suggested that treating hyperuricemia with allopurinol in combination with anti-hypertensive drugs and lipid-lowering therapies may synergistically act to reduce the risk of cardiovascular disease.[45] Additionally, using allopurinol to reduce uric acid exerts beneficial effects on inflammatory indices in patients with ischemic stroke.[46,47] Therefore, mice exhibiting hyperuricemia via UOX knockdown would help to better understand the associations between uric acid and diseases that are characterized by excessive oxidative stress.

Materials and Methods

Plasmid construction. Plasmid p*Silencer*4.1-CMVneo (Ambion, Austin, TX), was selected for stable expression of short hairpin RNA (shRNA) sequences in mammalian cells while conferring resistance to antibiotics. Sequences used for RNA interference were generated against mouse UOX and the following four sense sequences were chosen (1) 5'-AGC CUU CCG AAC AUU CAC U-3', (2) 5'-ACC UCA AGG UCU UGA AAA C-3', (3) 5'-GGA CUG AUC AAC AAG GAA G-3' and (4) 5'-ACC UAC ACG GUG AUA AUU C-3'. The loop sequence, TTCAAGAGA, joined the sense and antisense sequences in the hairpin structure. An additional "scrambled" shRNA sequence that was designed by Ambion to have limited similarity to the mouse genome database served as a negative control and was ligated into p*Silencer*4.1-CMVneo.

Cell culture and transfection conditions. A mouse hepatic cell line was purchased from ATCC (designation: FL83B, Manasss, VA) and grown per supplier's instructions in F-12K media (ATCC, Manasass, VA) supplemented with 10% fetal bovine serum and 500 I.U. penicillin/mL, and 500 µg streptomycin/mL. On the day of transfection, 6×10^4 cells were plated per well with antibiotic-free growth media in a 24-well plate with 48.5 µL Opti-MEM media (Invitrogen), 1.5 µL XP-sport (Ambion) and 0.5 µg p*Silencer*4.1-CMVneo with the shRNA to either UOX or the nonsense sequence per transfection reagent protocol. The transfection was completed for each of the four shRNA sequences for UOX knockdown and the nonsense shRNA sequence. Six hours post-transfection, the media was replaced with antibiotic containing growth media. Twenty-four hours post-transfection, cells were expanded to a 60-mm cell culture dish with growth media supplemented with 900 µg G418/mL media. Cells were exposed to G418 and surviving colonies were selected and expanded.

Real time RT-PCR. Real time RT-PCR was used to analyze mRNA knockdown in cell lines using acidic ribosomal protein (ARP) as a reference gene. A T-75 flask of cells was washed with 5 mL of cold Hanks balanced salt solution and layered with 3 mL Trizol LS (Invitrogen) and RNA was isolated according to manufacturer's protocol. RNA was quantified and the quality was determined using $A_{260}:A_{280}$. Two µg of RNA was reverse transcribed using oligo-dT primers (Promega) and MMLV (Promega) per manufacturer's protocol. Complementary DNA was diluted 1:4 with nuclease free water and 10 µL (for UOX PCR) or 5 µL (for ARP PCR) was used in a 50 µL PCR reaction with 25 µL 2X SYBR Green Supermix (BioRad, Hercules, CA), 1.25 µM forward ARP primer (5'-CAA CCC AGC TCT GGA GAA AC-3') and 1.25 µM reverse ARP primer (5'-GTG AGG TCC TCC TTG GTG AA-3') or 1.25 µM forward UOX primer (5'-TGG AGA CTT CAA CGG CTT CT-3') and 1.25 µM reverse UOX primer (5'-TGG CCC ATA GAT CTC CTT TG-3'). The real time RT-PCR protocol was performed on a BioRad iCycler IQ Detection System (Hercules, CA). The protocol began with a "hot-start" at 95°C for 5 minutes, followed by a cycle of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The cycle was completed forty times, followed by melt curve analysis. Real-time RT-PCR data was analyzed using the efficiency corrected relative expression method.[48] The cell line demonstrating the greatest UOX knockdown, the negative control cell line, and the wild type cell line were used in subsequent experiments to investigate the oxidative stress response.

Electron spin resonance. Electron spin resonance (ESR) spin trapping was used to detect short-lived reactive oxygen intermediates using an established method.[49] All ESR measurements were conducted using a Bruker EMX spectrometer (Bruker Instruments Inc., Billerica, MA) with a flat cell assembly and an Acquisit program was used for data acquisition and analysis. The ESR spectrometer settings were: receiver gain, 5.02×10^4; time constant, 40.96 ms; modulation amplitude, 0.50 G; scan time, 41.94 s; magnetic field 3480 ± 100 G. Experiments were performed at room temperature, under ambient air. Signal intensity was quantified by measuring average distance (mm) using the peak to peak method. Each sample (500 µL) contained 5×10^5 cells in phosphate buffered saline, 200 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and 0.0 or 200 µM hexavalent chromium (Cr(VI)) to

induce radical formation. Samples (n = 5) were incubated for 37°C for five minutes before loading into the flat cell assembly for free radical detection.

Cell viability. Cell viability was used to determine the ability of the cells to survive a reactive nitrogen species challenge. On day one, 1×10^4 cells were plated in a well of a 96-well plate and grown overnight. On day two, duplicate wells, the media was replaced with 100 µL media containing various concentrations of 3-morpholinosydnonimine hydrochloride (SIN-1). After 24 hours of SIN-1 exposure the cell viability was determined using the MTT assay (Cayman Chemical, Ann Arbor, MI) per manufacturer's protocol.

DNA fragmentation. Cells were seeded, 1.5×10^5 per well, in a 12-well plate and allowed to grow overnight. On day two, media was replaced with media that contained various concentrations of SIN-1. After three hours, cells were harvested and resuspended in 500 µL of ice-cold PBS. Fragmented DNA was detected using the Comet Assay per manufacturer's protocol (Trevigen, Gaithersburg, MD). The extent of DNA fragmentation was determined by measuring tail length beginning at center of the cells to the left rim of SYBR green fluorescence using Optimas 6.5 Image analysis software (Media Cybernetics, Silver Spring, MD).

Statistics. Data were analyzed by linear regression analysis or analysis of variance with PC-SAS general linear models procedure for significant differences among treatment means. In the event of a significant F value, the LSD procedure was used for means comparisons. Differences were considered significant at $p < 0.05$.

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