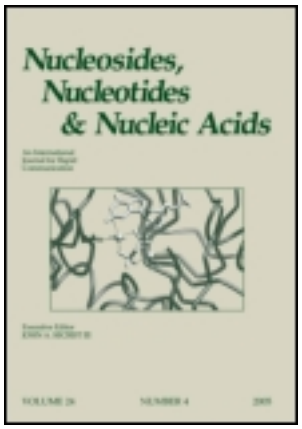

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Reactions of Peroxynitrite with Uric Acid: Formation of Reactive Intermediates, Alkylated Products and Triuret, and In Vivo Production of Triuret Under Conditions of Oxidative Stress

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REACTIONS OF PEROXYNITRITE WITH URIC ACID: FORMATION OF REACTIVE INTERMEDIATES, ALKYLATED PRODUCTS AND TRIURET, AND IN VIVO PRODUCTION OF TRIURET UNDER CONDITIONS OF OXIDATIVE STRESS

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□ *Hyperuricemia is associated with hypertension, metabolic syndrome, preeclampsia, cardiovascular disease and renal disease, all conditions associated with oxidative stress. We hypothesized that uric acid, a known antioxidant, might become prooxidative following its reaction with oxidants; and, thereby contribute to the pathogenesis of these diseases. Uric acid and 1,3-$^{15}N_2$-uric acid were reacted with peroxynitrite in different buffers and in the presence of alcohols, antioxidants and in human plasma. The reaction products were identified using liquid chromatography-mass spectrometry (LC-MS) analyses. The reactions generate reactive intermediates that yielded triuret as their final product. We also found that the antioxidant, ascorbate, could partially prevent this reaction. Whereas triuret was preferentially generated by the reactions in aqueous buffers, when uric acid or 1,3-$^{15}N_2$-uric acid was reacted with peroxynitrite in the presence of alcohols, it yielded alkylated alcohols as the final product. By extension, this reaction can alkylate other biomolecules containing OH groups and others containing labile hydrogens. Triuret was also found to be elevated in the urine of subjects with preeclampsia, a pregnancy-specific hypertensive syndrome that is associated with oxidative stress, whereas very little triuret is produced in normal healthy volunteers. We conclude that under conditions of oxidative stress, uric acid can form reactive intermediates,*

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including potential alkylating species, by reacting with peroxynitrite. These reactive intermediates could possibly explain how uric acid contributes to the pathogenesis of diseases such as the metabolic syndrome and hypertension.

Keywords Uric acid; methyluric acid; peroxynitrite; cardiovascular disease; endothelial dysfunction; triuret; ascorbate; alkylation; preeclampsia; hypertension; oxidative stress; reactive intermediates

INTRODUCTION

Uric acid is a purine generated during the degradation of RNA, DNA, and adenine nucleotides. Its immediate precursor, xanthine, is converted to uric acid by an enzymatic reaction with either xanthine oxidase or xanthine dehydrogenase. Xanthine oxidase generates the oxidants superoxide and hydrogen peroxide during this reaction. In most mammals, uric acid is further degraded to allantoin by the enzyme uricase; however, this enzyme was mutated in humans nearly 15 million years ago.[1] Thus, uric acid is considered an endproduct of purine metabolism in humans.

Uric and ascorbic acids are considered the two most important water soluble antioxidants in human plasma,[2,3] uric acid plasma levels being about 6 times that of ascorbic acids plasma levels. Uric acid can react with a variety of oxidants, including superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxynitrite. From among these oxidants, uric acid is known to react preferentially with peroxynitrite.[4,5] These reactions are thought to be initiated by the donation of an electron by uric acid to generate the urate radical, followed by its irreversible degradation to a variety of endproducts.[5,6] In this regard, the mechanism by which urate acts as an antioxidant is different from that of ascorbate in that ascorbate generates an ascorbyl radical in a reversible reaction such that ascorbate can be regenerated.[7]

The importance of the antioxidant properties of uric acid with respect to oxidants such as peroxynitrite has been demonstrated using in vitro cell culture systems.[4,8] Peroxynitrite is generated by the diffusion controlled reaction of superoxide and nitric oxide.[7] This reaction may occur in the vasculature, and particularly the endothelium, due to the presence of superoxide (generated by NADPH oxidase, xanthine oxidase, and other systems) and nitric oxide (generated by endothelial nitric oxide synthase; eNOS). Peroxynitrite has been shown to cause oxidative damage via the nitration of tissues that has been hypothesized to contribute to the development of vascular disease.[7] Incubation of endothelial cells with peroxynitrite rapidly causes oxidative stress, leading to the oxidation of tetrahydrobiopterin,[7] which is critical in the enzymatic reaction by which eNOS generates nitric oxide.[8] The addition of uric acid to cells along

with the peroxynitrite prevents this oxidative damage and partially restores nitric oxide generation. Complete restoration of nitric oxide production by eNOS occurs when both uric acid and ascorbate are added along with the peroxynitrite to cells.[4] Additionally, uric acid may help to maintain extracellular levels of the antioxidant enzyme superoxide dismutase, an important enzyme in protecting the extracellular milieu from oxidative stress.[9] Taken together, these studies suggest that uric acid is beneficial. This has led to the conclusion that elevated uric acid levels in cardiovascular disease may represent a beneficial host response to the oxidative stress known to occur in this disease process.[2,10]

Elevated serum uric acid levels are strongly associated with hypertension, obesity, the metabolic syndrome, renal disease, and cardiovascular disease. As discussed above, most authorities have viewed the elevation of uric acid as a secondary or compensatory phenomenon. This assumption has evolved due to the difficulty in demonstrating uric acid to be an independent risk factor in cardiovascular disease.[11] However, the increasing evidence that uric acid elevations often precede the development of hypertension, obesity, and metabolic syndrome[12–14] have raised questions as to the validity of this assumption. However, the increasing evidence that uric acid elevations often precede the development of hypertension, preeclampsia, obesity, and metabolic syndrome[12–15] have raised questions as to the validity of this assumption.

In recent years, our group has demonstrated in a series of cell culture, animal, and human studies that uric acid may be a true risk factor for hypertension, renal disease, and the metabolic syndrome.[16–22] Raising uric acid levels in rats with an uricase inhibitor rapidly results in the development of hypertension and renal disease.[16–18] We have also found that nearly 90% of early onset essential hypertension in adolescents have elevated serum uric acid levels (>5.5 mg/dL). In contrast, none of the control subjects in this study had elevated serum uric acid levels. The serum uric acid levels reported in this study were independent of obesity and renal function.[19] Preliminary trials in humans have also reported that lowering uric acid levels can lower blood pressure.[20,23] We have also reported that lowering serum uric acid levels in a rat model of the metabolic syndrome corrected most of the features of the metabolic syndrome.[21]

In this regard, several studies have reported that uric acid can become a prooxidant under the right conditions.[24–27] This can be explained by the formation of a urate radical under conditions of oxidative stress.[28–30] These data,[24–30] when taken together with the studies from our group, have led us to hypothesize that uric acid may combine with reactive oxygen species and nitrogen species in vivo to produce oxidation products that mediate deleterious effects on the body and contribute to the pathogenesis of hypertension, the metabolic syndrome, preeclampsia, renal disease, and cardiovascular disease.

A number of groups have investigated the reaction of uric acid with peroxynitrite and the biological implications for this reaction. Kuzkaya et al.[4] have reported that the scavenging of peroxynitrite by uric acid is important in preventing the oxidation of tetrahydrobiopterin, which would lead to the uncoupling of nitric oxide synthase. Skinner et al.[31] have reported a unique uric acid derivative which was detected in peroxynitrite reactions. Mass spectroscopic analysis indicated that this product was a nitrated uric acid derivative 2-nitrito-4-amino-5-hydroxyimidazoline. This unique product of the uric acid/ONOO- reaction was reported to result in endothelium-independent vasorelaxation of rat thoracic aorta. Thus, it was proposed that the uric acid nitration/nitrosation product may play a pivotal role in human pathophysiology by releasing ·NO, which could decrease vascular tone, increase tissue blood flow, and thereby constitute a role for uric acid not previously described. Robinson et al.[5] have reported triuret as a product of the chemical reaction of uric acid with peroxynitrite. Santos et al.[28] have reported the production of an aminocarbonyl radical in the reaction of uric acid with peroxynitrite. They also demonstrated that this radical is likely to be the species responsible for the effects of urate in amplifying peroxynitrite-mediated oxidation of liposomes and LDL. We also have identified by ESR two carbon-based radicals in the reaction of uric acid with peroxynitrite.[32] The quantitative significance of the radicals detected to final products of the reaction of uric acid with peroxynitrite is unknown and it is also unknown if other pathways are important for the reactions of uric acid with peroxynitrite. Given the background that these studies provide, our group chose to characterize the products and intermediates of the reaction of uric acid with peroxynitrite under a variety of conditions using LC-MS and LC-MS/MS as the primary analytical tools. LC-MS was used because it permitted the determination of the distribution of uric acid among the various products (determine the quantitative significance of various pathways) as well as their characterization. We demonstrate for the first time that uric acid can generate alklyating (substitution of the OH hydrogen of alcohols by dehydroxyallantoin group) species.

The principal objective of this study was to define the reactions of UA with peroxynitrite under a variety of experimental conditions with the view to identify products and intermediates.

MATERIALS AND METHODS

Chemicals, Preparation of Reagents and General Experimental Conditions

All chemicals and reagents, unless otherwise specified, were obtained from Sigma. $LiOH{\cdot}1H_2O$ and 1-methyl uric acid were purchased from Fluka. Distilled, deionized water (metal ion free), and EDTA (500 mM)

were purchased from Gibco (Langley, OK, USA). 1,3-$^{15}N_2$-Uric acid, d3-methanol and d4-methanol were purchased from Cambridge Isotopes (Andover, MA, USA). N-*tert*-butyl-α-phenylnitrone (PBN) was purchased from Alexis Biochemicals (San Diego, CA, USA). Diethylenetriaminepentaacetic acid (DTPA) was purchased from Sigma. Peroxynitrite was purchased from Cayman Chemical (Ann Arbor, MI, USA). The distilled water purchased from Gibco was used in solution preparation. Uric acid (100 mM), 1,3-$^{15}N_2$-uric acid (100 mM) and L-tyrosine were prepared by dissolving these compounds in KOH (300 mM). Lithium urate (30 mM) and lithium 1,3-$^{15}N_2$-urate (30 mM) solutions were prepared by dissolving uric acid and $^{15}N_2$-uric acid, respectively, in a LiOH solution (35 mM, pH = 8.05), ascorbate (100 mM), tetrahydrobiopterin (100 mM), glutathione (100 mM), cysteine (100 mM), and LiOH·1H_2O (35 mM) were prepared by dissolving these compounds in distilled water. EDTA (500 mM) was diluted to 10 mM using distilled water. Potassium phosphate buffer (300 mM) was prepared by dissolving 16.00 g monobasic potassium phosphate and 31.70 g dibasic potassium phosphate in distilled water. The pH of the resulting final solution was adjusted to pH = 7.4 or 9.0 using KOH (300 mM) or 1:1 phosphoric acid. The final volume of the potassium phosphate buffer was 1.0 L. In all experiments peroxynitrite was shielded from light and kept on ice and reactions were performed protected from light. The human studies were approved by the Institutional Review Board at the University of Florida. The reactions are summarized in Figure 1.

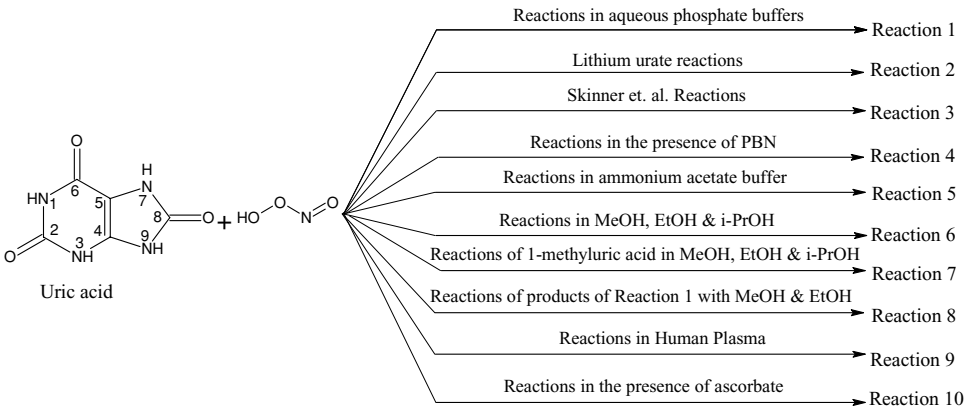

FIGURE 1 The summary of the reaction of peroxynitrite with uric acid and 1,3-$^{15}N_2$-uric acid. Reactions 1–8 were conducted as a pair with labeled and unlabeled uric acid to aid in the mass spectrometry analysis and identification of products. The effect of multiple pHs (7.4 and 9.0) were also investigated in Reactions 1 and 2 and these did not alter the formation products determined by mass spectrometry. Reaction 7 employed 1-methyluric acid instead of uric acid. For the structures of products see Figure 3.

Determination of Peroxynitrite Concentration

In preliminary studies, we have used PN prepared using two different published methods[33,34] and two commercial products (Cayman Chemical and Upstate Biotechnology, Lake Placid, NY). All produced the same products. After the initial testing, we used the peroxynitrite from Cayman Chemical for all the studies reported here.

Peroxynitrite concentration was determined prior to its use (as per the instructions from Cayman Chemical) in each experiment as follows: Peroxynitrite (Cayman) was placed on ice to thaw. A 25 μL aliquot of the peroxynitrite stock solution was diluted with 975 uL of KOH (300 mM). The absorbance of the resulting solution at 302 nm was then measured, using KOH (300 mM) as a blank. The concentration of peroxynitrite was then calculated using the molar extinction coefficient of 1670/M·cm. The volume of peroxynitrite needed to yield the desired concentration of peroxynitrite for a given experiment was then calculated.

Reactions of Uric Acid with Peroxynitrite in Aqueous Buffers [Reaction 1]

Uric acid stock solution (300 μL of a uric acid stock solution) was added to potassium phosphate buffer (pH 7.4) containing EDTA. One equivalent of peroxynitrite (in some experiments both 1 and 3 equivalents were used) was then added to the reaction mixture. The stock peroxynitrite concentration varied in each lot of commercially available peroxynitrite. As a result, the volumes of potassium phosphate buffer and peroxynitrite would vary by experiment, but the total reaction mixture volume was held constant at 3.00 mL, the added volume of uric acid stock solution was always 300 μL (concentration of the uric acid stock solutions was varied such that this volume of added uric acid remained constant). The final concentrations of uric acid varied from 300 μM (physiological) to 10 mM and peroxynitrite varied from 300 μM 10 mM, and these reagents were used in a 1:1 molar ratio unless otherwise specified. Final concentration of EDTA was 0.1 mM. Reactions in aqueous buffer were conducted at pHs 7.4 and 9.0. The reaction mixture was vortexed for 10 seconds and allowed to incubate at room temperature for 3 minutes. After 3 minutes at room temperature, the reaction mixtures were placed in ice and transferred to LC-MS vials on ice via Pasteur pipettes. The vials were immediately placed at -80°C until mass spectroscopic analysis could be performed. Please note that Reaction 1 was our "model reaction" upon which all other reaction procedures were based.

Reactions of Lithium Urate with Peroxynitrite [Reaction 2]

Lithium urate (300 μL of a prepared stock solution) reactions with peroxynitrite were conducted using conditions and volumes of reagents as in Reaction 1. Final concentrations of Li urate ranged from 0.3 to 10 mM.

Final concentrations of peroxynitrite ranged from 0.3 to 10 mM. Lithium urate were conducted at pHs 7.4 and 9.0.

Skinner Reactions [Reaction 3]

We have duplicated some of the reactions reported by Skinner et al.[31] with view to determine the product formed under their reaction conditions. Uric acid stock solutions were prepared in 100 mM potassium phosphate buffer containing 0.1 mM DTPA. Peroxynitrite (0, 0.25, 0.5, 0.75, or 1.0 mM) was then added to potassium phosphate buffer solution and allowed to incubate by itself in the buffer solution for 15 minutes. The total volume of the peroxynitrite/potassium phosphate buffer mixture was 2.70 mL. The volume of the potassium phosphate buffer was varied to account for variances in the peroxynitrite stock solution concentration. Next, 300 μL of one of the uric acid stock solutions was added to each of the peroxynitrite/potassium phosphate buffer solutions. This was repeated using three different stock solutions of uric acid such that at a final volume of 3.0 mL, the final concentrations of uric acid were 0.1, 0.2, and 0.3 mM. These reaction mixtures were allowed to incubate at room temperature for another 15 minutes, and were then placed in LC-MS vials and stored at −80°C until LC-MS analysis.

Reactions of Uric Acid with Peroxynitrite in the Presence of PBN [Reaction 4]

Uric acid (300 μL of 100 m M stock solution) was added to potassium phosphate buffer containing DTPA (0.1 mM final concentration) and the spin trapping agent, PBN. PBN concentrations used were 10 or 50 mM. Final reaction volumes were held constant at 3 mL (see Reaction 1). One equivalent (final concentration of 10 mM) of peroxynitrite was then added to the reaction mixture. The reaction mixture was transferred to LC-MS vials and stored at −80°C to await LC-MS analysis.

Reactions of Uric Acid with Peroxynitrite in Ammonium Acetate Buffer [Reaction 5]

Uric acid was weighed out into a vial (5.04 mg) to which a mixture of 50% ammonium acetate and 50% acetonitrile was added. The volume of the ammonium acetate:acetonitrile mixture and the volume of peroxynitrite to be added to the reaction mixture was varied to account for variances in the peroxynitrite stock solution concentration. To the heterogeneous mixture of uric acid in ammonium acetate:acetonitrile was added 1 equivalent of peroxynitrite, such that the final concentrations of uric acid and peroxynitrite were each 10 mM. The mixture was vortexed, and allowed to sit for

3 min at room temperature. It was then transferred to LC-MS vials to await analysis.

Reactions of Uric Acid with Peroxynitrite in Methanol, Ethanol, and Isopropanol [Reaction 6]

Uric acid powder (5.04 mg) was added to methanol, d4-methanol [CD_3OD; some reactions were also conducted with d3-methanol (CD_3OH)], ethanol or isopropanol and mixed. Uric acid only partially dissolved under these conditions. One equivalent of peroxynitrite (diluted to 1.5 ml) was then added to the reaction mixture (final volume was 3 ml of 50:50 alcohol:water). The final concentrations of uric acid and peroxynitrite were each 10 mM. The reaction mixture was vortexed and allowed to incubate at room temperature for 3 minutes. Uric acid dissolved completely as the reaction proceeded. After 3 minutes, at room temperature, the reaction mixtures were placed in ice and transferred to LC-MS vials on ice and stored at −80°C to await LC-MS analysis.

Reactions of 1-Methyluric Acid with Peroxynitrite in Methanol, Ethanol, and Isopropanol [Reaction 7]

The above series of reactions (see Reaction 6) were carried out in the same manner using 1-methyluric acid in the place of uric acid in order to elucidate the mechanism of reaction of peroxynitrite with uric acid.

Reaction of Products of Reaction 1 with Methanol and Ethanol [Reaction 8]

To an ice-cold solution of the reaction products from Reaction 1 (aqueous buffers), two times the volumes of either methanol or ethanol was added and vortexed. This cold solution was immediately analyzed by LC-MS.

Reactions of Uric Acid with Peroxynitrite in Human Plasma [Reaction 9]

$^{15}N_2$-uric acid was also reacted with peroxynitrite using human plasma diluted (1:3 plasma:buffer) with potassium phosphate buffer (pH 7.4) rather than in potassium phosphate buffer alone. This set of reactions was run following the procedure for Reaction 1 with the following exceptions: $^{15}N_2$-uric acid, at one of three different concentrations, was added to a 1:3 mixture of human plasma and potassium phosphate buffer. Final concentrations of $^{15}N_2$-uric acid were 0.3 mM (normal physiological concentration), 1.0 mM or 10 mM. Final concentrations of peroxynitrite were 1, 3 and 30 mM, respectively. The volume of the plasma:buffer mixture and the volume

of peroxynitrite to be added to the reaction mixture was varied (as above) to account for variances in the peroxynitrite stock solution concentration. All final reaction volumes were 3 mL. Once the reactions were complete, 3.0 mL of ice-cold 20% TCA was added to each of the reaction mixtures in order to deproteinize the mixture prior to LC-MS analyses. The resulting reaction mixture:TCA slurries were centrifuged (20 minutes at 4°C) in Eppendorf tubes (1.5 mL volume). The supernate was pipetted off of the centrifuged mixtures and was filtered using TCA-pretreated 0.2 μM centrifuge filters. The supernate was filtered for 10 min at 4°C, and was then transferred to LC-MS vials and stored at −80°C to await LC-MS analysis.

Reactions of Uric Acid with Peroxynitrite in the Presence of Ascorbate [Reaction 10]

Uric acid (300 μL) was added to potassium phosphate buffer (pH 7.4) containing EDTA. One equivalent of ascorbate (300 μL, see "Preparation of Reagents") was then added to the reaction mixtures. Next, 1 and 2 equivalents (separate reactions) of peroxynitrite were added to the reaction mixtures. The reaction mixtures were then vortexed for 10 seconds and allowed to incubate at room temperature for 3 minutes. Final reaction volume was 3.0 mL in each case. Final concentrations of uric acid and ascorbate were 10 mM in each case. After 3 minutes at room temperature, the reaction mixtures were placed in ice and transferred to LC-MS vials on ice. The vials were immediately placed at −80°C until LC-MS analysis could be performed.

The Preparation of 4,5-Dimethoxy-4,5-dihydrouric Acid (3K)

Compound 3K was prepared based on the method of Biltz and Heyn[35] by passing a rapid stream of chlorine (Matheson Tri-Gas) into a suspension of uric acid in MeOH, the temperature being kept below 15°C.

LC-MS Analyses of Reaction Mixtures

The reaction mixtures were analyzed by LC-MS in the fullscan (both positive and negative), tandem mass spectrometry (MS/MS) and single reaction monitoring (SRM) modes using atmospheric pressure chemical ionization (APCI) method. In addition, some samples also were further analyzed using the electrospray ionization (ESI) method. This enabled us to detect both positive and negative ions present as well as the unique fragmentation pattern of each intermediates/products present. The LC-MS analyses were performed using a Finnigan triple quadrupole mass spectrometer model TSQ 7000 and an Agilent quaternary pump HPLC

model 1100 equipped with a Phenominex Luna C18 column (either 150 or 250 × 4.6 mm). Samples were kept cold until analysis to minimize the decomposition of labile intermediates. The mobile phases used in the LC separation of the uric acid reaction products included NH_4OAc/AcOH and methanol (as gradient), formic acid/methanol gradient or NH_4OAc/AcOH /acetonitrile gradient. Typical HPLC analyses were carried out in a gradient elution mode using an aqueous mobile phase A (5 mmol/L ammonium acetate and 0.1% by volume of AcOH in water) and MeOH as organic mobile phase B. Mobile phase flow was 0.6 mL/min. Two typical HPLC gradients used are as follows: Gradient 1 (10 minutes, HPLC run): The gradient began at 90% A. The composition was linearly ramped to 75% A over the next 9 minutes, it remained constant for 0.5 minutes, and then it was reversed to the original composition of 90% A over 0.5 minutes. Gradient 2 (16 minutes HPLC run): The gradient began at 95% A. The composition was linearly ramped to 80% A over the next 9 minutes and to 10% A over the next 3 minutes, it remained constant for 2 minutes, and then it was reversed to the original composition of 95% A over 1 minute, after which it was kept constant for 1 minute to re-equilibrate the column.

Evaluation of the molecular weight and fragmentation patterns of the intermediates and products was performed using the mass spectrometer after the compounds had been separated on the LC column. The mobile phase flow was 0.6 mL min^{-1}, and the injection volume was 20 μL. Uric acid concentrations were determined in SRM mode using APCI source in the negative mode (reactions monitored for uric acid were m/z 166.9→95.9 at 25V and 166.9→123.9 at 25V and for $^{15}N_2$-uric acid were m/z 168.9→96.9 at 25V and 168.9→124.9 at 25V). Methylallantoin, allantoin and triuret were quantitated in the APCI positive mode using their respective SRM ions. In the TSQ 7000 instrument, nitrogen was used as both the sheath (80 psi) and the auxiliary (10 units) gases. For APCI mode, the vaporizer was kept at 500°C, and the heated capillary temperature was maintained at 200°C. The corona current was set to 3 kA by applying approximately 4 kV to the corona needle. The second quadrupole was used as a collision chamber with argon as a collision gas at a pressure in vicinity of 2.5 × 10^{-3} Torr. The LC-MS/MS ran on Xcalibur software, which is a flexible Windows NT PC-based data acquisition system that allowed complete instrument control. The operations of the LC-MS and data analyses were performed using the ThermoFinnigan Xcalibur software.

We have developed validated LC-MS/MS methods for the quantitation of uric acid and its metabolites triuret, allantoin, and 6-mainouracil. The draft of these methods has been submitted elsewhere for publication. The calibrations for the quantitations were performed using commercial standards of the metabolites and UA. The correlation coefficients were 0.991∼0.999, from the calibration curve. The intra- and interday precision

(RSD.%) of the metabolites ranged from 0.5% to 13.4% and 2.5% to 12.2%, respectively. For UA, the correlation coefficient was 0.9998∼1.0000 and intra- and interday precision ranged 1.30∼3.07 and 0.61∼3.68.

Determination of Triuret Levels in Healthy Volunteers, Patients with Normal Pregnancy, and Patients with Preeclampsia

The triuret concentration in the urine samples determined by LC-MS/MS developed specifically for the determination of uric acid metabolites in urine. Urine samples from volunteers or patients were stored at −80°C until analysis in cryo-vials. Before analysis the samples were diluted with water and mixed with water and filtered through 0.2 μM filter. The LC-MS/MS analyses were carried out with APCI interface operated in positive-ion mode. Liquid chromatography analyses were performed in a gradient elution mode using Phenomenex Luna 5μ C18 (2) 100Å (150 mm × 4.6 mm) column and ammonium acetate/acetic acid and methanol as mobile phases. The mobile phase flow was 0.6 mL min^{-1}, and the injection volume was 20 μL. In mass spectrometry, nitrogen was used as both the sheath (80 psi) and the auxiliary (10 units) gases. The vaporizer was kept at 500°C, and the heated capillary temperature was maintained at 200°C. The corona current was set to 3 kA by applying approximately 4 kV to the corona needle. The ions (parent M+1 ion at m/z 147) selected for SRM were *m/z* 130, 104. and 87.1. at 25V. The quantitation was achieved by using external calibration standard. The calibration range was 0.004 to 10.0 mg/dL and it was linear with the R^2 value of 0.9964. The operation of the LC-MS and data analysis was performed using the ThermoFinnigan Xcalibur 1.4 software.

RESULTS

In the past, the purine uric acid was considered to be one of the endproducts generated during the degradation of RNA, DNA, and adenine nucleotides. We found, however, that urate reacts with peroxynitrite to yield reactive intermediates that further react with alcohol to yield uric acid derived products containing the alkoxyl groups. When the reaction mixture was left overnight (in about 60 minutes most of the uric acid had reacted) at ambient temperature before LC-MS analysis, triuret was identified as the sole final product of the reaction. Formation of triuret occurs in plasma and *in vivo* under pathological conditions. Reactions conducted in the presence of MeOH, EtOH, and i-PrOH resulted in the alkylation of uric acid intermediates to yield O-alkylated allantoin. To our knowledge, this alkylation to yield O-alkylallantoin has not been described in the literature in uric acid's reactions with peroxynitrite. This

alkylation potentially could be an alternate pathway by which peroxynitrite-activated uric acid could exert biological effects under pathological conditions. The peroxynitrite reactions with uric acid are summarized in Figure 1.

Reactions of Uric Acid with Peroxynitrite in Aqueous Buffers

A series of reactions of uric acid with peroxynitrite were conducted with both unlabeled and labeled uric acid and analyzed by LC-MS. The parallel use of labeled and unlabeled uric acid permitted the identification of uric acid derived products by LC-MS and LC-MS/MS. Uric acid reactions were conducted typically at pH 7.4. Some of the reactions were conducted simultaneously at pHs 7.4 and 9.0 and the pH had no effect on the products suggesting that the reaction occurs with the mono anion of uric acid. The final concentrations of uric acid varied from 300 μM (the high end of the human physiological concentration) to 10 mM and peroxynitrite concentrations were 1 and 3 equivalents to uric acid. In the ranges studied, the uric acid and peroxynitrite concentrations had no effect on the products formed. Treatment with one equivalent of peroxynitrite resulted in the complete or nearly complete reaction of uric acid.

Figure 2 shows the full scan LC-MS total ion trace of one such reaction in which kinetic studies were conducted at refrigeration temperature (4°C). The reaction initially produced two intermediates: one with a $[M+NH_4]^+$ or $[M+H]^+$ (see discussions later) ion at m/z 216 *{*2B [3H (or 3R); Figure 3]; RT = 4.93 minutes*}* and second with a $[M+H]^+$ ion at m/z 231 [2D (3K); RT = 6.01 minutes]. At 0 minutes (3 minutes reaction at ambient temperature, followed by refrigeration), 41% uric acid had reacted to yield 21% 2B and 20% 2D as determined by LC-MS. At 30 minutes, 60% uric acid had reacted to yield 31% 2B, 23% 2D, and 6% triuret. By 1hr at room temperature 75% of uric acid had reacted to produce 3% 2B, 6% 2D, 33% triuret and 33% 2E (3O, m/z 173, RT 3.74 minutes). After 16 hours at room temperature, all the starting materials and intermediates were converted to triuret (2C). From several studies conducted by us in aqueous medium, it is evident that the formation of triuret is a signature reaction of peroxynitrite with uric acid in aqueous medium. The LC-MS/MS identification of triuret is shown in Figure 4. Robinson et al.[5] had identified triuret as a product of the reaction of peroxynitrite with uric acid. Our studies indicate the formation of labile intermediates that undergoes further reaction with HPLC eluent (MeOH) to yield alkylated methoxy compounds. When allowed to further react in aqueous solution (without exposure to MeOH before reaction is complete), these intermediates undergo conversion to triuret in the reaction mixture. These intermediates could react with biomolecules with suitable functional groups to form products other than triuret. No significant quantities of

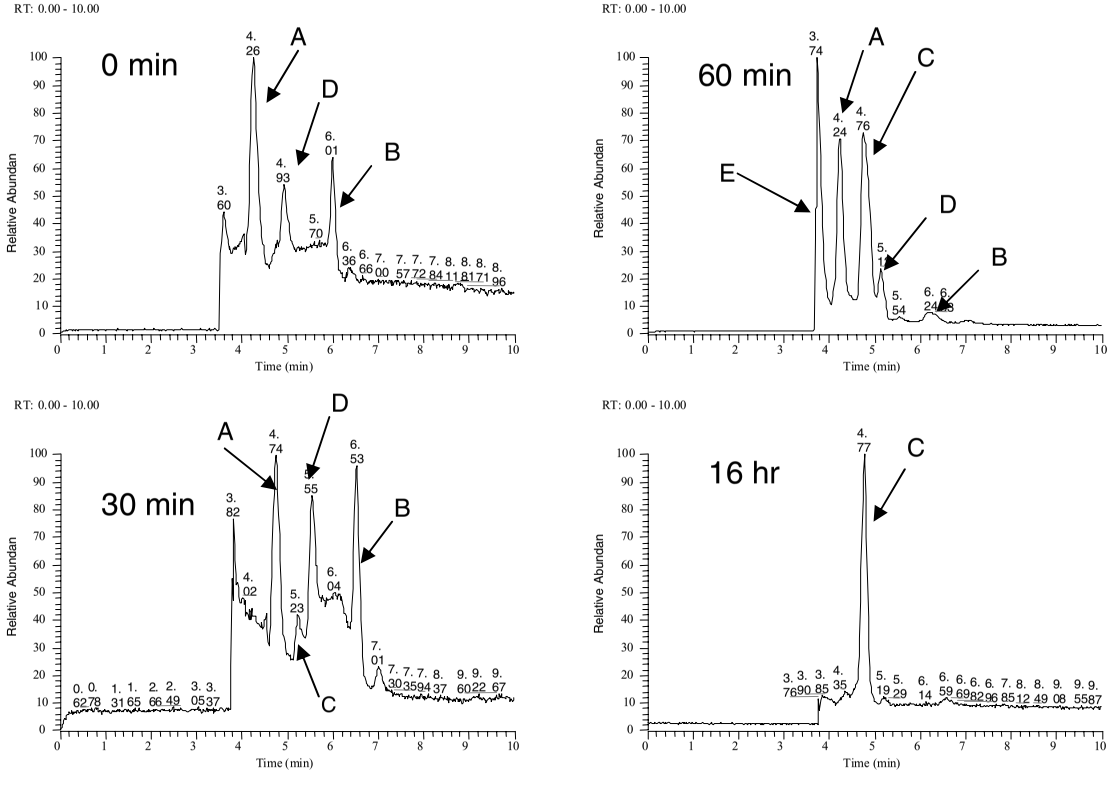

FIGURE 2 LC-MS analysis (total ion current; X axis = retention time; Y = relative abundance) of the time course of the reactions of uric acid with peroxynitrite. Reactions were carried out at ambient temperatures for 3 minutes, followed by rapid cooling in ice-water. Time 0 (actual start time is 4 minutes (3 minutes in ambient temperature and 1 minute in ice-water). Subsequent reactions were monitored from samples that were kept refrigerated (4°C). At 0 minute, 41% of uric acid had reacted with the formation of two intermediates B (21%, M+1 = 216) and D (20%, M+1 = 231). At 30 minutes, the intermediates B (32%) and D (23%) were present in higher concentrations, with a corresponding lowering of the concentration of the unreacted uric acid (40%). Also observable at this time point was the initial formation of triuret (C, 6%). At 60 minutes, it can be seen that the intermediates B (3%) and D (6%) had decreased and a higher concentration of triuret (33%) was observed compared to 30 min. Some unreacted uric acid (25%) was still observable at this time point. In addition, a new compound E (33%, M+1 = 173) was observable. At 16 hours, both B and D as well as all remaining unreacted uric acid were no longer observable as all have reacted completely to give triuret as the sole observable product of the reaction.

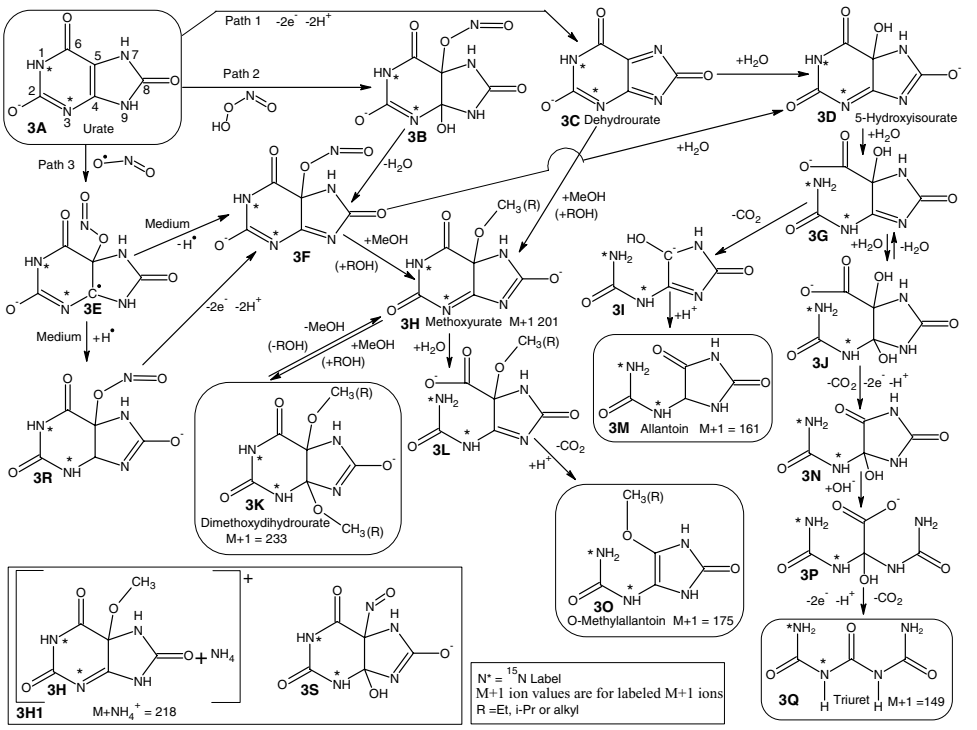

FIGURE 3 The schematic diagram proposed for the reaction of 1,3-$^{15}N_2$-uric acid with peroxynitrite in aqueous solutions (Reactions 1 and 2) and in the presence of methanol (Reaction 6; ethanol also produces similar products with the Et group substituting for Me group). The reactions with unlabeled uric acid (not shown) produced the corresponding unlabeled products. Reactions conducted in the presence of MeOH or when reaction product mixture in the cold was treated with MeOH, products 3K and 3O were produced. Two alternate pathways from uric acid are proposed. One involves the addition of peroxynitrite or the elements peroxynitrite across the C4-C5 double bond (path 2, Figure 3) of uric acid to form intermediate 3B and the second one involves the the addition of ·ONO radical to yield 3E, 3F or (and) 3R. Both pathways explain the formation of m/z 116. Based on several reports of the formation of ·ONO radicals from peroxynitrite, the pathway leading to 3E is the logical next step in its reaction with urate. One possibility is that 3B forms from a complex radical mediated process or from partially or completely formed radicals still enclosed in the solvent shell. In aqueous buffers triuret (3Q) is the final product with some reactions producing small quantities (1–2%) of allantoin (3M). Reactions in MeOH-H_2O mixtures produced O-methylated allantoin as the dominant final product (96%). This reaction indicates that uric acid activated by peroxynitrite can act as alklyating agent for biological molecules containing OH groups, and possibly other groups such as –SH, –COOH, $–NH_2$ and >NH.

alloxan or parabanic acid were detected in the reactions, although small quantities (1–2%) of allantoin were observed in some reactions.

MS/MS experiments showed that the peak at m/z 216 produced ions at m/z 199, 167, 156, and 124 at a collision energy of 25 V.

When 1,3-$^{15}N_2$-uric acid was used, all the products observed, including triuret were doubly labeled suggesting that they all retained nitrogens N1 and N3 of the six membered ring of uric acid.

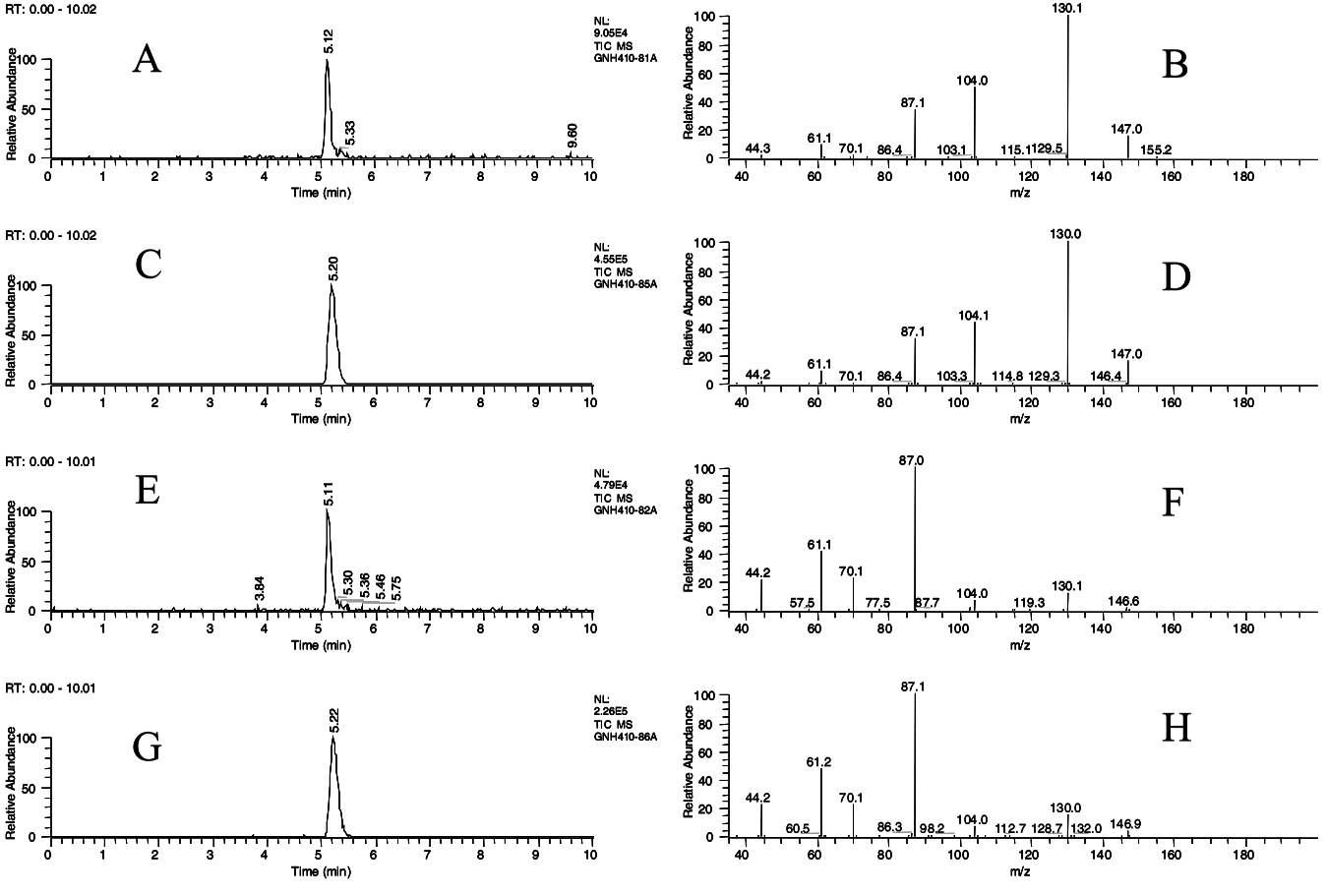

FIGURE 4 LC-MS identification (by MS/MS) of standard triuret and that from the reaction of uric acid with peroxynitrite [Reactions1]. Panel A is the total ion chromatogram of triuret standard and panel B is the MS/MS data generated at 15V. Panels C and D are the corresponding ones from Reaction 1. Panel E is the total ion chromatogram of triuret standard and panel F is the MS/MS data generated at 25V. Panels G and H are the corresponding ones from Reaction 1. As can be seen from retention times of 5.1 min and MS/MS data in panels B and D, and F and H, there is a perfect match between the standard and the reaction products.

Uric Acid Reactions with Peroxynitrite in Aqueous Buffers in the Presence of PBN

A series of reactions of uric acid with peroxynitrite were conducted with both unlabeled and labeled uric acid in the presence of excess PBN (5 times that of uric acid and peroxynitrite) and studied by LC-MS its effect on the products in Reaction 1. The idea behind the experiments was to see if trapping of potential urate radical by PBN would alter the products of reactions outlined in Figure 3. Analysis of the reaction products showed that there were no significant differences in the products between reactions conducted with and without PBN. The usual uric acid products analyzed by LC-MS (see Figure 2) were well separated from PBN and hence we were able to determine the effect of PBN on their formation. However, if small quantities of PBN adducts were formed under these conditions and they came under the PBN peak in LC-MS analysis, the existing method will not be able to determine them. Since these experiments were designed to test the effect of added PBN on the products in Figure 2, it posed no problem.

Uric Acid Reaction with Peroxynitrite in Ammonium Acetate Buffer

Reactions of uric acid with peroxynitrite were also conducted in ammonium acetate buffer and a 50:50 mixture of ammonium acetate buffer and acetonitrile. These reactions produced mainly triuret.

Skinner et al.[31] Reaction

We have repeated some of the reactions of uric acid and peroxynitrite reported by Skinner et al.[31] They have reported the product of the reaction to be 2-nitrito-4-amino-5-hydroxyimidazoline; whereas, we observed triuret as the only identifiable product in the LC-MS analyses of these reaction products (see Figure 4 for the tandem mass spectrometric identification of triuret).

Uric Acid Reaction Products with Peroxynitrite in Alcohol (MeOH, EtOH or i-PrOH)-Water (50:50)

Subsequent analyses of Reaction 1 product mixtures showed that the products 2B[3H or (3R)] and 2D(3O) were formed in the HPLC when MeOH was used as one of the mobile phases. Use of other common LC-MS solvents such as acetonitrile did not provide good separation of the reaction products, and thus were not pursued further. To further explore the mechanism of this reaction, we chose to expose urate to peroxynitrite in methanol containing aqueous buffer (from peroxynitrite solutions). This resulted in the initial formation of products, 3H (or 3R) (15%), unreacted

uric acid (12%), triuret (detected), 3K (23%), and 3O (50%). When this mixture was left at ambient temperature overnight, all of the unreacted uric acid and products (other than 3O) were converted to a mixture containing 96% 3O and 4% triuret. Reaction with labeled uric acid gave the same correspondingly labeled products. The tandem mass spectrometric identification of alkylallantoins is shown in Figure 5. Even though 3O contains the dehydroxyallantoin group, it is not derived form allantoin, but rather from methoxylated (alkoxylated) precursor 3H.

Uric acid reactions with peroxynitrite were also conducted in the presence of labeled MeOH (both d3- and d4-methanol gave same products). Reaction of unlabeled uric acid with peroxynitrite in d4-MeOH produced primarily d3-labeled 3O (6A; Figure 6) (M+1 = 176) (~88% of products) and a mixture of d3- and d6- [~12% of products; ratio of d3 (M+1 = 234) and d6(M+1 = 237) = 1.0:0.45] labeled 3K (6B and 6C), corresponding to the incorporation of the d3 substituted Me group to form d3- and d6-dimethyl-3K. Small amounts of 3H (3R) (M+1 = 216) were also present. Reactions of $^{15}N_2$-uric acid with peroxynitrite in d4-methanol produced primarily the corresponding d3- and $^{15}N_2$-labeled 3O (6D) (M+1 = 178) and d3 and d6 and $^{15}N_2$-[ratio of d3 (M+1 = 236) and d6 (M+1 = 239) = 1.0: 0.40]-labeled 3K (6E and 6F), corresponding to the incorporation of the d3 substituted Me group to form d3- and d6-dimethyl-3K. Small amounts of corresponding 3H (or 3R) isomer (M+1 = 218) were also present.

We also explored the reaction of uric acid with peroxynitrite in EtOH and i-PrOH that have greater degree of steric hindrance than methanol. LC-MS analysis of the EtOH reaction products showed a single peak (RT = 3.92 minutes) with an M+1 ion of m/z 187, corresponding to the O-Et-allantoin (3O with Et group instead of Me group). Similar LC-MS analysis of the reaction mixture in i-PrOH produced a dominant peak (8.07 minutes at 12 minutes run; 95%) at m/z 201, corresponding to the isopropyl substituted 3O. No detectable amount of triuret was formed in reactions conducted in the presence of EtOH and i-PrOH, even though these mixtures contained 50% (by volume) of water. When the ice-cold reaction mixtures of uric acid and peroxynitrite in aqueous buffer containing preformed reactive intermediates from previously completed Reaction 1 (3 minutes reaction at ambient temperature, followed by cooling in ice-water mixture) was treated with MeOH or EtOH, it resulted in the formation of the corresponding methylated or ethylated allantoin (3O) as determined by LC-MS analyses.

We also studied the reaction of 1-methyluric acid with peroxynitrite in MeOH to further confirm our identification of the products and to test the generality of the reaction of uric acids. LC-MS analysis showed mainly two peaks (3.60 minutes (26%), 7.05 minutes (74%); 15 minutes analytical run). The peak at 3.6 minutes had a $[M+H]^+$ ion at m/z 187, corresponding to the monomethylated 3O and the peak at 7.05 minutes had a $[M+H]^+$ ion

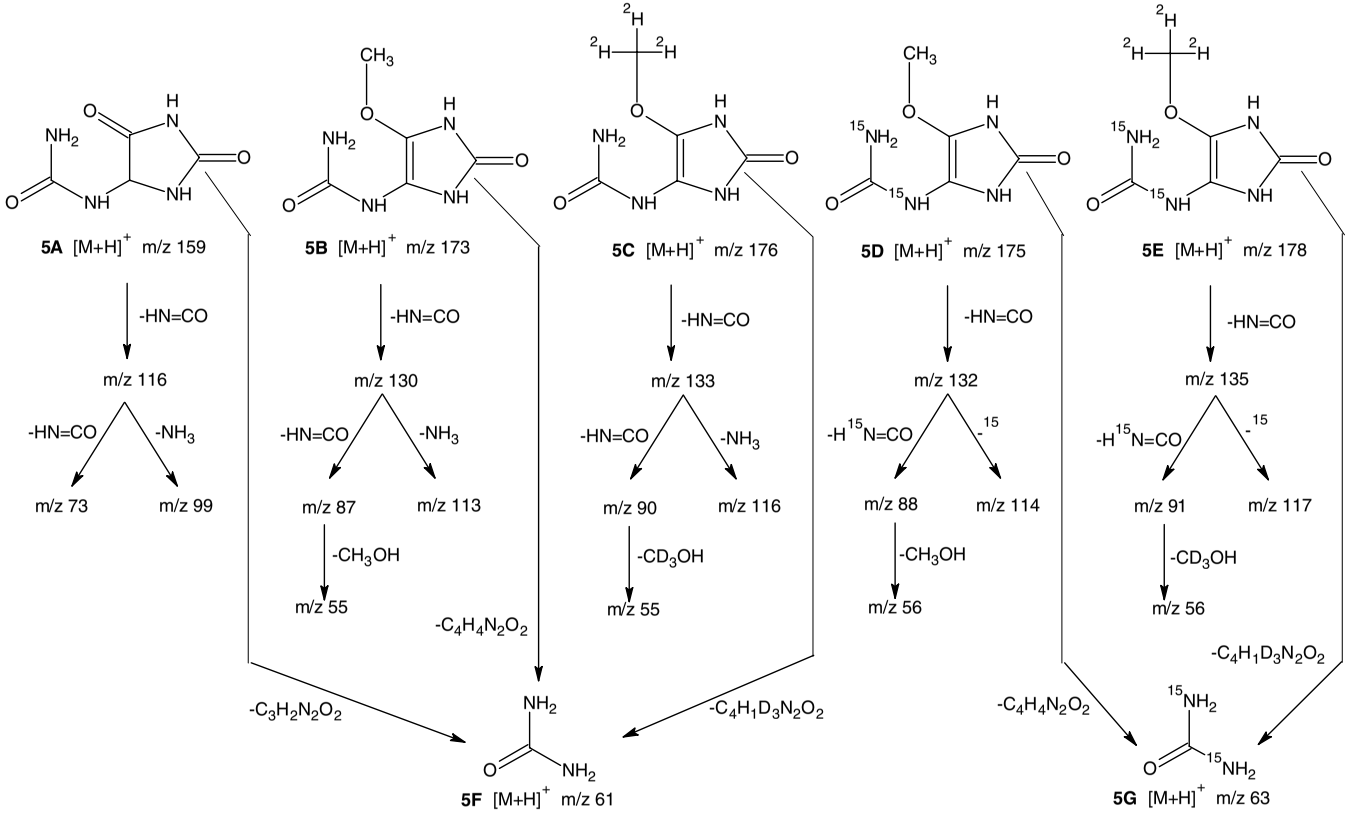

FIGURE 5 The main fragmentation patterns of protonated allantoin and labeled and unlabeled methylallantoins under CID conditions (collision energies used: 15 and 25V). Notations on scheme: 5A represents standard unlabeled allantoin; 5B represents O-methylated allantoin produced from the reaction of uric acid with peroxynitrite in the presence of CH_3OH; 5C represents deuterium labeled methylated allantoin produced from the reaction of uric acid with peroxynitrite in the presence of CD_3OD (CD_3OH also produced the same product); 5D represents $^{15}N_2$-labeled methylated allantoin produced from the reaction of 1,3-$^{15}N_2$-uric acid with peroxynitrite in the presence of CH_3OH; 7E represents deuterium and ^{15}N labeled methylated allantoin produced from the reaction of 1,3-$^{15}N_2$-uric acid with peroxynitrite in the presence of CD_3OD and 5F and 5G are unlabeled and labeled urea. The loss of unlabeled H-N=C=O from 5D and 5C in initial fragmentation shows that it occurs from the 5-memebered ring and that the formation of urea fragments and loss either labeled (5C and 5E) or unlabeled MeOH (5B and 5D) are further confirmation of the structures.

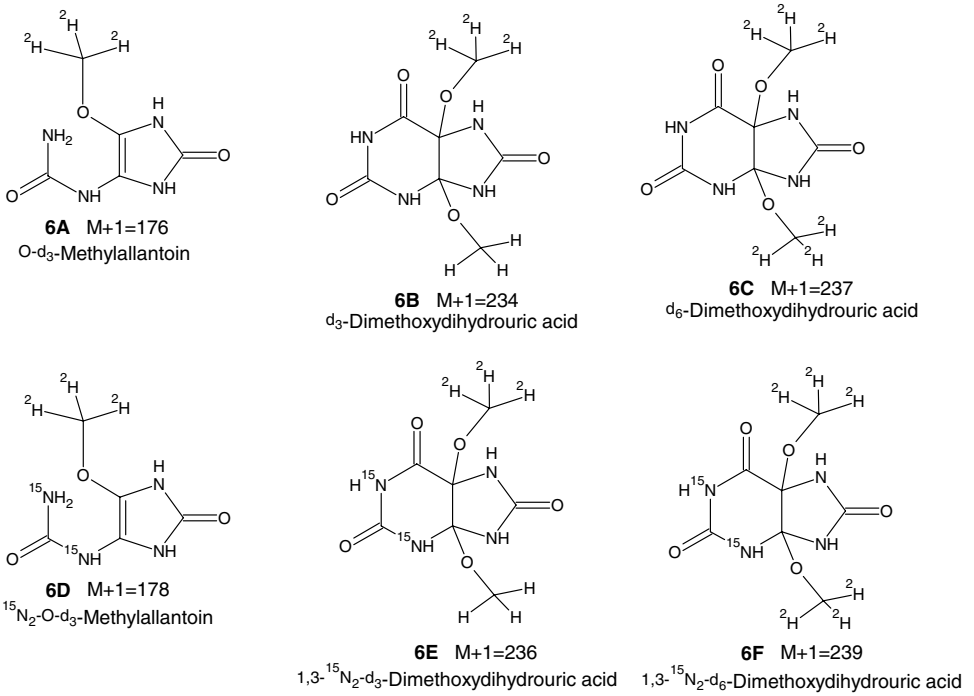

FIGURE 6 Structures of labeled 4,5-dimethoxy-4,5-dihydrouric acid and O-methylallantoin.

at m/z 251, corresponding to the monomethyl 3K. When the same reaction was conducted in d4-methanol, the peak at 3.6 minutes had an $[M+H]^+$ ion of m/z 190, corresponding to the incorporation of the d3 substituted Me group to form d3-methyl-3O and the peak at 7.05 minutes had an $[M+H]^+$ ion of m/z 251, corresponding to the incorporation of two d3-Me groups from solvent to form N-methyl-3K.

The unlabeled compound 3K was synthesized using the reaction of chlorine[35] with uric acid in MeOH. It had the same mass spectrum, retention time and fragmentation pattern as 3K in LC-MS analysis. This provided further confirmation of the structure of 3K.

Reactions of Lithium Urate with Peroxynitrite

We also investigated the reaction of lithium urate (more soluble at physiological pH). When urate was exposed to peroxynitrite under these conditions, the reaction proceeded in the same fashion as it did when the urate had been dissolved in KOH and then added to the phosphate buffer prior to being exposed to peroxynitrite.

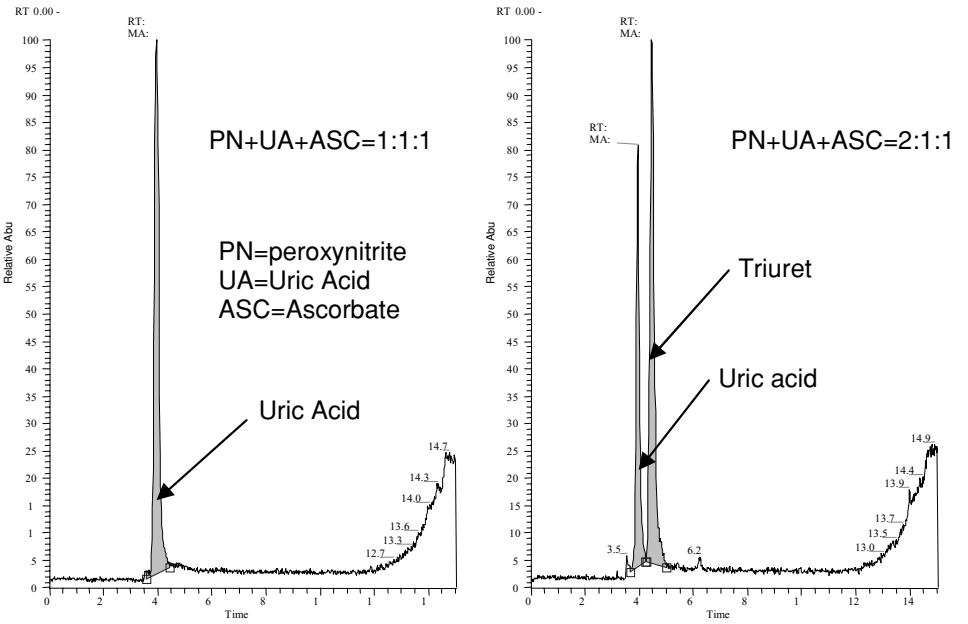

FIGURE 7 LC-MS analysis (total ion current) of the reaction of uric acid in the presence of 1 molar equivalent of ascorbate, and 1 (left) or 2 (right) molar equivalents of peroxynitrite. In a 1:1:1 molar ratio of ascorbate:urate:peroxynitrite, ascorbate appears to completely block the reaction of urate with peroxynitrite, as unreacted uric acid is observable, but the product triuret is not. In increasing the concentration of peroxynitrite, such that we have a 1:1:2 molar ratio of ascorbate:urate:peroxynitrite, the ascorbate is still able to partially block the reaction of urate with peroxynitrite, as some unreacted uric acid is observable, as well as the product triuret.

Reaction of Urate with Peroxynitrite in the Presence of antioxidants

We next decided to test the possibility that the reaction of urate with peroxynitrite could be blocked by ascorbate. To this end, we added one equivalent of ascorbate to the phosphate buffer containing urate prior to the addition of the peroxynitrite. In reactions conducted using a 1:1:1 molar ratio of ascorbate:urate:peroxynitrite, urate was well protected from oxidation by peroxynitrite by ascorbate. As illustrated in the full scan LC-MS total ion trace (Figure 7), neither the intermediates nor triuret were observable when the 1:1:1 molar ration of reagents was used. When a 1:1:2 molar ratio of ascorbate:urate:peroxynitrite was used, urate was only partially (70%) oxidized to triuret (Figure 7), and none of the reactive intermediates were observable.

Reaction of Urate with Peroxynitrite in Human Plasma

We added $^{15}N_2$-urate to human plasma (in order to distinguish any reaction products from endogenous reaction products of urate) and then

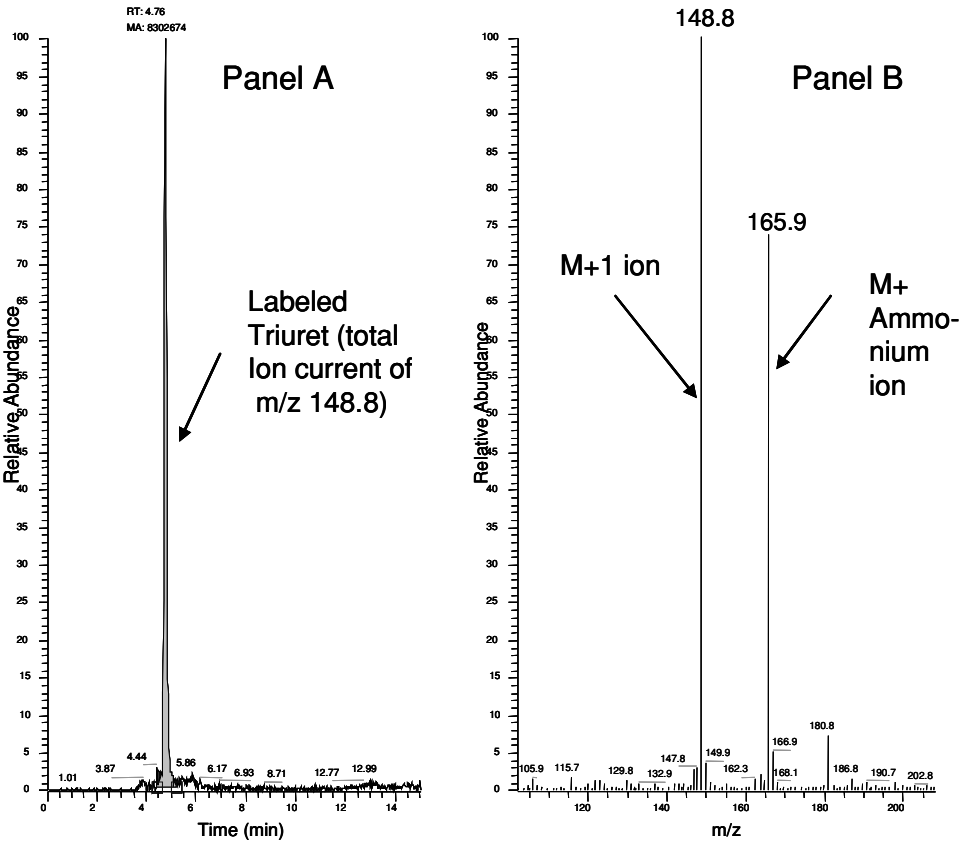

FIGURE 8 LC-MS identification of $^{15}N_2$-triuret from plasma reactions of $^{15}N_2$-uric acid with peroxynitrite: A) the total ion chromatogram of the plasma extract; B) plot of m/z 149; and C) the ions of triuret peak at 4.76 minutes (panel A). m/z 149 is the M+1 ion of labeled triuret and m/z 166 is the ammonium adduct of labeled triuret.

exposed the labeled urate to peroxynitrite. Figure 8 shows the identification of labeled triuret from plasma reactions of labeled uric acid with peroxynitrite by full scan LC-MS total ion trace. This was also confirmed by SRM analysis. The formation of triuret from the reaction of uric acid from peroxynitrite, even in the presence of plasma components suggests that measuring triuret concentration can indicate the extent of peroxynitrite reaction in vitro and in vivo.

Determination of Triuret Levels in Healthy Volunteers, Patients with Normal Pregnancy, and Patients with Preeclampsia

We have conducted a preliminary investigation with a limited number of subjects who are normal healthy volunteers, patients with normal pregnancy and patients with preeclampsia. The triuret concentration data from the

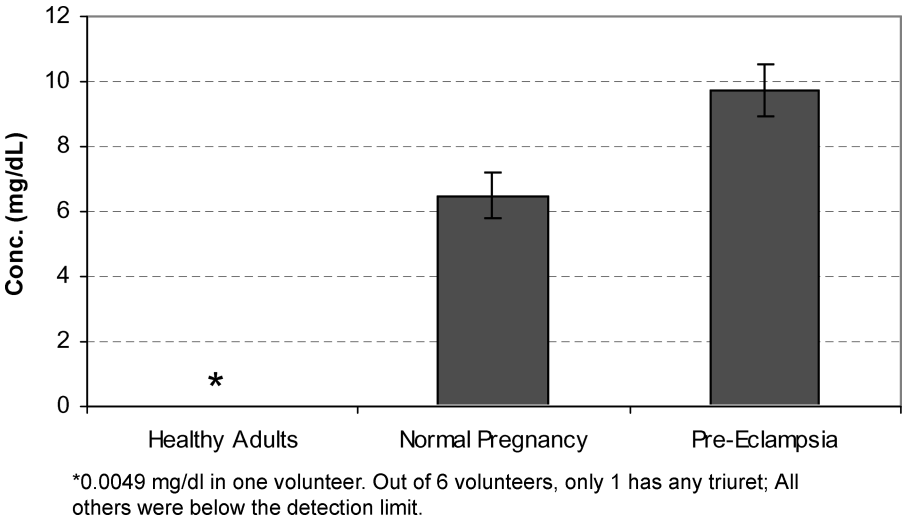

FIGURE 9 Triuret concentrations in the urine samples from normal healthy volunteers (N = 6), patients with normal pregnancy (N = 7), and patients with pre-eclampsia (N = 9).

LC-MS/MS analysis of urine samples is shown in Figure 9. As is evident there are significant differences in the amount of triuret in healthy volunteers and patients.

DISCUSSION

Peroxynitrite reacts with lipids, DNA and proteins via direct oxidative reactions or indirect radical-mediated mechanisms.[36] In this paper we report the formation of reactive intermediates and alkylated products from the reaction of peroxynitrite with uric acid. These intermediates further react in aqueous medium to form triuret or react with molecules with functional groups such as OH to form alkylated products. The study scheme is summarized in Figure 1.

While the reaction of uric acid with peroxynitrite might be beneficial as a mechanism for removing peroxynitrite, in this paper we demonstrate that reaction also leads to the formation of unique reactive intermediates that further react with alcohols to give alkylated products or react with aqueous medium to give triuret. The first labile product (2B) observed had an m/z 216 (218 for the labeled uric acid). This indicates that the molecular weight of the intermediate is 215, demonstrating that the intermediate has gained a nitrogen (a mass of 215 indicates odd number of nitrogens). The mass difference of 47 units (between uric acid and 2B) indicates a gain of elements of HONO group. We considered structures 3R and 3S shown in Figure 3 as potential compound 2B. MS/MS experiments at 25 V produced ions at m/z 199, 167, 156 and 124, representing the loss of NH_3

from 216, MeOH from 199, HN=C=O from 199 and HN=C=O from 167, respectively. This could be potentially could be the ammonium adduct of 3H (structure 3H1) (3H itself was not identified in the reaction mixtures). The formation of 3H can lead to the formation of additional products 3K and 3O that were observed in the reaction. Thus, the ammonium adduct 3H1 can be formed in the mass spectrometer (LC-MS analysis were conducted in ammonium acetate/acetic acid buffer) and the formation of such adducts are common in LC-MS analyses.[37] There is some doubt about 2B being 3H1, as 3H itself has not been identified and under the conditions we employed for LC-MS analysis, ammonium adduct formation, if present is a minor pathway. There is a possibility that the intermediate 2B is a mixture (unresolved peak) of 3R and the ammonium adduct of 3H (both will have the same M = 1 ion). Whether it is 3R or ammonium adduct of 3H, this intermediate is unstable and leads to triuret or alkylated allantoin 3O (depending on the medium). As stated, the attempt to isolate 2B by preparative HPLC resulted in its complete conversion to triuret (the isolated fraction contained only triuret).

One can visualize the addition of peroxynitrite or the elements peroxynitrite across the C4-C5 double bond (path 2, Figure 3) of uric acid to form intermediate 3B and 3B can lose the elements of water (from C4 and N9) to give the intermediate 3F.

3 F also can be formed directly by the addition of $^{\cdot}$ONO radical to urate followed by the loss of $H^{\cdot}$ to the medium (path 3) or the abstraction of hydrogen by the resulting radical from the medium to give 3R. 3F can also be generated from 3R by oxidation. 3F can exchange its ONO group with water or MeOH to yield 3D or 3H, respectively. These then can lead to other observed products. It has been reported[38–45] that peroxynitrite can dissociate either partially or completely to yield $HO^{\cdot}$ and $^{\cdot}$ONO radicals, in the following manner:

$$NO^{\cdot} + {}^{\cdot}O_2^- \rightarrow ONOO^-$$

$$ONOO^- + H^+ \rightleftharpoons ONOOH$$

$$ONOOH \rightarrow [{}^{\cdot}OH\text{---}{}^{\cdot}NO_2] \rightarrow {}^{\cdot}OH + {}^{\cdot}NO_2$$

In addition, others have reported or predicted the formation of $^{\cdot}NO_2$ radical from peroxynitrite or equivalent compounds.[46–48]

One possibility is that 3B forms from a complex radical mediated process or from partially or completely formed radicals still enclosed in the solvent shell.

For oxidation reactions of uric acid, other authors[5,6] had proposed a pathway involving two electron oxidations (path 1, Figure 3) to yield dehydrourate 3C which can further react with water to yield allantoin and

triuret. This mechanism may not be operative in peroxynitrite reactions and it is possible that path 3 (addition of ·ONO radical) or possibly path 2 could be operative as evidenced by the following. Methyl substitution at position N7 in methylated uric acids should prevent the reaction to form 3C equivalent because the double bond cannot be formed between C5 and N7. This may mean that the reaction may not proceed. But, our investigations (data not shown here) of the peroxynitrite reactions of a series of methyl-substituted uric acids (substituted at the nitrogen positions) showed that the reaction with peroxynitrite proceeds to produce peroxynitrite reaction products including the dimethoxy compound equivalent to 3K. We believe that the primary unstable intermediates formed in our reactions are 3E and 3F (path 3) as a result of the addition of the ·ONO radical to uric acid and they then lead to the products observed. In addition, it is possible that 3R is formed as an intermediate.

Therefore, we are proposing that 3H as a possible intermediate for 3K and 3O. As mentioned this intermediate is labile and has been identified only in ice-cold reaction mixtures. The attempted isolation of peak 2B by preparative HPLC resulted in its conversion to triuret (isolated fraction contained only triuret). In some reactions small quantities (1–2%) of allantoin was formed, likely through 3G and 3K that leads to triuret. Kahn and coworkers[49] identified 3G and 3J by NMR in urate oxidations by uricase. The pathway for the formation of triuret follows that of Robinson et al.[5]

The reactive intermediates further react to generate the end product of this reaction, triuret, which now appears to be a signature end product. The reaction occurs with 1 equivalent of peroxynitrite. Use of higher concentrations of peroxynitrite (up to 3 equivalents) did not alter the products observed by LC-MS. Within the range of pHs tested (pH 7.4 and 9), pH had no effect on the products suggesting that it is the mono anion of urate that reacts with peroxynitrite. In addition, the cations present (Li, K, or Na) had no effect on the products observed. While the observation that triuret is a product of the peroxynitrite and uric acid reaction had been previously shown,[5] to our knowledge this reaction product had not been documented in any biological system.

There is a possibility that nitric oxide can be generated from peroxynitrite and that this can further react with UA. As reported in our recent publication,[53] nitric oxide reactions with UA produce 6-aminouracil as the sole product. No 6-aminouracil was observed in any of the reactions of UA with peroxynitrite, thus excluding the possible generation of any significant amounts of nitric oxide.

The presence of MeOH used either as eluent or intentionally added to investigate the alkylation potential of uric acid activated by peroxynitrite, results in the partial conversion of 3H to 3K, the dimethoxy adduct of uric acid (4,5-dimethoxy-4,5-dihydrouric acid). Under these conditions, the

final stable product formed is the methylated allantoin (3O), likely formed through the intermediate 3H. We have not identified any direct reaction product of 3K. The final product of its reaction appears to be 3O formed through 3H. We have multiple levels of confirmation of the products 3O and 3K using studies with labeled uric acid, methyluric acid, d4-MeOH and reactions with other alcohols.

Since products 2B(3H), 2D(3K) and 2E(3O) shown in Figure 2 are likely to be generated by the reaction of MeOH from eluent (reactions conducted in ammonium acetate produced only triuret; see Reaction 5) with reactive intermediates formed before the HPLC analysis, the extent of their formation at a given time can be used as a approximate measure of the reactive intermediates in the solution that was injected in the HPLC. These intermediates could be 3B, 3C, 3C, or other unknown species that contain the elements of uric acid (all products contain all or part of uric acid). Thus, based on the above reasoning, one can estimate that 41% of reactive intermediate(s) remained in solution at T = 0 (3 min at room temperature), 54% at T = 30 minutes, 42% at T = 1 hour, and 0% at T = 16 hours.

In the reactions conducted with 1,3-$^{15}N_2$-uric acid, the corresponding dinitrogen labeled products H (or 3R), 3K, 3O and 3Q] were formed in all cases. This shows that the identified products all retained the nitrogens 1 and 3 of uric acid. The fact that the labels were retained also lends support to the proposed reaction scheme. The structure of 3K was confirmed by the preparation of the compound and comparison of its LC-MS data with that of the prepared compound. LC-MS/MS studies were conducted to further confirm the structures of labeled and unlabeled O-methylallantoin (3O). The fragmentation patterns are shown in Figure 5. The major fragmentation pattern arises from the loss of HN=CO group from the 5 membered ring (the lost HN=CO group does not contain any of the nitrogen labels) and other characteristic peaks arise from the loss of MeOH as well as urea. The mass spectrometry data lend support to the proposed structures. This is also indicative of the type of alkylation products that could be formed with biomolecules.

When the cold reaction mixtures of uric acid and peroxynitrite in aqueous buffer (from Reaction 1)(reaction time 3 min at ambient temperature, followed cooling in ice-water mixture) were treated with MeOH or EtOH, it resulted in the formation of the corresponding methylated or ethylated allantoin (3O), as determined by LC-MS analysis after the addition of MeOH or EtOH. This is significant because it suggests that the preformed intermediates in solution were able to alkylate alcohols and that the intermediates live long enough in solution to participate in further reactions.

As indicated, the reaction with alcohol is highly preferred even in the presence of excess water. It occurs even in the presence of low levels of

alcohols, as seen in the LC-MS studies using alcohol as one of the mobile phases (low organic content). We believe that compounds containing labile hydrogen such as OH can get alkylated once UA is activated by PN. There are numerous such groups in biological molecules. While it is true pure alcohols are not abundantly present in the biological milieu, the observation that uric acid can alkylate chemical species containing hydroxyl groups suggests that alkylation could be a novel mechanism by which uric acid acts. Further studies are required to determine which types of biological compounds might be targeted.

We also examined the products formed from the reaction of uric acid and peroxynitrite that had been reported by Skinner et al.[31] We observed only triuret when replicating the same reaction conditions, where the authors reported 2-nitrito-4-amino-5-hydroxyimidazoline as the product of the reaction. Our results are consistent with that of Robinson et al.[5]

We selected alcohols as model compounds for biomolecules containing OH and other active groups and investigated the reactions of uric acid with peroxynitrite in their presence. Reactions of uric acid with 1 equivalent of peroxynitrite conducted in methanol initially produced a mixture of 3H (or 3R) (14.6%), unreacted uric acid (11.6%), triuret (detected), 3K (23.1%), and 3O(50.6%). When this mixture was left at ambient temperature overnight, all of the unreacted uric acid and products (other than 3O) were converted to a final mixture containing 96% 3O and 4% triuret. The reactions produced 96% of the alkylation product 3O, when the medium contained about 50% (by volume; approximately 64% by mole concentration) water (from buffer) and 50% methanol (by volume; approximately 36% by mole concentration) and this suggest a high degree of affinity for the alkylation reactions versus the formation of triuret via the reaction with water.

Reactions of unlabeled uric acid with d4-MeOH produced primarily d3-labeled 3O (4A) (M+1 = 176) (∼88% of products) and a mixture of d3- and d6- (∼12% of products) (ratio of d3- (M+1 = 234; 4B) and d6-(M+1 = 237; 4C) = 1.0:0.45) labeled 3K and small quantities of (less than 1%) 3H (or 3R) (M+1 = 216). The fact that 4B and 4C are present at a ratio of about 2:1 rather than 4C alone suggests that one of the methoxyl groups in 4C is labile and that it partially exchanges with the methanol in the HPLC eluent. Such an exchange may go through the formation of monomethoxyl 3H. The presence of only small quantities of 3H (or 3R) suggests that it has already further reacted with the d4-methanol to give 3K or (and) 3O. Reactions of $^{15}N_2$-uric acid with peroxynitrite in d4-methanol produced similar results.

We also investigated the reaction of 1-methyluric acid with peroxynitrite in MeOH and it further confirmed our identification of the products (the corresponding methyluric acid products were formed) and it showed the generality of the reaction of uric acids with peroxynitrite.

When lithium urate was exposed to peroxynitrite under conditions of Reaction 1, the reactions produced the same products as when the urate had been dissolved in KOH and then added to the phosphate buffer prior to being exposed to peroxynitrite. This implies that the counter ion had no effect on the products of the reaction and that Li can be used when solubility of urate is a concern.

During the course of our investigations, we wondered whether the reaction of uric acid with peroxynitrite could be blocked by ascorbate. In reactions conducted using a 1:1:1 molar ratio of ascorbate:urate:peroxynitrite, urate was well protected from oxidation by peroxynitrite by ascorbate. As illustrated in the full scan LC-MS total ion trace (Figure 7), neither the intermediates nor triuret were observable when the 1:1:1 molar ratio of reagents was used. When a 1:1:2 molar ratio of ascorbate:urate:peroxynitrite was used, urate was partially (70%) oxidized to triuret (Figure 7), and none of the reactive intermediates were observable. It is unclear from our studies whether ascorbate degraded peroxynitrite, making it unavailable for further reaction or whether it preferentially reacted with peroxynitrite.

In order to determine whether or not the reaction of urate with peroxynitrite could occur in a biological milieu, we added $^{15}N_2$-urate to human plasma and then exposed the labeled urate to peroxynitrite. Figure 8 shows the identification of labeled triuret from plasma reactions of labeled uric acid with peroxynitrite by full scan LC-MS total ion trace. The formation of triuret from the reaction of uric acid from peroxynitrite, even in the presence of plasma components suggests that measuring triuret concentration can indicate the extent of peroxynitrite reaction *in vitro* and *in vivo*.

Preeclampsia is a pregnancy-specific disorder characterized by hypertension and proteinuria that is associated with increased oxidative stress and reduced antioxidant defenses.[50] As part of an ongoing study we have conducted the LC-MS/MS analysis of triuret in the urine samples from preeclampsia patients (Figure 9). In the limited subjects investigated, the triuret levels were: normal healthy volunteers $\ll$ patients with normal pregnancy $\ll$ preeclampsia patients. Normal pregnancy is associated with some oxidative stress,[51,52] which is amplified in preeclampsia patients. No one has previously reported the presence of triuret in any human, animal or cell culture studies. This data is indicative of the potential biological relevance of triuret pathway. A recent preliminary study by us also indicates that triuret levels are elevated in smokes compared to non smokers. More detailed studies with more subjects are needed to confirm and extend these findings.

When generated in vivo, the intermediates generated by the reaction of peroxynitrite with uric acid are potentially capable of alkylating molecules with suitable functional groups in their surroundings. The potential for such an alkylation of –OH, –SH, $-NH_2$, >NH and –COOH groups is

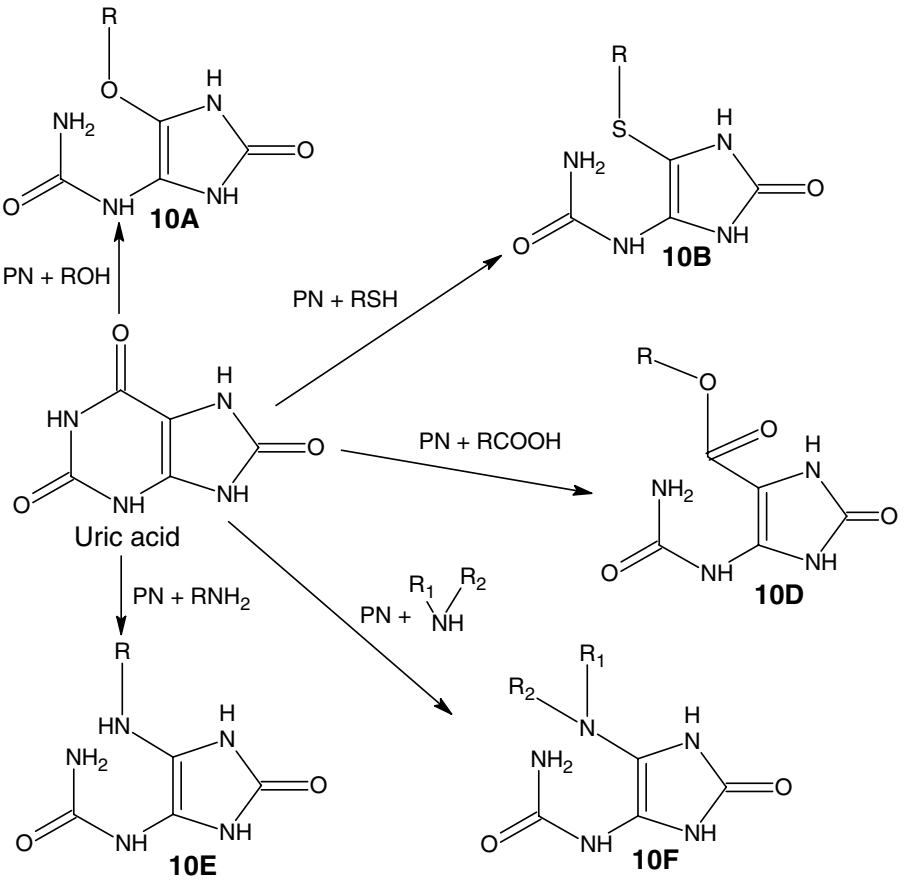

FIGURE 10 Proposed scheme for the potential peroxynitrite activated uric acid's alkylations of functionalized biomolecules.

proposed in Figure 10. This could be of particular biological significance, as enzymes, DNA, and RNA, as well as other biomolecules could be potential targets of these intermediates. This alkylation potential of biomolecules is undergoing further study in our group. Taken together, these results lead us to conclude that under conditions of oxidative stress, uric acid can form reactive intermediates. These reactive intermediates might be important in explaining how uric acid contributes to the pathogenesis of diseases such as hypertension, metabolic syndrome, preeclampsia, renal disease, and cardiovascular disease; as well as how it might contribute to the characteristic endothelial dysfunction associated with these conditions. A possible scenario is that the reactive intermediates can further react with components of endothelial cells and cause damage and disruption to their normal function. Perhaps part of this damage, such as alkylation, could be irreversible and cumulative.

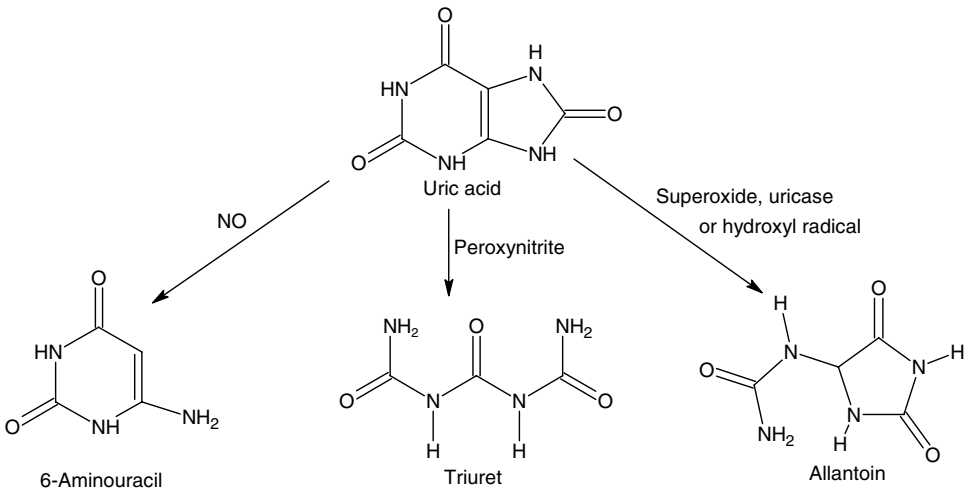

FIGURE 11 Degradation of urate by different oxidants to yield potential signature products. Oxidation of uric acid to allantoin had been reported to occur with uricase (urate oxidase)[49] and hydroxyl radicals.[55] Our studies with superoxide and UA showed the formation of allantoin as the primary product of the reaction. It is possible that the superoxide reaction proceeds though the formation of hydroxyl radical. As reported in our recent publication,[53] nitric oxide reactions with UA produce 6-aminouracil as the sole product. PN reaction with UA in aqueous media generates triuret.

Recently, we reported[53] that uric acid reacts with nitric oxide to yield 6-aminouracil. In this paper we report that uric acid reacts with peroxynitrite to form triuret and alkylated products. It has been known for sometime[54] that uric acid reacts with uricase, superoxide, OH radical and other similar oxidants to yield primarily allantoin. Our studies with superoxide and UA showed the formation of allantoin as the primary product of the reaction. It is possible that the reactions of superoxide proceeds through the formation of OH radical. Thus, there are three distinct pathways exists (Figure 11) for uric acid's reaction with oxidants and measurements of these signature metabolites (allantoin, 6-aminouracil, and triuret) under pathological conditions associated with elevated uric acid levels and oxidative stress will provide insights into the significance of each of these pathways in a given disease. We have identified all three metabolites in human pathological conditions (some data are presented in this article and in the recent report).[53] Further work to explore these pathways in vivo is underway.

In conclusion, our studies indicate a potential pathway by which uric acid's reaction with peroxynitrite might lead to deleterious effects: via the formation of reactive intermediate(s) and their potential alkylation of biomolecules containing reactive functional groups. Such effects can be partially blocked by ascorbate. We also have identified significant amounts of triuret in patients under oxidative stress, whereas very little, if any, triuret is produced in normal healthy volunteers. Further mechanistic studies

investigating the role of pro-oxidative effects of uric acid in cardiovascular disease are indicated.

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