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Immunological detection of nitrosative stress-mediated modified Tamm–Horsfall glycoprotein (THP) in calcium oxalate stone formers

V. PRAGASAM, P. KALAISELVI, K. SUMITRA, S. SRINIVASAN, P. ANANDKUMAR, & P. VARALAKSHMI

Department of Medical Biochemistry, Dr. ALM PGIBMS, University of Madras, Taramani Campus, Chennai, India

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Abstract

The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in hyperoxaluric condition has been proved experimentally. This may result in the formation of the cytotoxic metabolite peroxynitrite, which is capable of causing lipid peroxidation and protein modification. The presence of nitrotyrosine in proteins has been associated with several pathological conditions. The present study investigated the presence of nitrotyrosine in the stone formers Tamm–Horsfall glycoprotein (THP). *In vitro* nitration of control THP was carried out using peroxynitrite. New Zealand white rabbits were immunized with peroxynitrated THP at 15-day intervals. Antisera collected following the third immunization were assayed for antibody titres using solid-phase ELISA. Antibodies were purified by affinity chromatography. The carbonyl content of control, stone formers and nitrated THP were determined. Western blotting was carried with control, stone formers and nitrated THPs. Immunodiffusion studies demonstrated cross-reaction with nitrated bovine serum albumin. Significant amounts ($p < 0.001$) of carbonyl content were present in both stone formers and nitrated THPs. Western blot analysis confirmed the presence of nitrated amino acid 3-nitrotyrosine in stone formers, which could bring about structural and functional modifications of THP in hyperoxaluric patients. A cross-reaction with nitrated bovine serum albumin confirms that the raised antibody has certain paratopes similar to the epitope of nitrated protein molecules. Detection of 3-nitrotyrosine in stone formers THP indicates that it is one of the key factors influencing the conversion of THP to a structurally and immunologically altered form during calcium oxalate stone formation.

Keywords: *3-Nitrotyrosine, hyperoxaluria, enzyme-linked immunosorbent assay, nitrosative stress*

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Introduction

Oxidative/nitrosative stress occurs when ROS/RNS exceeds the natural antioxidants' capacity of the organism. During oxidative/nitrosative stress, unconditional ROS/RNS attack, denature or modify lipids, proteins, carbohydrates, DNA and other molecules.

Correspondence: Viswanathan Pragasam, Department of Medical Biochemistry, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai — 600 113, India. Tel: 91-44-2487-0767, 91-44-2492-5317. Fax: 91-44-2492-6709. E-mail: pvishvanath@yahoo.com

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Oxidative stress is a key feature of hyperoxaluric condition and its presence is evidenced by the reported elevation of the lipid peroxidation product malondialdehyde (MDA), depressed antioxidant capacities, and impaired antioxidant enzyme activities in the plasma and the kidneys of nephrolithic patients and animals (Selvam 2002). Increased ROS activity results in non-enzymatic production of reactive carbonyl compounds, which in turn reacts with and modifies structural and functional proteins leading to the formation of advanced glycation end products.

It has been documented that RNS can mediate the same kind of chemistry as ROS (Beckman & Koppenol 1996). Toblli et al. (1999) showed that nitric oxide metabolites are present in hyperoxaluric conditions. Oxidative stress induced by RNS is mediated primarily by two nitrogen oxide species: peroxynitrite and nitroxyl anions. Peroxynitrite originates from the reaction between nitric oxide ($NO^{\cdot}$) and superoxide anion, while NO can result from various biochemical pathways. Peroxynitrite ($ONOO^{-}$) is a powerful oxidant (Huie & Padmaja 1993) that nitrates free and protein-associated tyrosine and other phenolic moieties (Halliwal & Gutteridge 1989). Excessive protein nitration has been associated with various pathological conditions including neurological disorders including Parkinson's disease, multiple sclerosis, viral infection, etc. (Sawa et al. 2000). Recently, the present authors reported on the structural and functional modification of nitrated THP, which is similar to the THP obtained from the renal stone formers (Pragasam et al. 2005b).

THP is an abundant urinary protein (Kumar & Muchmore 1990) in humans. Considerable attention has been focused on its normal function as well as on its involvement in nephrolithasis in recent times. Bachmann et al. (1995) confirmed the presence of NO synthase in the thick ascending loop of Henle, which also synthesizes THP. In support of this, the present authors recently demonstrated increased expression of inducible nitric oxide synthase (iNOS) in the kidney of experimentally induced hyperoxaluric animals, one of the key enzymes involved in generation of excess amount of nitric oxide under nitrosative stress condition (Pragasam et al. 2005a). Based on our previous observation that considerable functional homology exists between nitrated and stone formers THP, the present study was designed to produce antibodies against nitrated non-stone formers THP and its subsequent utilization to detect the presence of such modification in THP derived from hyperoxaluric patients.

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Materials and methods

Materials

Diatomaceous earth and bovine serum albumin (BSA; fatty acid free) were obtained from Sigma Biochemical (Dorset, UK); anti-rabbit IgG antibodies were obtained from the National Institute of Immunology (New Delhi, India). Amplified alkaline phosphatase immunoblotting kit and complete Fruends adjuvant's (CFA) was purchased from Genei (Bangalore, India). Phenyl methyl sulfonyl fluoride (PMSF) and all the other chemicals, reagents and solvents used were of analytical grade from Sisco Research Laboratories (Mumbai, India).

Collection of urine samples from healthy and stone formers

Urine samples (24 h) from healthy individuals ($n = 50$) and stone formers ($n = 67$) attending the Urologic Clinics of Royapettah Medical College and Hospital were collected.

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Isolation and purification of THP

Isolation and purification of human urinary THP was carried out by the method of Serafini-Cessi et al. (1989) and Gokhale et al. (1997) and stored at 4°C using PMSF as a preservative for further studies.

Nitration of THP and BSA

Peroxynitrite preparation. Peroxynitrite was prepared by the method of Radi et al. (1991). Solutions of 0.6 M $NaNO_2$ and 0.6 M HCl plus 0.7 M H_2O_2 were pumped at 26 ml min^{-1} into a T-junction and mixed in a glass tube. The acid-catalysed reaction of nitrous acid with H_2O_2 to form peroxynitrous acid was quenched by pumping 15 M NaOH at the same rate into a second T-junction at the end of the glass tube. Excess H_2O_2 was removed by passage over a 1×5-cm column filled with 4 g MnO_2 (granular). The solution was frozen at -20°C for as long as 1 week. Peroxynitrite forms a yellow top layer due to freeze fractionation, which was retained for further studies. This top layer typically contained 170–220 mM peroxynitrite as determined by absorbance at 302 nm ($\varepsilon_{302} = 1670\ M^{-1}\ cm^{-1}$).

Nitration of proteins was carried out by using control THP and BSA, which was incubated with a fixed concentration of peroxynitrite (1 mM) for 10 min. The reaction was arrested by extensive dialysis for 48 h to remove salts from nitrated THP and BSA.

Determination of the carbonyl content in nitrated and stone formers THP

The carbonyl content of the protein was estimated by the method of Levine et al. (1990).

Production of rabbit anti-nitrated THP polyclonal antibodies

Polyclonal antibodies were raised against nitrated THP in rabbits according to the method of Horton et al. (1988).

A total of 1 mg of the nitrated THP in 0.5 ml saline was emulsified with 0.5 ml complete Freund's adjuvant by passing through a syringe (total volume of the mixture 1.0 ml). This mixture was injected at two sites intramuscularly (0.5 ml each). The procedure was repeated at an interval of 15 days for four more booster doses. Rabbits were periodically bled and the antibody titre was checked using ELISA. Pooled sera were fractionated in ammonium sulphate and dialysed overnight. Unwanted antibodies against native THP were eliminated using Sepharose-4B affinity column coupled to native THP. Specific anti-nitrotyrosine antibodies were affinity purified using a Sepharose-4B column coupled to 3-nitro-L-tyrosine.

Specificity and competition analysis of anti-nitrotyrosine antibodies using ELISA

Polyclonal anti-nitrotyrosine antibodies were assessed for specificity and cross-reactivity by ELISA and Western blot. Briefly, 96-well polystyrene flat-bottomed ELISA plates were coated with either native or nitrated THP (30 μg ml^{-1}) in carbonate buffer (50 mM, pH 9.5) and incubated overnight at 4°C. After washing, plates were blocked with 5% gelatin in PBS plus 0.05% Tween 20 (v/v) for 1 h at 37°C. After washing, anti-nitrotyrosine polyclonal antibodies in PBS-0.05% Tween

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20 was added and left for 2 h at 37°C. The plates were washed and incubated with horse radish peroxidase conjugated with goat anti-rabbit IgG for 2 h at 37°C. The plates were developed with O-phenylenediamine dihydrochloride and absorbance was read at 492 nm using a microplate reader.

Cross-reaction with native and nitrated BSA was assessed by Immunodiffusion

Immunodiffusion was carried in 1.5% low melting agarose as described by Kabat and Mayer (1961).

SDS-PAGE and Western blotting

Nitrated and stone formers THP were subjected to SDS-PAGE on 10% polyacrylamide gels (Haddad et al. 1994). Bands were transferred to nitrocellulose by semi-dry blotting. Non-specific sites in the nitrocellulose were blocked with BSA for 16 h at 4°C and overlaid with the raised polyclonal anti-nitrotyrosine rabbit antibody, followed by biotinylated anti-rabbit IgG plus biotinylated alkaline phosphatase and developed using diamino benzidine (DAB) detection system.

Statistical analysis

Data are expressed as mean $\pm$SD. Statistical analysis was performed using SPSS package with a significance of $p < 0.05$.

Results

Determination of carbonyl contents in nitrated BSA/THP

Figure 1 shows the carbonyl content of nitrated BSA/THP along with stone formers THP. The carbonyl content was significantly high ($p < 0.001$) in nitrated BSA/THP when compared with the native THP. In stone formers THP, carbonyl content was at $p < 0.001$ when compared with native THP.

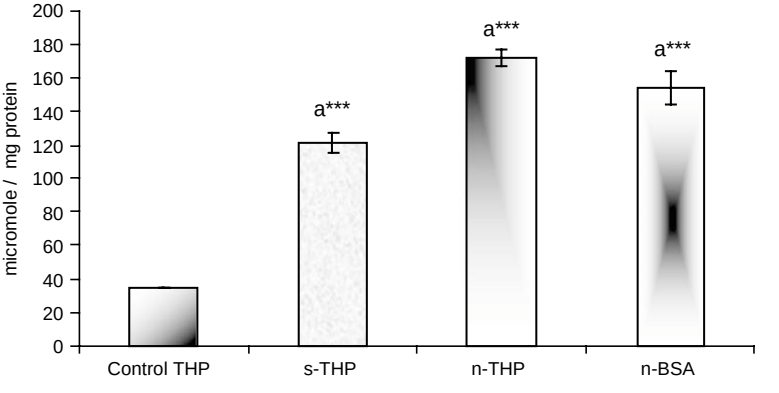

Figure 1. Levels of carbonyl content for different forms of THP as described in the Materials and methods. Comparisons are made between those columns marked 'a' (control) versus various THPs at a significance level of ***$p < 0.001$.

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Determination of antibody titres

The THP anti-serum and the pre-immune rabbit serum were assayed for antibody titres. Figure 2 shows the titre levels for the antibody raised against nitrated THP in rabbit serum and its reactivity with antigen by ELISA. The concentration of the antibody following three booster injections attained maximum by 60 days. After the fourth booster injection, no further increase in the concentration of antibody was observed (Figure 2). Hence, serum was collected after the fourth booster injection (after 75 days) and used for further studies.

Purification of antibody by affinity column chromatography

Figure 3 shows the elution profile of the antinitrated THP antibodies from rabbit serum partially purified by affinity column chromatography. Elution was initially carried out with phosphate buffered saline pH 7.4 (first ten fractions) for the complete removal of unbound proteins and then with glycine-HCl buffer (pH 2.2) to elute the anti-nitrated THP antibodies. The absorbance of the eluted fractions was monitored at 280 nm. The elution profile showed a major protein peak in the glycine eluate at third, fourth and fifth fractions.

Determination of optimum antibody dilution using ELISA

Figure 4 represents the antigen-antibody reactivity of the purified antibody as assayed by ELISA. The optimal dilutions of immunoreactants, i.e. the greatest dilutions yielding the required sensitivity and linearity through the desired range of concentration, were determined empirically. Antibody showed maximal reactivity up to the dilution of 1:5000. Above the dilution of 5000, it declined slowly and did not show any immunoreactivity above the dilution of 1:30 000. For further studies, 1:5000 diluted antibodies were used.

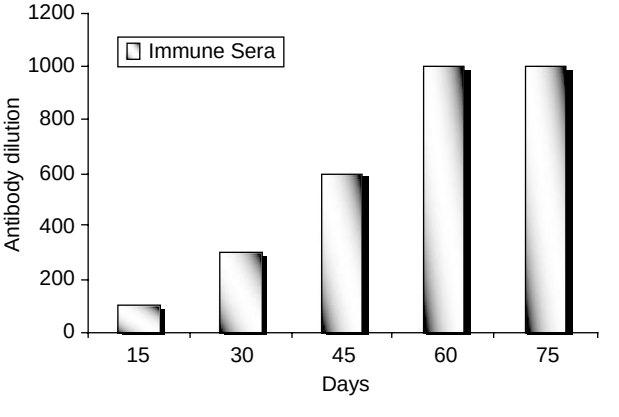

Figure 2. Antibody titre of immune sera of rabbits after different weeks of immunization as described in the Materials and methods.

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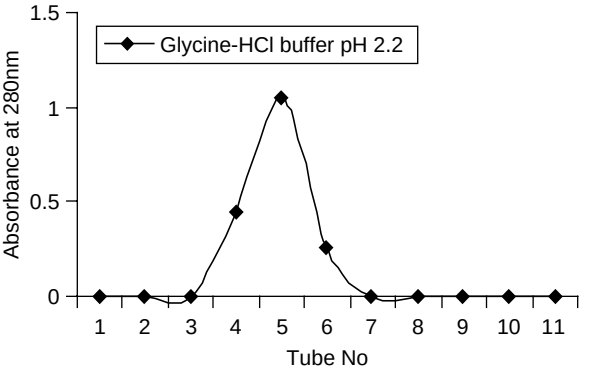

Figure 3. Purification of antinitrotyrosinated THP antibodies by affinity chromatography as described in the Materials and methods.

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Cross-reactivity of anti-nitrotyrosine with nitrated BSA

The validity of the antibody for use with a range of other proteins was investigated. BSA was nitrated *in vitro* using peroxynitrite. Cross-reactivity was determined by immunodiffusion using immobilized nitro-BSA for the anti-nitrotyrosine antibody (Figure 5). The nitrated BSA had low affinity for the antibody than nitrated THP. Before nitration, the nitrotyrosine present in these proteins was undetectable by the immunodiffusion method as seen in Figure 5. Nitrated BSA expressed partial immuno-reaction with anti-nitrotyrosine antibodies, hence the arc in the agarose gel appeared lightly when compared with the arc in the nitrated THP.

Detection of 3-nitrotyrosine in stone formers THP by Western blotting

The presence of the nitrated THP in stone formers' urine was confirmed by SDS-PAGE immunoblotting of untreated urine and THP was isolated from stone formers' urine. Figure 6 shows an immunoblot with the anti-nitrotyrosine antibody; the major protein bands stained by the antibody have molecular masses of 90 kDa. For a crude urine sample of stone formers, the band (lane 4) appears lighter when compared with

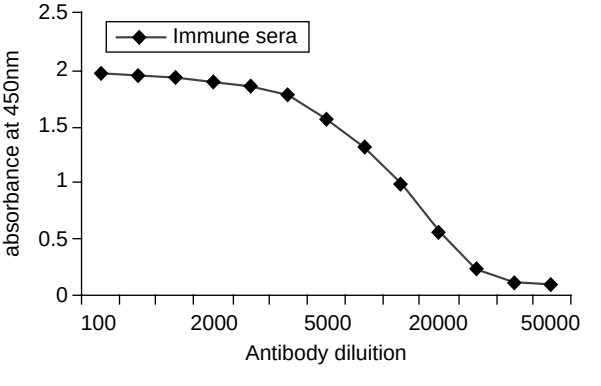

Figure 4. Determination of the optimum antibody dilution of antinitrotyrosinated antibody as described in the Materials and methods.

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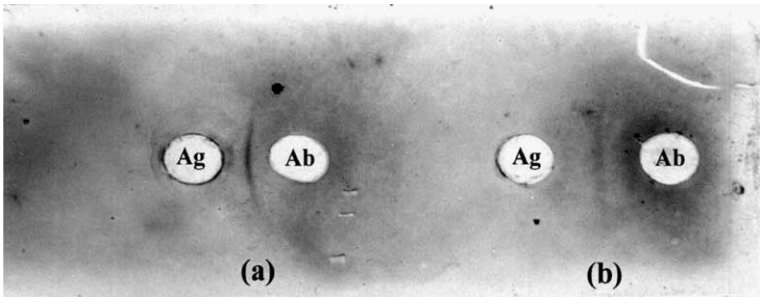

Figure 5. Immunodiffusion studies for nitrated THP and BSA as described in the Materials and methods: (a) a strong precipitation reaction of antinitrated THP with nitrated THP and (b) a minimal cross-reaction with nitrated BSA.

the nitrated protein sample (lane 2) and anti-3-nitrotyrosine showed no reaction with THP isolated from control subjects (lane 3).

Discussion

Due to the transient nature of free radical species and their often broad range of reactivity, it becomes challenging to define the mechanisms of protein damage in processes of oxidant stress when a diverse spectrum of reactive species are produced (Dean et al. 1997). Nitric oxide has proven to be a ubiquitous signal transduction molecule and a potent mediator of tissue injury owing to its low molecular mass,

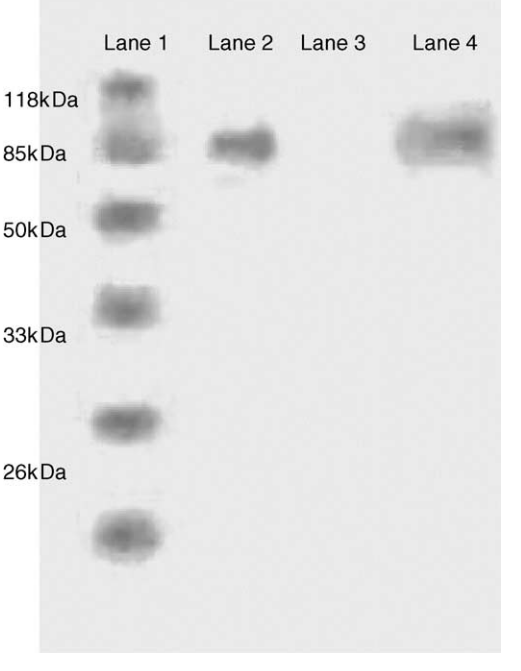

Figure 6. Immunoblot for the antinitrotyrosinated antibody for both stone formers and nitrated THP. Lanes 1, molecular weight markers; 2, *in vitro* nitrated THP; 3, THP isolated from normal subjects; and 4, THP isolated from stone formers' urine showing a positive reaction for nitrated tyrosine residues.

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volatility, lipophilicity, free radical nature and diverse reactivities. However, excessive NO production may contribute to several renal diseases, including immune-mediated glomerulonephritis, obstructive nephropathy and renal allograft rejection (Kone 1997).

Peroxynitrite is a potent oxidant as well as a nitrating agent produced by the reaction of nitric oxide with superoxide and having a biological half-life of <0.1 s at pH 7.2 and 37°C (Beckman et al. 1990). It readily nitrates phenolic compounds such as tyrosine residues in proteins, thereby leaving a footprint that has been detected in a number of human tissues (Crow & Ischiropoulos 1996). Tyrosine nitration can modify proteins and alter enzyme activities (Radi 2004). Nitrotyrosine was first discovered as a marker for *in vivo* nitration (Ohshima et al. 1990). As assessed by nitrotyrosine detection, peroxynitrite has been suggested to be involved in the pathogenesis of a wide range of diseases, including human atherosclerosis, hypertension, diabetes, heart failure and aging (Koeck et al. 2004). Immunological approaches (the production of polyclonal antibodies) against the nitrated proteins aid as a perfect alternative tool to assess the structural and functional changes in proteins derived from control and various pathological conditions as they are more cost-effective, reliable and less laborious when compared with the production of monoclonal antibodies, which are cumbersome and highly expensive.

The ability of anti-nitrotyrosine antibodies to discriminate between nitrotyrosine residues in different environments and proteins may be useful in raising antibodies to specific motifs containing tyrosine residues, e.g. at the active site of enzymes (Beckman & Koppenol 1996) and at tyrosine phosphorylation sites. It is essentially a semi-quantitative method with different nitrated proteins since the specific antibody may not bind to all the nitrated tyrosine residues in a sample either as a result of a lack of access to them or because of the fact that antibody binding may be influenced by the environment of the tyrosine residues.

Experimental evidence indicates that there is involvement of THP in calcium oxalate stone formation (Khan & Hackett 1993). Biochemical and quantitative comparison of urinary THP excretion in normal subjects and stone formers has also shown variable results (Olczak et al. 1999). It has already been established by the present authors that peroxynitrite induces structural and functional modification of THP, which is comparable with stone formers THP, and have identified nitrosative stress to be a key factor in the formation of calcium oxalate stones in urolithic patients (Pragasam et al. 2005b). This suggests that elevated levels of nitrotyrosine-modified THP will be found in oxalate lithiasis involving oxidative damage where in a higher concentration of nitric oxide and superoxide anions might be expected.

At present, there is minimal insight into the aetiological role of 3-nitrotyrosine in renal stone formation. Oxidative modification of proteins occurs in urolithic conditions (Govindaraj & Selvam 2001) and proteins represent elective targets of oxidant-mediated injury (Davies 1987). Selvam and Kalaiselvi (2001) demonstrated that COM binding proteins derived from peroxidized renal tissue act as a promoter of the nucleation and aggregation phases in a crystal growth medium. They concluded that depletion of thiol content in proteins contributed to promoting activity of the calcium oxalate aggregation and poor inhibition of nucleation in a crystal growth system. The prominence of nitration reflects its relative stability, making this post-translational modification a potentially useful marker of extended exposure of proteins to RNS (Haqqani et al. 2002).

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Biochemical and structural modifications of proteins induced by oxidative attack may lead to functional alterations and in particular to the progressive loss of their metabolic, functional or immunological properties (Davies 1987). Earlier reports confirm that the presence of nitric oxide metabolites such as nitrite and nitrate are present in the hyperoxaluric conditions (Toblli et al. 1999). Although extensive work has been carried out in ROS-mediated damage to renal lithogenic proteins (Asokan et al. 2004), meagre evidence is available about the presence of RNS-mediated damage to proteins. Amino acid modifications that are likely to arise through reactions with peroxynitrite are oxidation products of cysteine, methionine (Pryor & Stone 1993) and oxidized/nitrated tryptophan (Padmaja et al. 1996).

The ELISA technique for nitrated proteins is a sensitive method for determining the relative amounts of nitrated THPs in urine, which will be particularly useful for the measurement of a large number of nitrosative stress-induced pathological conditions. The present study describes an ELISA technique that is specific for the detection of nitrotyrosine residues in proteins. The level of nitrated protein in some pathological conditions was considerably lower than achieved by nitration of proteins under *in vitro* by peroxynitrite.

An elevated level of 3-chlorotyrosine has been observed during oxidation of LDL isolated from human atherosclerotic intima (Hazen & Heinecke 1997). It has two orders of magnitude lower affinity for the anti-nitrotyrosine antibody than for the free nitrotyrosine. The antibody is therefore unlikely to cross-react with chlorotyrosine containing epitopes in normal or inflammatory conditions.

Carbonyl content is the index of protein oxidation. In stone formers and nitrated THP, the carbonyl contents were significantly elevated. Increased protein carbonyl contents have been reported in chronic renal failure patients (Himmelfarb et al. 2000). The present results clearly confirm that protein carbonyls are also elevated under urolithic conditions. Endogenous nitrotyrosine residues were detected in stone formers THP in the present study. The results are coherent with the identification of increased carbonyl contents in stone formers THP, implying the hand-in-hand roles of RNS and ROS in modifying its inhibitory activities.

Affinity-purified anti-nitrated THP was examined with *in vitro* nitrated BSA. The results suggest that it has a minimal cross-reaction with nitrated BSA. Furthermore, we investigated the binding of various low molecular weight compounds (L-tyrosine, L-phenylalanine and 3-aminotyrosine) by ELISA. The antibody specifically recognized nitrated protein, but not other tyrosine-related compounds (data not shown). Further extending it for other amino acids including O-phosphotyrosine and 4-nitro phenylalanine, which have nitrotyrosine-like structures, showed no cross-reaction with respective antibodies, unlike monoclonal 3-nitrotyrosine antibodies. The production of polyclonal antibodies against nitrated THP, and their subsequent utilization, has shown the role of nitrated THP in calcium oxalate stone-forming condition.

Thus, from the results of present study, we conclude that the presence of 3-nitrotyrosine in stone formers THP will be one of the key factors influencing the conversion of THP into a structurally and immunologically altered form during calcium oxalate stone formation. Cross-reaction with nitrated BSA further confirms that the raised antibody has certain paratopes, similar to the epitope of nitrated protein molecules. Hence, further studies are required to assess whether this antibody can be used to identify recurrent calcium oxalate stone formers.

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