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Uric Acid Mediates Photodynamic Inactivation of Caprine Alpha-2-Macroglobulin

SHAKIL A. KHAN and FAHIM H. KHAN*

Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh – 202 002, INDIA

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Uric acid (2,6,8 trioxopurine), the end product of purine metabolism in mammalian systems, has shown a wide range of antioxidant properties including scavenging of hydroxyl radical and singlet oxygen. In this study we show that in the presence of visible light, uric acid disrupted caprine alpha-2-macroglobulin (α_2M) structure and antiproteolytic function *in vitro*. Proteinase cleaves the bait region of caprine inhibitor inducing major conformational changes and entrapping the enzyme within its molecular cage. In contrast to native α_2M, modified antiproteinase lost half of its antiproteolytic potential within 4 hours of uric acid exposure. The changes in uv-absorption spectra of the treated protein suggested possible spatial rearrangement of subunits or conformational change. Analysis of the mechanism by which α_2M was inactivated revealed that the process was dependent on generation of superoxide anion and hydrogen peroxide. Our findings suggest that antiproteolytic activity of caprine α_2M could be compromised via oxidative modification mediated by uric acid. Moreover, low concentrations of α_2M were found to stimulate superoxide production by some unknown mechanism.

Keywords: Alpha-2-macroglobulin, proteinase inhibitor, uric acid, free radicals, active oxygen species

INTRODUCTION

In the course of acute inflammatory reactions, infiltrating phagocytes generate oxygen metabolites and release proteinases that inactivate key members of the antiproteinase barrier[1]. Antiproteinase activities of virtually every known plasma proteinase inhibitor have been shown to be suppressed following oxidative or proteolytic attack[2–4]. The only plasma proteinase inhibitor believed to be resistant to physiological inactivation has been multifunctional α_2M[4].

α_2M, one of the key member of the antiproteinase barrier is found in the body fluids of mammals and vertebrates[5]. It is also a major antiproteinase of extra-vascular fluid such as lymph, colostrum, synovial fluid, pleural fluid, bronchoalveolar lavage fluid and aqueous humor[6–8]. α_2M can be distinguished from other antiproteinases by its ability to inhibit proteinases possessing various catalytic mechanisms without direct blockage of their active site[5]. The unique mechanism is initiated by proteolytic cleavage of the bait region ~ a 30 amino acid residue stretch, that is susceptible to cleavage by a majority of proteinases. Bait region cleavage triggers a conformational change that encages the proteinase and hinders its access to large molecular weight substrates and inhibitors[9]. We have recently shown that the caprine α_2M, a homotetramer of 146 KDa subunits, resembles other

* To whom correspondence is to be addressed. Tel: +91–571–400741.

tetrameric α_2Ms in quaternary structure, proteinase inhibition and several other properties[10].

The unusual inhibitor mechanism of α_2M and its apparent resistance to inactivation appear to support its role as "fail-safe" antiproteinase at inflammatory sites[11]. Recent studies have demonstrated that halogenated oxidants can irreversibly destory the structure and function of human α_2M[12]. However, halogenated oxidants represent the only known class of physiologically relevant α_2M inactivator. In this article we describe experinents to show that uric acid (2,6,8 trioxopurine), a physiological antioxidant, can also compromise functional activity of caprine α_2M, a homologue of human α_2M. Although uric acid exposed caprine α_2M still entrapped proteinase and underwent its characteristic "slow" to "fast" transformation on trypsinization, its structure underwent more subtle changes from reactive species generated by uric acid. To our surprise, low concentrations of α_2M stimulated free radical production by uric acid by some unknown mechanisms.

MATERIALS AND METHODS

Trypsin, phenylmethylsulphonylfluoride (PMSF), soyabean trypsin inhibitor (STI), N-benzoyl-DL-arginine-p-nitroanilide (BAPNA), nitroblue tetrazolium (NBT), Ethylene diaminetetraacetic acid (EDTA), bovine erythrocyte superoxide dismutase, catalase and uric acid were from Sigma Chemical Co. (St. Louis, MO). Sephacryl-S300 HR was purchased from Pharmacia, Sweden. All the other reagents used were of highest purity commercially available.

Purification of α_2M

α_2M was isolated from goat blood by the method of Khan et al.[10]. Goat blood was routinely collected at slaughter (within few minutes after the animals were killed) into bottles containing 1/10 volume of acid citrate dextrose containing 1 mM PMSF/STI (50 mg/l of blood). Caprine α_2M can be purified in high yields by a simple two step procedure. Goat plasma was fractionated with ammonium sulfate and the fraction precipitating between 20–40% saturation was dialysed against 50 mM sodium phosphate buffer, pH 7.4 containing 50 mM KCl. This was subsequently chromatographed on Sephacryl-S300 (82 × 1.5 cm) in the same buffer. The fractions containing inhibitory activity against trypsin were pooled and concentrated. The purified protein gave single band on 5% polyacrylamide gel electrophoresis (PAGE) and two distinct bands in SDS-PAGE as reported earlier[10].

Reaction of uric acid with α_2M

α_2M (110 – 200 nmol) was incubated with uric acid (in 50 mM sodium phosphate buffer pH 7.4) in final volume of 1000 µl at room temperature. Uric acid solution was freshly prepared at 5 mM concentration in 50 mM sodium phosphate buffer, pH 7.4. Free radical scavengers (superoxide dismutase, catalase, thiourea, sodium benzoate) were included in some experiments. Incubation at room temperature (28–30°C) was performed under illumination of 500 flux from a fluorescent lamp. Following varying incubation periods, antiproteinase activity of α_2M and/or the free radical generated was quantitated spectrophotometrically.

α_2M antiproteolytic activity

Functional α_2M was assessed on the basis of the ability of the antiproteinase to protect the amidolytic activity of trypsin from an excess of STI as described by Ganrot[9].

Polyacrylamide gel electrophoresis

Samples of native or uric acid treated α_2M were examined by electrophoresis directly or following incubation with 3-fold molar excess of bovine trypsin for 15 min at 37°C. Native PAGE using 5% gel were run essentially as outlined by Laemmli[13] using tris-glycine buffer pH 8.3.

Assay of superoxide radical

Superoxide was detected by the reduction of nitroblue tetrazolium as described by Nakayama et al[14]. A typical assay contained in total volume of 3.0 ml, 50 mM sodium phosphate pH 7.4, 33 mM NBT, 0.1 mM EDTA and 0.06% triton x-100. The reaction was started by addition of uric acid under the illumination from fluorescent lamp and absorbance at 560 nm was measured under various experimental condition against a blank which did not contain uric acid. To confirm the formation of superoxide anion, SOD was introduced into the solution before adding uric acid.

Other methods

UV absorption spectra were measured in DU-40 at 28°C with cells having 1 cm path length per compartment and with α_2M concentration of 130 nmol. α_2M incubated with uric acid (final concentration 5 mM) was photoilluminated for 4 h at room temperature. The reaction mixture was extensively dialysed against 50 mM sodium phosphate buffer pH 7.4 and uv-absorption spectra was measured. To rule out possible contribution of urate or its degradation product in the absorbance changes, the reaction mixture was read against a blank of photoirradiated urate (5 mM).

Protein estimation was carried out by the method of Lowry et al[15]. The zinc content of α_2M was determined according to the method of Song et al[16].

RESULTS

Uric acid induced inactivation of caprine α_2M

To directly quantitate the effect of uric acid exposure on α_2M functions, its antiproteolytic potential was assessed in the amidase assay. As shown in Figure (1), α_2M (128 nmol) exposed to photoilluminated uric acid (final concentration 5 mM) for varying time period gradually lost antiproteinase activity i.e. its ability to bind proteinase in a manner that shielded the entrapped enzyme from STI under the assay condition. Moreover, α_2M incubated with increasing concentration of uric acid also displayed expected loss in antitrypsin activity of the caprine inhibitor (Table I). These results suggest that α_2M exposed to photoilluminated uric acid rapidly lost the bulk of its antiproteinase activity. Control experiments in which α_2M was exposed to uric acid (5 mM) in the dark did not result in any significant (less than 2%) loss in antiproteolytic activity. Neither uric acid nor catalase, superoxide dismutase, thiourea, sodium benzoate at the maximum concentration used in these studies had any effect on α_2M assay.

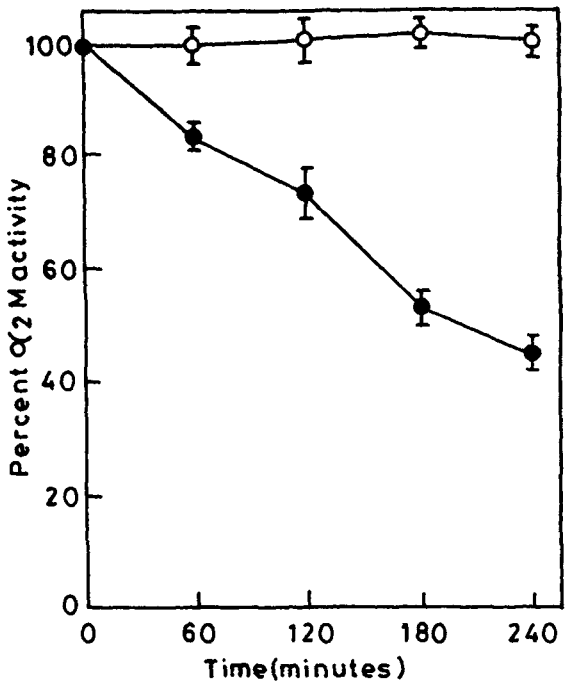


FIGURE 1 Disruption of α_2M function by uric acid. Native α_2M (128 nmol) was incubated with uric acid (5 mM) for increasing periods in a final volume of 1 ml at room temperature under fluorescent light (•). α_2M was assayed for loss in antiproteinase activity by amidase assay as described in the text. Untreated α_2M (◦) is shown for comparison purposes. Results represent the mean±SEM calculated from three separate experiments

TABLE I Inhibition of α_2M antiproteolytic activity after treatment with uric acid

Conditions	*α_2M activity° (Units)*
Native α_2M	118.2 ± 6.7
α_2M + 0.5 mM uric acid	80.1 ± 7.8
α_2M + 1.0 mM uric acid	72.4 ± 2.7
α_2M + 2.0 mM uric acid	67.8 ± 1.1
α_2M + 5.0 mM uric acid	49.2 ± 2.6

Concentration of uric acid shown are final reaction concentration. All incubations were in light for 4 h (28°C) in 50 mM phosphate buffer pH 7.4. α_2M activity was assayed by the method of Ganrot (9).
Results represent the mean±SEM calculated from three separate experiments.

Involvement of oxygen free radical in the reaction

It has recently been demonstrated that photoilluminated uric acid can generate active oxygen species[17], and so the potential role of these species in α_2M inactivation was determined. Uric acid-induced α_2M inactivation was inhibited by several radical scavengers. Superoxide dismutase and catalase remove superoxide anion and hydrogen peroxide respectively. Thiourea and sodium benzoate eliminate hydroxyl radicals. Addition of native superoxide dismutase or catalase almost completely preserved α_2M activity suggesting possible involvement of superoxide and hydrogen peroxide (Table II). The lack of protection by benzoate or thiourea suggests that the damaging agent is not hydroxyl radical. Together these findings suggest that superoxide and hydrogen peroxide played a critical role in α_2M destruction.

Structural analysis of dysfunctional α_2M

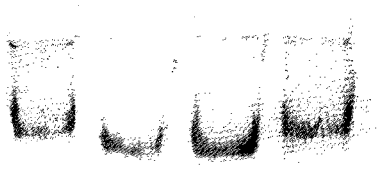
To determine the conformational and structural status of α_2M exposed to uric acid, samples of treated antiproteinase were subjected to PAGE analysis. As shown in Fig. 2, lane d, caprine α_2M exposed to 5 mM uric acid retained its native conformation. Proteolysis of the bait region in the native α_2M precipitates a series of rapid conformational changes which serves to entrap the attacking proteinase[6]. After the addition of trypsin to uric acid exposed α_2M, the resultant complex migrated with increased mobility under non-denaturing condition (i.e. fast form of α_2M) similar to trypsinized native α_2M (Fig. 2, lane c). Since treated α_2M migrated normally in PAGE and could be converted to the "fast" form following the addition of trypsin, it appears that caprine α_2M exposed to uric acid did not undergo extensive structural modifications.

TABLE II Percent residual α_2M activity after treatment with uric acid (5 mM) in the presence of scavengers

Conditions	*Percent α_2M activity*
Native α_2M	100.0 ± 2.4
α_2M + uric acid	51.3 ± 1.6
α_2M+uric acid + SOD (0.1 mg/ml)	111.1 ± 7.8
α_2M + uric acid + catalase (0.1 mg/ml)	115.5 ± 3.1
α_2M + uric acid + thiourea (50 mM)	45.0 ± 3.6
α_2M + uric acid + sodium benzoate (50 mM)	47.4 ± 1.2

Concentration of scavengers shown are final reaction concentrations. All incubations were in light for 4 h (28°C) in 50 mM phosphate buffer pH 7.4. α_2M activity was assayed as described by Ganrot (9).
Results represent the mean±SEM calculated from 4 independent experiments and are expressed as a percentage of the native α_2M activity.

To determine whether α_2M underwent more subtle damage, absorbance spectra of native and uric acid exposed α_2M were measured in the near uv-wavelength region. The overall shape of spectra of α_2M exposed to uric acid indicates a red shift accompanied by an increase in absorption. Uric acid exposed α_2M showed a distinct maximum around 285 nm instead of at 280 nm as observed in native α_2M (Fig. 3). The near uv-spectroscopic changes associated with the conformation of α_2M are primarily due to perturbation of tryptophan residues[18] of the caprine inhibitor. The increase in λ_{max} and absorption red shift, observed in uric acid exposed α_2M, indicates that tryptophan residues are transferred to a more hydrophobic environment[18,19], reflecting altered conformation of the inhibitor. Thus α_2M might have undergone small spatial rearrangement of α_2M subunits after reaction with uric acid without any gross conformational change.



a b c d

FIGURE 2 Reactivity of trypsin with native and uric acid exposed α_2M. α_2M (128 nmol) was incubated with 5 mM uric acid under appropriate condition as described in the text. Native and uric acid exposed α_2M was treated with 3-fold molar excess of trypsin at 37°C for 10 min and reaction was stopped with 1 mM PMSF. Lane a contains native α_2M; lane b contains trypsinized native α_2M; lane c contains trypsinized uric acid exposed α_2M, and lane d contains uric acid exposed α_2M

Caprine α_2M enhances reactive oxygen species generation by uric acid

As reported previously[20] uric acid, generated superoxide anion on photoillumination, which can be assayed by incorporating NBT in the incubating medium. Surprisingly this response was considerably enhanced in presence of low concentration of α_2M (Fig. 4). The fact that NBT was genuinely assaying superoxide was confirmed by superoxide dismutase inhibiting the reaction.

In the presence of α_2M (128 nmol), uric acid generated a dose dependent increase in superoxide generation (Fig. 5A). However, in presence of 5 mM uric acid, dose dependent increase in α_2M concentration showed biphasic effect. Low concentration of α_2M had a stimulatory effect on superoxide radical generation and higher concentration (greater than 128 nmoles) inhibited the response (Fig. 5B). Control experiments containing caprine α_2M alone did not show any free radical generation on photoillumination. Moreover, α_2M (128 nmol) exposed to uric acid in dark did not result in stimulation of superoxide radical by α_2M. Therefore, in presence of visible light, low concentration of caprine α_2M seems to be facilitating the reaction by some unknown mechanism.

DISCUSSION

Caprine α_2M is a broad spectrum antiproteinase capable of inhibiting endopeptidases of various

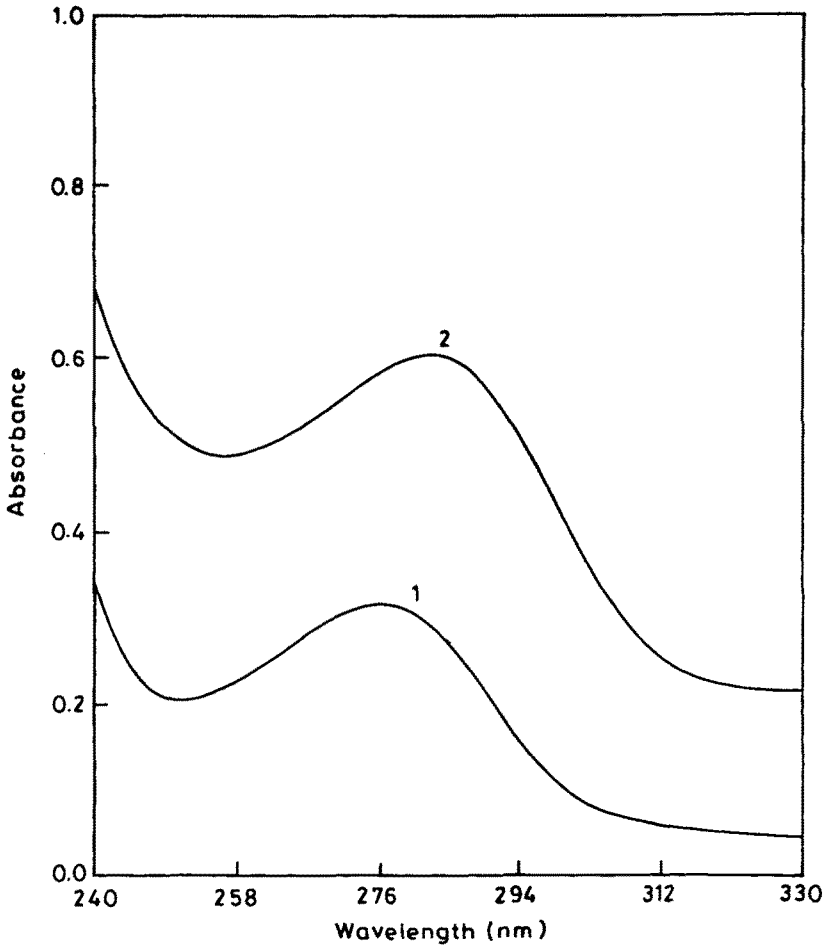


FIGURE 3 UV absorption spectra of native and uric acid exposed α_2M. The uv spectra of native and uric acid treated α_2M were taken as described in the materials and methods. Trace 1 is the absorption spectrum of α_2M; trace 2 corresponds to absorption spectrum of uric acid treated α_2M

classes or substrate specificity[10]. Following proteolytic cleavage within its bait region, the antiproteinase undergoes a series of conformational changes that encages the attacking proteinase and hinder its access to large molecular weight substrate and inhibitors. Simple two step purification procedure with high yield of α_2M coupled with its resemblance to human α_2M in quaternary structure, proteinase inhibition and other properties makes it obvious choice for these studies.

Uric acid (2,6,8 trioxopurine) is produced in mammalian systems as the end product of purine metabolism. The normal physiological concentration of uric acid in human plasma is 0.2 -.35 mM[21]. Uric acid can act as an antioxidant both by binding iron and copper ions in forms that do not accelerate free radical reactions, by directly scavenging oxidizing species such as singlet O_2, hydroxyl radical, HOCl, peroxyl radicals[22] and by inhibiting peroxynitrite dependent tyrosine nitration[23]. Increased uric acid concentrations have been observed in body fluids under certain diseased conditions such as hyperuricemia, gout and arthritis. Recent studies have indicated that uric acid can itself generate

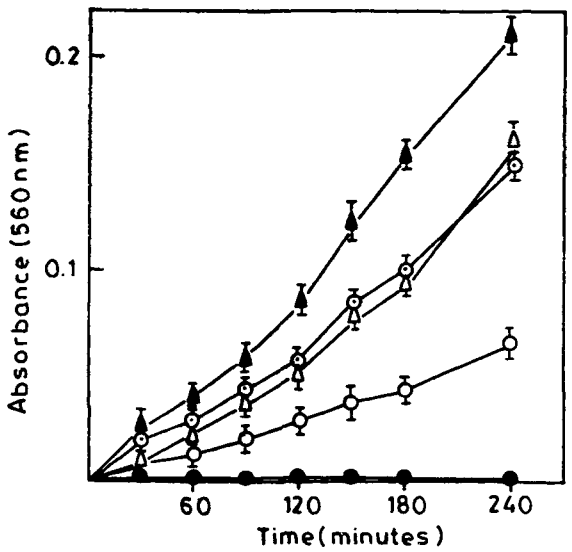


FIGURE 4 Photogeneration of superoxide anion on illumination of uric acid and effect of incubation in the presence of α_2M. Superoxide was determined by the reduction of nitroblue tetrazolium to formazan as referred in the text. The concentration of α_2M was 128 nmol. Uric acid 2 mM (○) and 5 mM (○); α_2M + 2 mM uric acid (△), α_2M + 5 mM uric acid (▲) and α_2M + 5 mM uric acid + SOD (0.1mg/ml) (•). Data represent the mean±SEM calculated from three separate experiments. Error bars appear only when they are larger than symbols

reactive species that could cause biological damage[17,24]. Free radical induced damage to the antiproteinase barrier has been implicated in the pathogenesis of several diseases including emphysema, gout and arthritis[1]. Since uric acid is a major antioxidant of the body fluids and α_2M is a key member of the antiproteinase barrier, these investigations on uric acid induced damage to α_2M may yield important information about the etiology of these diseases.

Following exposure to uric acid, α_2M was unable to completely shield entrapped trypsin from inhibition by STI. Antiproteinase activity of α_2M declined with increasing length of exposure to uric acid. It lost ~50% of its antiproteolytic potential following uric acid exposure within 4h. An analysis of mechanism involved in functional disruption of α_2M revealed that the antiproteinase was protected from uric acid induced damage by agents that consumed H_2O_2 (catalase) or decreased steady state concentration of superoxide (superoxide dismutase). It has been shown that uric acid generates O_2^- which in turn can give rise to OH^-radicals by modified Haber-Weiss reaction[25]. The two hydroxyl radical scavengers present in large excess (Table II) should have protected antiprotease against damage by the hydroxyl radical. The lack of protection by benzoate and thiourea suggests that the oxidizing agent is not the hydroxyl radical. The probable mechanism of α_2M inactivation involves O_2^- radical. The superoxide radical might directly participate in α_2M inactivation[26] or may form uric acid free radical through reaction of uric acid with superoxide anion[27]. The free radical metabolite of uric acid is known to inactivate certain enzymes[24] and antiproteinase[28] and may therefore be capable of α_2M damage.

It is important to note that although caprine α_2M has bound Zn^{2+} (535.33 ± 6.38 μg/g of protein), addition of metal-chelating agent, EDTA (final concentration 2 mM) did not have any effect on α_2M inactivating response elicited by uric acid. The superoxide stimulatory effect induced by caprine α_2M on photoilluminated uric acid also remained unaffected by EDTA treatment. (Data not shown).

In contrast to the loss of α_2M antiproteolytic activity, the native conformation of α_2M was not significantly altered following uric acid exposure. Retention of "slow" form of α_2M and its ability to undergo "slow" to "fast" transformation after uric acid treatment rules out direct oxidative attack on the thiolester bond of α_2M. Scission of thiolester bond results in the loss of α_2M antiproteolytic activity by trigging the transition of the inhibitor to "fast" conformation. The antiproteolytic function of treated α_2M could possibly be undermined via oxidation of cleavage site within the bait region or spatial rearrangement of subunits of α_2M occuring after uric acid treatment may "expose" the entrapped proteinase. It is conceivable that uric acid treated

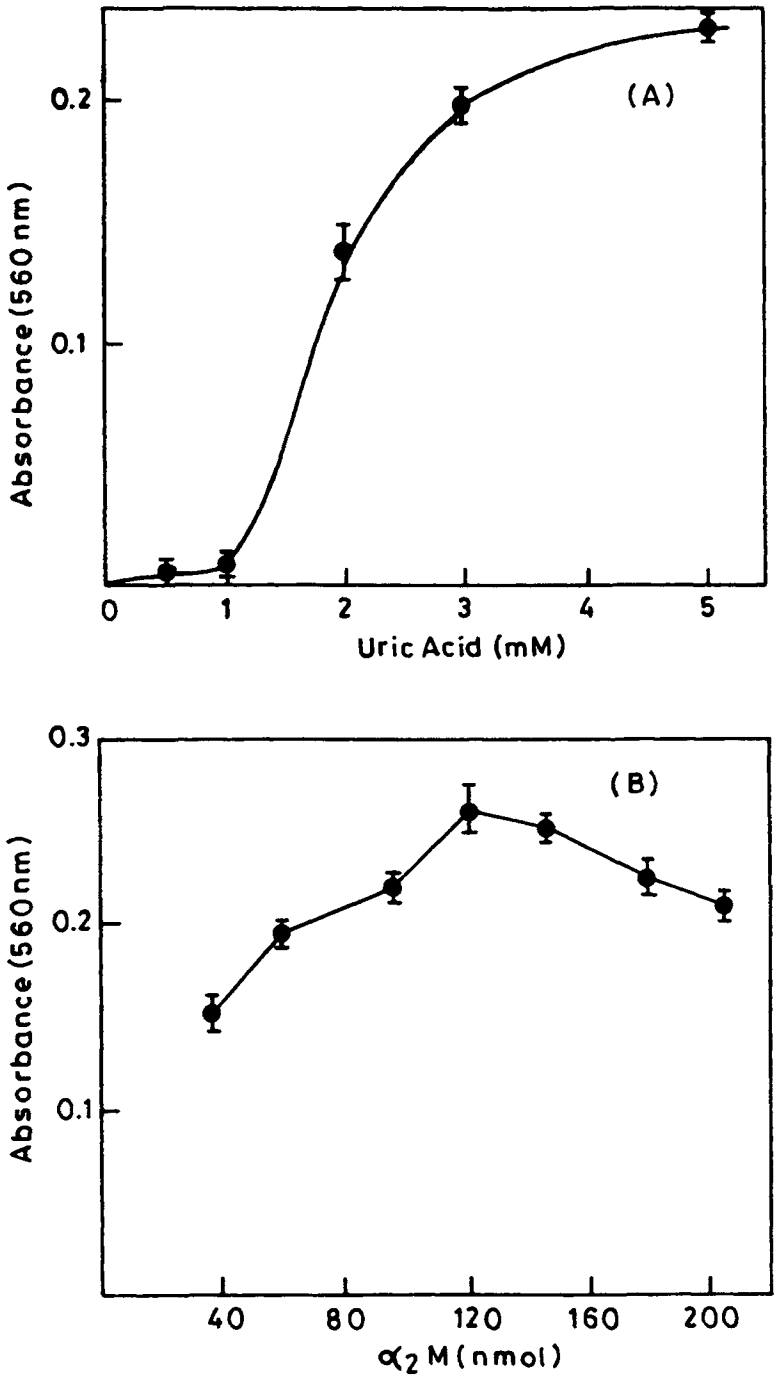


FIGURE 5 Effect of increasing uric acid or α_2M concentration on NBT reduction by superoxide anion. Generation of superoxide anion by photoilluminated uric acid was determined by reduction of NBT to formazan as described in the text. Reactions were carried out under fluorescent light for 4h at room temperature. (A) α_2M (128 nmol) was exposed to varying concentration of uric acid. (B) Uric acid (5mM) was incubated with increasing concentration of α_2M. The results represent the mean±SEM calculated from three separate experiments

α_2M retained the ability to entrap the proteinase, albeit in manner that no longer afforded steric hindrance. These data are not meant to detract from the established antioxidant role of uric acid in biological fluids. Nonetheless, our studies support the idea that several of the antioxidant are themselves capable of inducing biological damage under appropriate conditions.

α_2M induced enhancement of superoxide radical production by uric acid is an unexpected result for which we cannot offer mechanistic explaination with available data. Protein exposed to free radical systems in the presence of oxygen yields hydroperoxide[29]. These hydroperoxides can further give rise to reactive free radicals on spontaneous degradation or rearrangement[30]. Superoxide or its protonated form is not effective in protein peroxidation[29]. Moreover, vulnerability of O_2^- enhancement by catalase (which removes hydrogen peroxide but has no effect on protein hydroperoxide) rules out direct involvement of protein hydroperoxide in enhancement of O_2^- production by α_2M.

α_2M has been subject of considerable attention currently because of its role in controlling proteolytic events in tissues during inflammation. Until recently, it was the only plasma inhibitor believed to be resistant to proteolytic or oxidative events[12]. The sensitivity of the α_2M to high dose of uric acid suggests that uric acid elevated during certain inflammatory diseases such as gout and arthritis may also inactivate α_2M and unleash a whole cascade of tissue destructive proteinase. Recent studies demonstrating the presence of inactive α_2M in proteins recovered from inflammatory sites *in vivo*[31,32] lend support to the physiological relevance of our studies in pathophysiological setting.

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