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Measurement of intracellular generation of hydrogen peroxide by rat glomeruli *in vitro*

NORISHI UEDA, BERTRAND GUIDET, and SUDHIR V. SHAH

Department of Internal Medicine, Division of Nephrology, University of Arkansas for Medical Sciences and John L. McClellan Memorial Veterans Administration Hospital, Little Rock, Arkansas, USA

Sequential reduction of oxygen along the univalent pathway leads to the generation of superoxide anion, hydrogen peroxide, hydroxyl radical, and water [1, 2]. These partially reduced oxygen intermediates have been implicated as important mediators in various models of ischemic, toxic and immune-mediated tissue injury including glomerular injury [3]. Reactive oxygen metabolites have been shown to affect several biological processes potentially important in glomerular diseases, and their role in both inflammatory as well as non-inflammatory glomerular diseases has recently been demonstrated [3]. Evidence for the importance of reactive oxygen metabolites in experimental models of glomerular disease is based largely on the protective effects of scavengers of reactive oxygen metabolites.

Although the extracellular generation of reactive oxygen metabolites by glomerular cells [3] has been examined in *in vitro* studies, there is no information on the *in vitro* measurement of intracellular hydrogen peroxide under normal conditions or in response to agents in which reactive oxygen metabolites have been implicated in causing glomerular injury. The measurement of these partially reduced intermediates is difficult because of the existence of efficient scavenging enzymes and/or their high reactivity. However, recent studies have shown the ability to measure normal [4] and enhanced intracellular generation of hydrogen peroxide *in vivo* [5, 6]. The method employed to detect hydrogen peroxide is based on the inhibition of endogenous catalase by 3-amino-1,2,4-triazole. Catalase is inactivated by aminotriazole only in the presence of hydrogen peroxide, because aminotriazole reacts with the catalase intermediate, compound I, to form an inactive covalent complex with the protein [7, 8]. The inactivation of tissue catalase by aminotriazole can therefore be interpreted as a qualitative measure of hydrogen production [4–8]. The reaction sequence is shown in Equations (1) and (2):

$$\text{catalase} + \text{hydrogen peroxide} = [\text{catalase-hydrogen peroxide}]\text{compound I} \quad \text{(Eq. 1)}$$

$$\text{compound I} + \text{aminotriazole} \rightarrow \text{irreversible inhibited catalase} \quad \text{(Eq. 2)}$$

Both the *in vitro* and *in vivo* inactivation of catalase can be prevented by ethanol because ethanol rapidly reduces compound I and effectively competes with the aminotriazole reaction [4–8].

$$\text{compound I} + \text{hydrogen peroxide} \rightarrow \text{catalase} + O_2 + 2H_2O \text{ (catalatic activity)} \quad \text{(Eq. 3)}$$

$$\text{compound I} + \text{ethanol} \rightarrow \text{catalase} + \text{acetaldehyde} + 2H_2O \text{ (peroxidatic activity)} \quad \text{(Eq. 4)}$$

In our previous studies, using aminotriazole-induced inhibition of catalase, coupled with the prevention of the aminotriazole-induced inhibition by ethanol, we have provided evidence for *in vivo* generation of intracellular hydrogen peroxide by rat renal cortex and glomeruli under normal conditions [4] and by renal cortex from rats with glycerol-induced acute renal failure [6]. In the present study we examined the utility of this method to demonstrate the intracellular generation of hydrogen peroxide *in vitro* by freshly isolated rat glomeruli under normal conditions and in response to agents known to enhance intracellular content of hydrogen peroxide.

Adriamycin (an anthracycline antibiotic used in cancer chemotherapy) undergoes a one-electron reduction to a free radical, semiquinone species [3] which in the presence of molecular oxygen is rapidly reoxidized in a process which generates reactive oxygen metabolites. A single intravenous injection of Adriamycin causes nephrotic syndrome in rats [3] with morphological and functional changes similar to those seen in minimal change disease in humans. Enhanced intracellular generation of reactive oxygen metabolites have been implicated in Adriamycin-induced nephrotic syndrome [9]. We also examined whether enhanced intracellular generation of hydrogen peroxide by freshly isolated glomeruli could be demonstrated in response to Adriamycin.

Methods

Measurement of intracellular hydrogen peroxide generation by rat glomeruli in vitro

Kidneys from adult male Sprague-Dawley rats (250 to 300 g) were perfused with ice-cold Hank's balanced salt solution

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(HBSS) to remove the blood as described in our previous study [4]. Glomeruli were isolated by a combination of differential sieving and centrifugation as in our previous studies [4]. Freshly isolated glomeruli were resuspended in ice-cold HBSS (pH 7.4) and distributed randomly in order to have approximately 0.5 mg of protein per tube. Following an incubation for 15 or 30 minutes at 37°C in a Dubnoff metabolic shaking water bath, aminotriazole was added to the tubes. When the effect of ethanol (0.17 M) to prevent aminotriazole-induced inhibition of catalase was assessed, it was added to the tubes 10 minutes before the addition of aminotriazole. The final volume of each suspension was 1 ml, with final concentration of aminotriazole ranging from 2.5 to 50 mM. The tubes were incubated for an additional period of time as indicated in **Results** and **Discussion.** At the end of the incubation period, tubes were centrifuged at 700 g at 4°C for five minutes, supernatant removed, and glomeruli were washed once with ice-cold HBSS. The glomeruli were then resuspended in 1 ml of cold 50 mM phosphate buffer (pH 7.0) containing 0.1% Triton X-100, 0.1 mM EDTA, and 0.17 M ethanol. The addition of ethanol at the end of the incubation period prevented any further effect of aminotriazole present within the cells. The glomeruli were disrupted three times by sonication for five seconds on ice at a power output of 40 W, and the samples were centrifuged at 700 g for 10 minutes. The aliquots of the supernatants were used for determination of catalase activity, aminotriazole content and protein measurements. Since ethanol and Triton X-100 were already present in the sonication buffer, they were not added, as recommended by Cohen, Dembiec and Marcus [10], to the samples before performing the catalase assay. The catalase activity in the glomeruli was measured as described by Aebi [11]. The samples were diluted with cold phosphate buffer (50 mM, without Triton) to achieve a fourfold dilution. A total of 0.8 ml of the diluted samples were distributed in the reference cuvette and the test cuvette, then 0.2 ml of phosphate buffer was added to the reference cuvette and the reaction was started by the addition of 0.2 ml of 50 mM hydrogen peroxide in the test cuvette. The final concentration of hydrogen peroxide in the test cuvette was 10 mM, and its disappearance was followed for 30 seconds using a spectrophotometer at a wavelength of 240 nm (extinction coefficient of 43.6 $M^{-1}cm^{-1}$) as previously described [11]. The assay contained sufficient sample to achieve a stable decrease of optic density between 0.04 and 0.20 per 30 seconds, as recommended by Aebi [11]. Under the conditions described, the decomposition of hydrogen peroxide by catalase contained in the samples follows a first order kinetic as given by the equation:

$$k = 2.3/t * \log Eo/E$$

where k is the first order kinetic rate constant, t is the time over which the decrease of hydrogen peroxide due to catalase activity is measured (30 seconds), Eo and E are the optical density (OD) at time 0 and 30 seconds respectively. Protein concentration in the supernatants was determined by the method of Lowry et al [12] and the results were expressed as k/mg protein.

In order to examine the sensitivity of catalase-aminotriazole system for measurement of hydrogen peroxide we measured the inhibition of catalase when various concentrations of hydrogen peroxide were added to the reaction mixture directly as previously described [8]. The various concentrations of hydrogen peroxide (0 to 40 nmol) were added to the reaction mixture containing phosphate buffer (pH 7.0), 0.1% Triton X-100, beef liver catalase (0.25 mg/ml), and aminotriazole (2.5 mM) in a total volume of 1 ml, and incubated for 10 minutes at room temperature. Then 10 μl of ethanol (final concentration, 0.17 M) was added to decompose complex I and stop reaction with aminotriazole and incubated for 10 minutes at room temperature. The reaction mixture was diluted with equal volume of ice-cold phosphate buffer, and the residual catalase activity was determined by measuring OD at 240 nm against the blank containing phosphate buffer alone in the same manner as the measurement of hydrogen peroxide in glomeruli. The percentage of inhibition of catalase by hydrogen peroxide was calculated by the equation:

$$\% \text{ inhibition of catalase} = 100 \times (1\text{-}OD_{sample}/OD_{control})$$

where OD_{sample} represents the OD reading in the reaction mixture with hydrogen peroxide and $OD_{control}$ is the OD reading in the reaction mixture with phosphate buffer alone in the same volume of hydrogen peroxide.

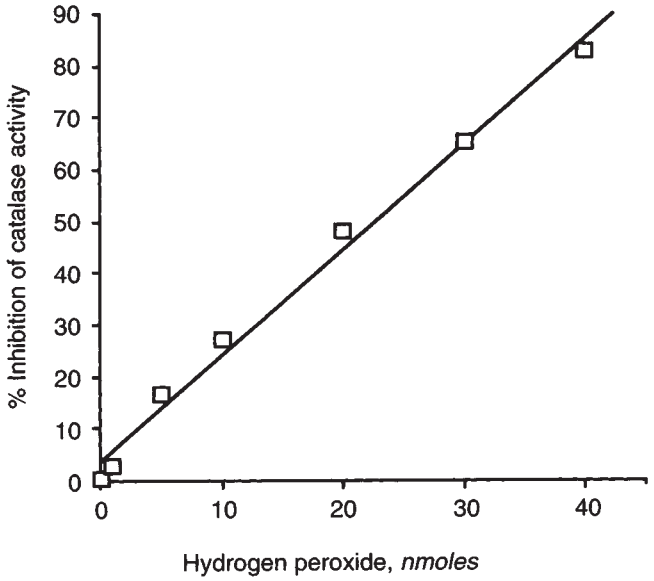

Fig. 1. *Sensitivity of the catalase-aminotriazole system for measuring hydrogen peroxide.* The various amounts of hydrogen peroxide (1, 5, 10, 20, 30, and 40 nmol) were added to the reaction mixture containing phosphate buffer (pH 7.0), 0.1% Triton X-100, 0.25 mg/ml catalase, and 2.5 mM aminotriazole in a total volume of 1 ml. Incubations and measurement of residual catalase activity were carried out as described in **Methods.** Results are expressed as % inhibition of catalase compared to that in absence of hydrogen peroxide, $N = 2$.

As shown in Figure 1, a linear relationship was observed between the amounts of hydrogen peroxide added and the catalase inhibition in the reaction mixture. The data confirmed that this assay system can detect nanomole range of hydrogen peroxide in the reaction mixture. This assay has a fairly broad pH optimum (pH 6.8 to 7.5) [11]. Taken together, the assay employed in our study is sensitive and has a general application for the measurement of intracellular hydrogen peroxide in biological settings.

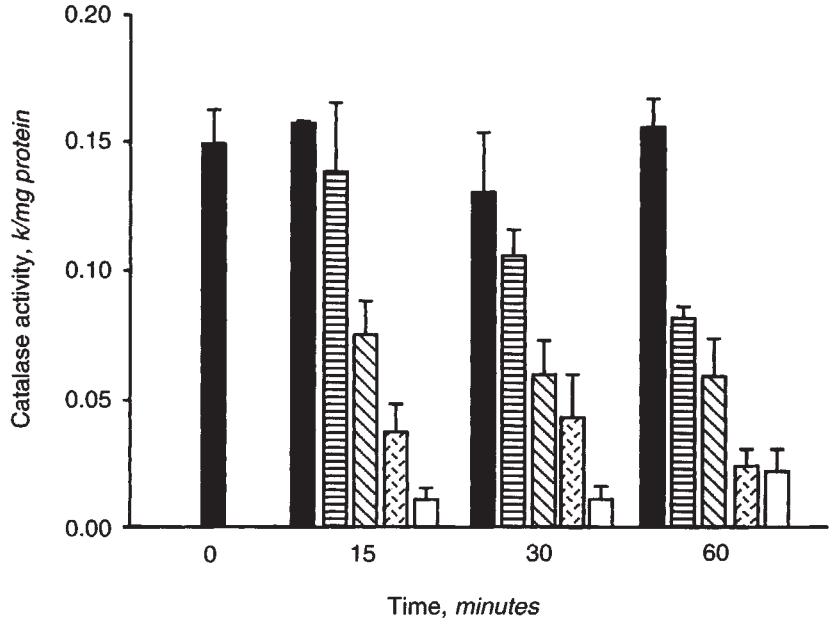

Fig. 2. *Effect of different doses of aminotriazole on catalase activity at various time points in rat glomeruli under normal conditions.* After 30 minutes incubation, glomeruli (■) were incubated with various doses of aminotriazole [AT; (▤) AT 5 mM; (▧) AT 10 mM; (▨) AT 20 mM; (□) AT 50 mM] for variable periods (15 to 60 min). Results are means ± SE, $N = 5$. Inhibition of glomerular catalase activity was proportional to the dose of aminotriazole used and incubation period.

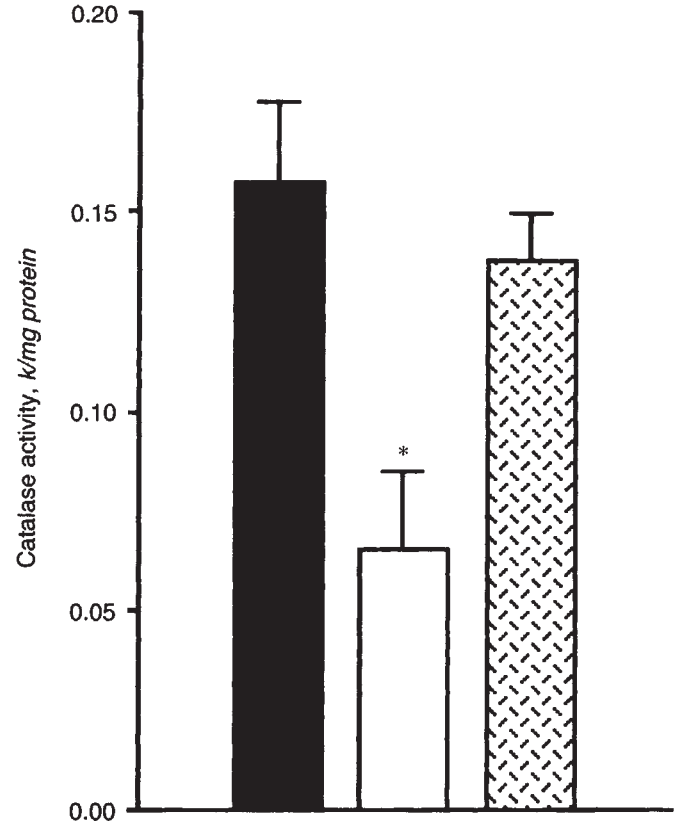

Fig. 3. *Effect of ethanol on aminotriazole-induced catalase inhibition in rat glomeruli under normal conditions.* After 30 minutes incubation, glomeruli (■) were incubated with 5 mM aminotriazole (AT) for 60 minutes with (▨) or without (□) 0.17 M ethanol. Results are means ± SE, $N = 5$. *$P < 0.001$, compared to glomeruli alone or glomeruli + AT + ethanol.

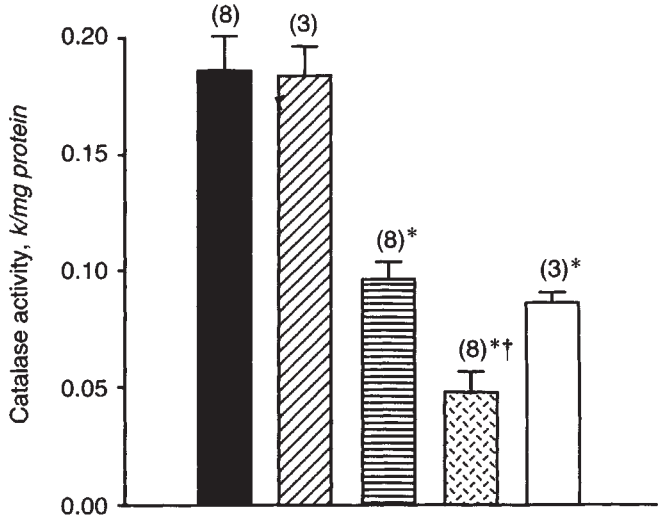

Fig. 4. *Effect of glucose/glucose oxidase on the generation of intracellular hydrogen peroxide by rat glomeruli.* After 30 minutes incubation, glomeruli (■) were incubated with either glucose (G, 5 mM)/glucose oxidase (GO, 0.5 U/ml) alone (▨) or 5 mM aminotriazole (AT; ▤) for 60 minutes with G/GO (▨) or G/heat-inactivated glucose oxidase (inact. GO, 0.5 U/ml; □). Results are means ± SE, and the number of experiments are shown in parentheses. *$P < 0.001$, compared to glomeruli alone or glomeruli + G/GO, †$P < 0.01$, compared to glomeruli + AT or glomeruli + G/inact. GO + AT.

We examined the utility of the aminotriazole-induced inhibition of catalase assay to demonstrate enhanced intracellular hydrogen peroxide when glomeruli are exposed to glucose/glucose oxidase, a system that generates hydrogen peroxide or 2-methyl-1,4-naphthoquinone (menadione) which enhances intracellular generation of hydrogen peroxide [7, 13]. Glucose (5 mM)/cold or heat-inactivated glucose oxidase (0.5 U/ml), or menadione (175 μM) were added at the beginning of the incubation period.

Using this method we examined whether Adriamycin enhances intracellular generation of hydrogen peroxide by freshly isolated rat glomeruli *in vitro*. The various doses of Adriamycin (10 to 250 μM) were added 15 minutes before the addition of

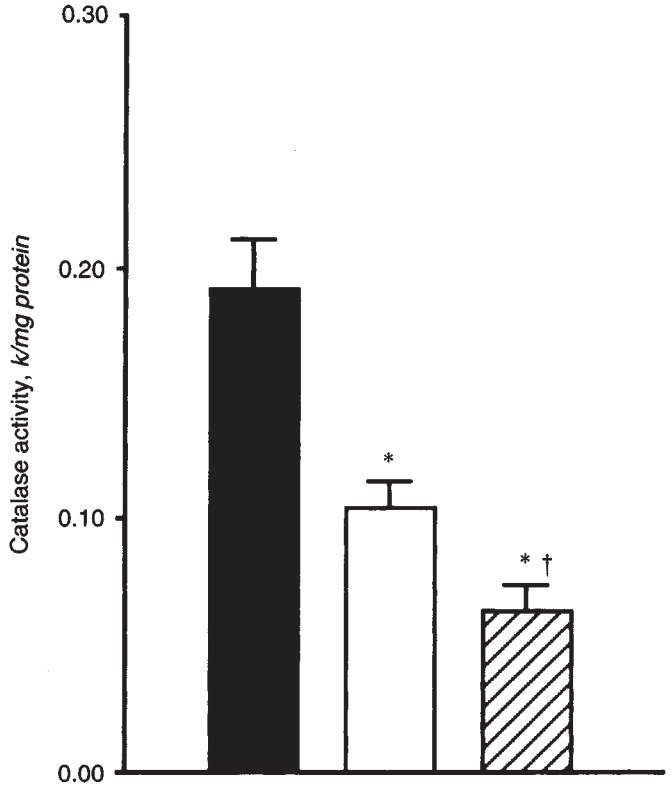

Fig. 5. *Effect of menadione on intracellular generation of hydrogen peroxide by rat glomeruli.* After 30 minutes incubation, glomeruli (■) were incubated with 5 mM aminotriazole (AT) for 60 minutes with (▨) or without (□) menadione (175 μM). Results are means ± SE, $N = 9$. *$P < 0.001$, compared to glomeruli alone, †$P < 0.01$, compared to glomeruli + AT.

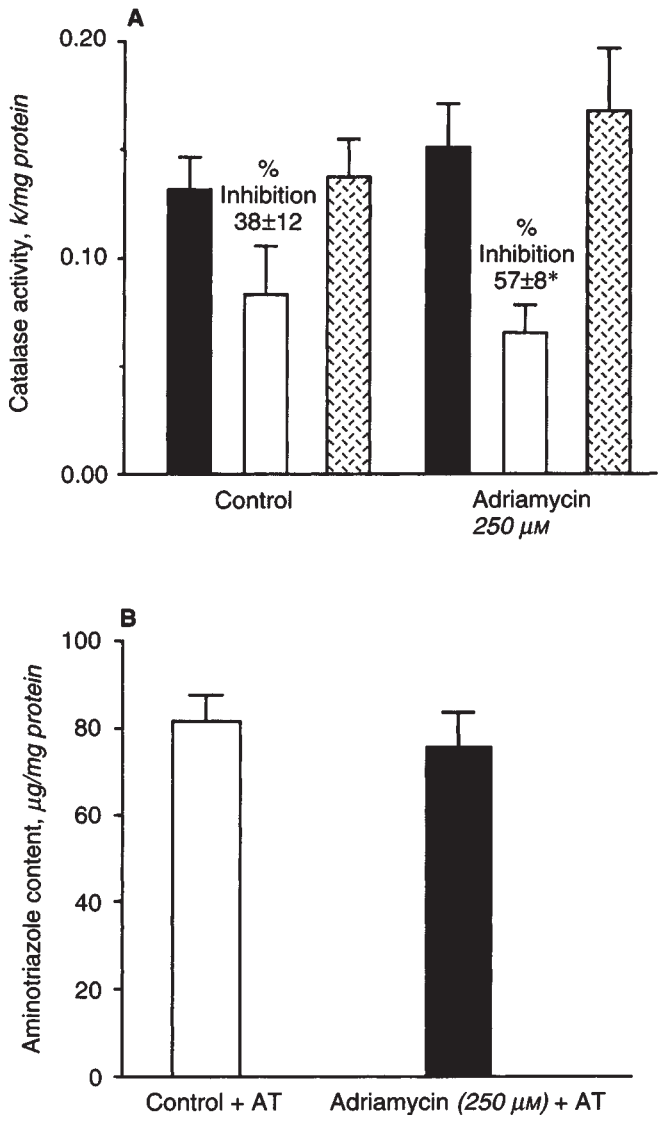

Fig. 6. A. *Effect of Adriamycin on intracellular generation of hydrogen peroxide by rat glomeruli.* After 15 minutes incubation with or without Adriamycin (250 μM), glomeruli (■) were incubated with 2.5 mM aminotriazole (AT) for 45 minutes with (▨) or without (□) 17 M ethanol. Results are means ± SE, $N = 4$. *$P < 0.01$, compared to control glomeruli + AT. **B.** Effect of Adriamycin on aminotriazole content in rat glomeruli. After 15 minutes incubation with or without Adriamycin (250 μM), glomeruli were incubated with 2.5 mM aminotriazole (AT) for 45 minutes. Results are means ± SE, $N = 3$.

aminotriazole (2.5 mM) or HBSS buffer in the same volume as aminotriazole. The total incubation time with Adriamycin was 60 minutes. The concentrations of Adriamycin used in our study are similar to those used in previous *in vitro* studies [14, 15] in which the doses used were also based on the levels obtainable in the kidney *in vivo* [15]. To determine whether aminotriazole-induced inhibition of catalase activity is reversed by ethanol, 0.17 M ethanol was added 10 minutes before the addition of aminotriazole in some tubes.

Aminotriazole assay

The aminotriazole content of the supernatant was determined colorimetrically with some modifications by coupling of diazotized aminotriazole to 4,5-dihydroxy-2,7-naphthalene disulfonic acid (chromotropic acid) to form a colored derivative read at 525 nm [5, 16]. The supernatants were mixed with 0.33 M trichloroacetic acid to diazotize aminotriazole [16] in proportion to 1:1 (vol/vol) and used for the assay. The aminotriazole content in the diluted samples was determined based on a standard curve which was constructed using the known amount of aminotriazole (0 to 20 μg/ml) in the same mixture of phosphate buffer (which contained 0.1% Triton X-100, 0.1 mM EDTA and 0.17 M ethanol) and 0.33 M trichloroacetic acid (1:1, vol/vol). The results were expressed as μg/mg protein.

Statistics

Results are means ± SE, and statistical significance was determined by unpaired Student's *t*-test and a two-way analysis of variance.

Results and Discussion

Increased generation of hydrogen peroxide leads to a greater inhibition of the catalase activity by aminotriazole [4–8]. This observation has been used as a method to demonstrate enhanced *in vivo* generation of hydrogen peroxide in normal [4] and pathological conditions [5, 6]. If the catalase activity is

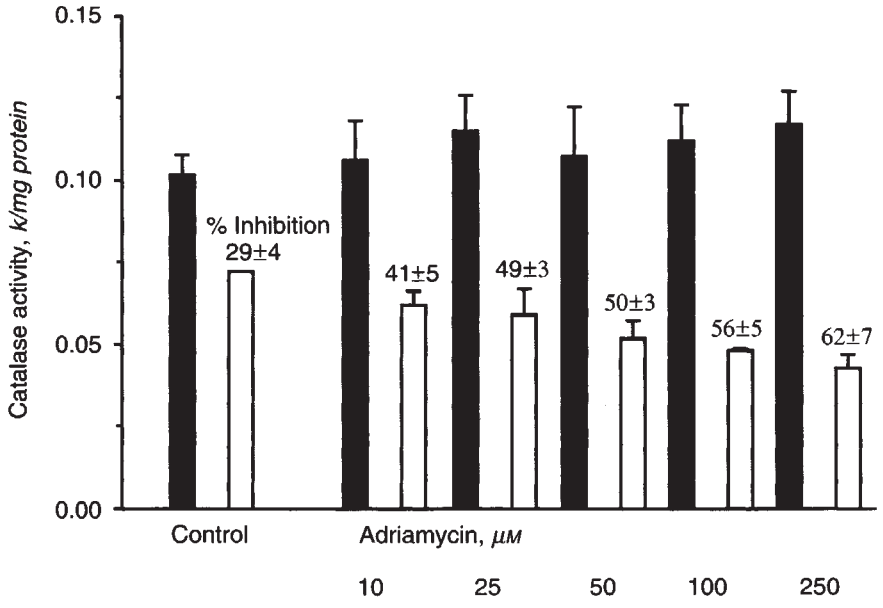

Fig. 7. *Concentration-dependent effect of Adriamycin on the generation of intracellular hydrogen peroxide by rat glomeruli.* After 15 minutes incubation with or without various doses of Adriamycin (10 to 250 μM), glomeruli were incubated with (□) or without (■) 2.5 mM aminotriazole for 45 minutes. Results are means ± SE, $N = 3$.

maximally inhibited by aminotriazole, it would not be possible to demonstrate increased inhibition. To use the aminotriazole-induced inhibition of catalase to demonstrate enhanced *in vitro* generation of hydrogen peroxide, a dose and/or time points where aminotriazole does not maximally inhibit catalase is necessary. We, therefore, first examined the effect of various doses of aminotriazole (5 to 50 mM) on glomerular catalase activity at different time points (15 to 60 min). As shown in Figure 2, the inhibitory effect of aminotriazole at different time points was proportional to the dose used, and the inhibition of glomerular catalase activity was exponential with respect to the time.

The inhibition of tissue catalase by aminotriazole, coupled with its prevention by ethanol, has been used to demonstrate the generation of hydrogen peroxide [4–8]. Based on the data for the inhibitory effect of aminotriazole at different time points and doses on glomerular catalase activity, the effect of ethanol on aminotriazole-induced inhibition of catalase activity was assessed at 60 minutes after incubation of aminotriazole (5 mM). Ethanol was able to prevent completely aminotriazole-induced inhibition of catalase activity in the glomeruli (Fig. 3), indicating that ethanol reverses the aminotriazole-induced inhibition of catalase. In the present study the kidneys were perfused prior to the isolation of glomeruli to remove the blood as described in our previous study [4]. We have previously shown that there is little if any contribution of hydrogen peroxide generated by red blood cells or leukocytes in the kidney [4]. Thus our data represent the production of intracellular hydrogen peroxide by glomeruli rather than bone marrow-derived cells. Taken together, these data demonstrate intracellular generation of hydrogen peroxide by freshly isolated glomeruli under normal conditions.

We next examined the utility of this method to demonstrate increased intracellular hydrogen peroxide by exposing glomeruli to glucose/glucose oxidase, a system that generates hydrogen peroxide directly. Glucose/glucose oxidase, in the absence of aminotriazole had no effect on the glomerular catalase activity. However, in the presence of aminotriazole glucose/glucose oxidase (but not heat-inactivated glucose oxidase) caused a significantly greater inhibition of catalase indicating increased intracellular hydrogen peroxide (Fig. 4).

It has been shown that the cytotoxicity of menadione, a quinone, is the result of oxidative stress caused by the redox cycling of the drug in its target cells [13]. Exposure of endothelial cells to menadione was shown to result in increased content of hydrogen peroxide in the medium [13]. Menadione caused a significantly greater inhibition of catalase, indicating increased intracellular hydrogen peroxide by the glomeruli (Fig. 5). Taken together, the data with glucose/glucose oxidase and menadione demonstrate the utility of this method to detect enhanced intracellular hydrogen peroxide in isolated rat glomeruli.

We then attempted to demonstrate enhanced generation of intracellular hydrogen peroxide in response to Adriamycin, a drug of relevance to glomerular pathophysiology because a single intravenous injection of Adriamycin induces nephrotic syndrome in rats [3]. We utilized 2.5 mM aminotriazole with 45-minute incubation period. In Adriamycin (250 μM)-treated glomeruli, aminotriazole resulted in much greater inhibition ($P < 0.01$) of catalase activity (residual catalase activity in control glomeruli, 0.083 ± 0.022 k/mg protein; Adriamycin-treated glomeruli, 0.065 ± 0.013 k/mg protein, $N = 4$; Fig. 6A). The inhibition of catalase by aminotriazole was 38 ± 12% in control glomeruli and 57 ± 8% in Adriamycin-treated glomeruli ($N = 4$, $P < 0.01$), indicating that Adriamycin enhances intracellular generation of hydrogen peroxide by freshly isolated rat glomeruli. The aminotriazole-induced inhibition of catalase was reversed by ethanol, thus verifying that aminotriazole-induced inhibition of catalase is due to its action on compound I (Fig. 6A). The aminotriazole content in Adriamycin-treated glomeruli (75.4 ± 8.2 μg/mg protein, $N = 3$) was similar to control glomeruli (81.7 ± 5.8 μg/mg protein, $N = 3$; Fig. 6B), indicating that the greater inhibition of catalase by aminotriazole in Adriamycin-treated glomeruli is not due to the greater content of aminotriazole within glomeruli. As shown in

Figure 7, Adriamycin in the presence of aminotriazole caused dose-dependent inhibition of catalase.

In summary, using the method based on the aminotriazole-induced inhibition of catalase we have demonstrated the generation of intracellular hydrogen peroxide by freshly isolated glomeruli *in vitro* under normal conditions. The assay employed in our study is sensitive with a fairly broad pH optimum and has a general application for the measurement of intracellular hydrogen peroxide in biological settings. We provided evidence for a dose-dependent enhanced generation of intracellular hydrogen peroxide by Adriamycin in rat glomeruli. Our results demonstrate the utility of this method to examine enhanced generation of intracellular hydrogen peroxide *in vitro*.

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Reprint requests to Sudhir V. Shah, M.D., University of Arkansas for Medical Sciences, 4301 W. Markham, Slot 501, Little Rock, Arkansas 72205, USA.

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