SIGNIFICANCE OF HEPATIC XANTHINE OXIDASE AND URIC ACID IN AGED AND DIETARY RESTRICTED RATS

Hae Young Chung¹ and Byung Pal Yu^{2,3}

¹ Vascular Aging Research Group,
Department of Pharmacy and

² Department of Molecular Biology,
Pusan National University, Gumjung-ku, Pusan,
609-735, Korea;

³ Department of Physiology, The University of Texas
Health Science Center at San Antonio, USA.

ABSTRACT

Xanthine oxidase (XOD), one of the major intracellular sources of superoxide production, is well characterized as a causative factor in ischemia/reperfusion related damage. In the present study, we investigated age-effect on the status of XOD, an enzyme interconvertible with xanthine dehydrogenase (XDH) under oxidative stress. We also examined the modulation of the enzyme using the anti-oxidative action of dietary restriction (DR). We obtained evidence showing XOD activity to be significantly increased by DR, peaking at 24 months, although no progressive, age-related changes were noticed. On the other hand, while XDH activity decreased in ad libitum fed rats with age, DR maintained higher activity levels at 18 and 24 months of age. During aging, the conversion of XDH to XOD was slightly increased, as indicated by the XOD/XDH ratio. One novel finding of the present study is DR's ability to elevate the uric acid level, which likely augments the anti-oxidative defense system, thereby buffering against oxidatively stressed conditions during aging. Based on what is known about the antioxidative abilities of DR and uric acid, we propose that the high uric acid levels we observed in DR rats may well serve as part of a defense strategy to protect redox balance.

KEYWORDS:

xanthine oxidase, xanthine dehydrogenase, oxidative stress, aging, uric acid

INTRODUCTION

Xanthine oxidoreductase (XO: xanthine dehydrogenase + xanthine oxidase) exists in separate but interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XOD) (1). Ubiquitously distrib-

uted, XDH and XOD are commonly known as the rate-limiting enzymes in nucleic acid degradation and purine catabolism. However, their wide involvement is also found in the antimicrobial activity in various tissues (2-4), in iron metabolism (5), and in the hepatic detoxification process (6). Furthermore, XDH and XOD are implicated in certain pathological conditions such as xanthinuria (7), ethanol toxicity (8), influenza virus infection (9), atherosclerosis (10), and gout (11).

XOD is capable of enzymatically oxidizing a wide variety of organic compounds, including some reactive aldehyde species. Among the many reactive aldehydes, the by-products of lipid peroxidation process of long polyunsaturated fatty acid and 4-hydroxy-2-alkenals are of special interest (12). 4-Hydroxy-2-nonenal has received a recent attention because of its potent cytotoxic properties (13-15). From the standpoint of oxidative stress, it is important to recognize that XOD is involved in catabolism of these reactive aldehydes to acetate, thereby reducing cytotoxicity (16).

Uric acid is an XO reaction end-product, which was regarded simply as a waste product from purine catabolism, without having any beneficial biological role. However, recent anti-oxidant biochemistry studies show uric acid as one of the most effective free radical scavengers (17). Accumulated evidence led to the acceptance of uric acid as a physiological antioxidant (17, 18). According to these studies, uric acid has a potent defense function against a wide variety of the cellular oxidative damage of lipids, proteins, and nucleic acids (17). Such effective protective defenses are based on uric acid's broad reaction against various reactive species such as hydroxyl radical (1), singlet oxygen (2), peroxynitrite (3), and hydroperoxyl radicals. Furthermore, uric acid is an efficient iron chelator (4), which certainly can play a major role in the over-all suppressive action against oxidative stress (19-21).

Data on the function of XO as a major cellular source of superoxide radical production (1) and its putative role as a causative factor for ischemia/reperfusion damage are well accepted (2). However, little is known about the status of this enzyme in relation to the aging process, although an increased conversion of XDH to XOD in the aged kidney was previously reported from this laboratory (22). Thus, DR's ability to increase uric acid can be

*To whom all correspondence should be addressed:

Byung Pal Yu, Ph. D.

Department of Molecular Biology

Pusan National University, Gumjung-ku, Pusan, Korea, 609-735

Telephone: 82-51-510-2212

Facsimile: 82-51-581-1509

E-mail: yubp@hyowon.pusan.ac.kr

important to the proper maintenance an XOD/XDH balance and to the age-related oxidative process.

In this present report, we documented the modulation of hepatic XOD/XDH activities and the formation of uric acid by DR during aging.

MATERIALS AND METHODS

Animals and dietary regimen: Specific pathogen-free Fischer 344 male rats (Charles River Laboratories) were used. All rats were fed *ad libitum* until 6 weeks of age, at which time they were separated into two groups: *ad libitum*-fed (AL) control and diet-restricted (DR) group, whose food intake was restricted of 60% of the food intake of control. Detailed maintenance of animals and dietary procedures are published previously (23).

Rats were sacrificed by decapitation and the livers were quickly removed and rinsed in ice-cold buffer, pH 7.4 (100 mM Tris, 1 mM EDTA, 10 mM dithiothreitol, 0.2 mM phenylmethyl-sulfonylfluoride, 1 μ M pepstatin, 2 μ M leupeptin, 80 mg/l trypsin inhibitor. Tissues were immediately immersed in liquid nitrogen and stored at -80°C .

Tissue preparation: Rat liver was homogenized in 10 volumes of ice-cold buffer (pH 7.4) containing 0.5 mM PMSF, 1 mM EDTA, 80 mg/L trypsin inhibitor, and 1 μ M leupeptin. The crude nuclear fraction was removed by centrifugation at 900 g for 15 min at 4°C . The resulting supernatant was re-centrifuged at 12,000 g for 15 min at 4°C and at 105,000 g for 1 hr at 4°C to yield cytosol fraction.

Isolation of XOD/XDH by chromatography: Size exclusion chromatography was used to remove endogenous low molecular weight inhibitors. Briefly, prepacked Sephadex G-25 columns (942 cm, Pharmacia LKB) were equilibrated with 50 mM potassium-phosphate buffer (pH 7.4) containing 0.1 mM EDTA. Cytosolic fraction was centrifuged at 4°C to at 900 g for 2 min in a swinging-bucket rotor. One milliliter of cytosolic sample was added to each column, centrifuged at 900 g for an additional 2 min and the eluant collected (24). Aliquots of column fraction were saved for protein assay as described (13).

XOD/XDH assay method: The spectrophotometric assay based on the production of uric acid at 295 nm at 25°C was used (26). In brief, a reaction medium containing 50 mM xanthine, 100 μ M EDTA, and 50 μ M potassium-phosphate, pH 7.4, and the isolated sample was incubated for XOD activity in the absence of NAD^{+} . The total activity of XO (i.e., XDH+XOD) was measured under the same conditions in the presence of 500 mM NAD^{+} . Total enzyme activity was reported as international units (1 mol/min of urate formed at 25°C and pH 7.4). The percent of XDH conversion was obtained from the ratio of XOD/XOH+XOD.

Determination of uric acid: The metaphosphoric acid extracts of liver was diluted with 0.1M borate buffer (pH 9.5) prior to analysis for uric acid by the method of Scheibe (24).

Statistical analysis of data: Data shown are the mean $\pm$ SEM. Data were analyzed using the statistical package Super ANOVA; $p < 0.05$ was considered significant.

RESULTS

To assess the intracellular status of XOD and XDH, it was necessary first to measure xanthine oxidoreductase (XO) activity, i.e. XOD plus XDH. As shown in Fig. 1-A, XO activity showed age-related changes, decreasing in AL rats. However, this decrease in XO activity was prevented in DR rats, with the difference between the AL and DR groups being more significant at 18 and 24 months of age.

Changes in XDH due to age-effect and DR are illustrated in Fig. 1-B. First, age-related declines of XDH were detected in AL rats. As for a DR effect, although the XDH activity did not increase with age, DR rats showed well-sustained activity levels during aging, comparable to those of 6 month-old rats. Next, XOD was assayed. Data on changes in XOD activity for AL and DR rats during aging are illustrated in Fig. 1-C. Although the XOD activity in AL rats changed little with age, DR rats showed significant increases in XOD activity at 12 and 24 months of age compared to AL rats. This difference was more pronounced than the case with XO.

Data on the effect of age on the conversion of XDH to XOD and its modulation by DR are shown Fig. 2. The extent of conversion is expressed in the percent of change as determined by the ratio obtained from XOD divided by the combined activities of XOD and XDH. Fig. 2 shows the conversion of XDH to XOD during aging and dietary influence. We observed a slight increase during aging without any discernable dietary modulation.

We directed the last phase of our study to the measurement of uric acid levels at different ages and in DR rats. No noticeable age-related influence on the uric acid production was found; however, the dietary increase in uric acid production was quite remarkable. The DR group showed significantly higher levels of uric acid throughout the lifespan compared to the AL group (Fig. 3).

DISCUSSION

Oxidative stress has been implicated in the pathogenesis of the many injuries and tissue damage (25). One experimental model in which oxidative stress-related injuries are clearly shown is ischemia-reperfusion model developed for liver (26), kidney (26), heart (27), brain (28) and small intestine (29). It is well accepted in the literature that cellular damage by ischemia followed by perfusion is accentuated by superoxide generation through the activation of xanthine oxidase system (1). The evidence showing the involvement of XOD as the enzymatic source of free radical generation in ischemic tissues is documented mostly by the use of XOD inhibitors, which ameliorate ischemic injury and free radical activity (30, 31). However, the exact role and significance of the status of XOD in the pathophysiology of reperfusion injury in aged and particu-

Fig. 1. Effect of age and dietary restriction on hepatic xanthine oxidoreductase, xanthine dehydrogenase and xanthine oxidase activity. A, xanthine oxidoreductase; B, xanthine dehydrogenase; C, xanthine oxidase. The values represent the mean $\pm$ SEM of 8 rats. Statistical significance: * $p < 0.05$, ** $p < 0.01$, versus *ad libitum* rats at the same age.

larly in calorie restricted animals are not well explored at present time (31).

Under normal conditions, XDH catalyzes hypoxanthine to xanthine with production of uric acid in presence of NAD^+ as electron acceptor (32). In ischemic perfused tissues, however, XDH undergoes proteolytic conversion to XOD, using O_2 as its electron acceptor (33). According to the scheme of McCord and Roy, XOD reduces molecular oxygen to generate $\text{O}_2^{\cdot-}$ and H_2O_2 in the presence of hypoxanthine or xanthine (34).

In the present study, we assessed whether aging is a contributing factor in the conversion of XDH to XOD, and whether the XOD activity increases the intracellular ROS

status during aging. Our data clearly showed no increase of this enzyme activity in AL rats. However, the activity of XOD in DR rats significantly increased with age, showing a 76% increase at 24 months of age. The increased XOD activity was a somewhat surprising finding because of DR's expected role in suppressing potential conditions that favor increased oxidative stress, as seen with aging.

On the other hand, XDH levels of the DR group were higher than those of AL rats at 18 and 24 months of age. This dietary modulation by DR was taken as an indicator of DR's ability to balance XDH/XOD. Thus, it seems that during aging, DR's action to maintain high levels of XDH for the overall oxidative status may be part of its anti-oxidative strategy.

Fig. 2. The conversion and modulation of xanthine oxidase to dehydrogenase in liver. The values represent the mean $\pm$ SEM of 8 rats.

One novel finding of our study is the increased uric acid in DR rats, shown in Fig. 3. This consequence creates an interesting phenomenon from the standpoint of the free radical defense because of uric acid's strong anti-oxidant ability (35). Uric acid is an effective antioxidant in its ability to scavenge hydroxyl radicals, hypochlorous acid, and peroxynitrite (3). There is now evidence available showing such processes not only *in vitro* with isolated organs (36), but also *in vivo* (37). In isolated organ preparations, uric acid protects against reperfusion damage induced by free radical generating granulocytes (38). The benefits of uric acid also include the prevention of oxidative inactivation of endothelial enzymes such as cyclooxygenase, angiotensin converting enzyme, and free radical mediated vascular dilatation under oxidative stressed conditions (35). Work by Ames et al. clearly show that uric acid is a far better free radical scavenger than the most commonly known antioxidants (7). This means that increased uric acid by DR can serve as an added anti-oxidative source for its already enhanced enzymatic defenses.

Based on evidence, one could view the XO system in its dual function, one, for its $O_2^{\cdot-}$ producing potential and, two, for its antioxidant urate-generating activity. Under certain conditions, stoichiometrically, XOD is estimated to produce about 3 times more uric acid than $O_2^{\cdot-}$, making the anti-oxidative role of XOD system quantitatively more significant (39). Therefore, the DR rat's remarkable resistance to oxidative stress could be explained in part by their quantitatively much higher uric acid levels at all ages.

It seems likely in aged tissues where the incidence of focal hypoxic conditions occur, the production of urate by XDH/XOD system can be an extra-protective mechanism against oxidative damage. This notion is in line with the findings of Koog, et al. (40), who showed that in

Fig. 3. Effect of age and dietary restriction on uric acid level in the liver. The values represent the mean $\pm$ SEM of 8 rats. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus *ad libitum* rats at the same age.

oxidatively stressed epithelial and endothelial cells, the XDH/XOD system was considered to be a major protector against oxidative insults (40). Based on a similar rationale, we propose that the high XOD and XDH levels maintained by DR during aging may be a physiologically significant strategy for boosting the antioxidant defense system against overall age-related oxidative stress.

To further substantiate our proposal, future research on XOD and XDH should be extended to include other major organs and tissues for their contribution to the overall redox status. Included in this endeavor are investigations of uric acid metabolism and changes in uricase activity during aging.

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