Molecular and Cellular Biochemistry **296:** 11–16, 2007.
DOI: 10.1007/s11010-005-9068-4

Effect of bilateral *in vivo* ischemia/reperfusion on the activities of superoxide dismutase and catalase: Response to a standardized grape suspension

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Received 14 September 2005; accepted 27 October 2005; Published online: 3 January 2007

Abstract

Purpose: Ischemia/reperfusion (I/R) is a major etiological factor in the bladder dysfunctions observed in men with lower tract obstruction, women with postmenopausal incontinence and with aging. A standardized grape suspension protects the rabbit urinary bladder from both the contractile dysfunctions and the morphologic changes mediated by I/R. Using a model of *in vivo* bilateral ischemia/reperfusion, the current study investigated the effect of this grape suspension on the endogenous antioxidant defense systems. *Materials and methods:* 24 NZW rabbits were separated into 6 groups of 4. Groups 1–3 were treated by gavage with aqueous grape suspensions; groups 4–6 received sugar-water vehicle. Groups 3 and 6 were controls. Groups 1 and 4 were subjected to bilateral ischemia for 2 h (I). Groups 2 and 5 underwent bilateral ischemia for 2 h and reperfusion for 1 week (I/R). For all rabbit bladders, the muscle and mucosa were separated by blunt dissection and analyzed separately. The effects of the various treatments on bladder antioxidant systems of cytoplasmic superoxide dismutase (Cu-Zn superoxide dismutase; SOD), and catalase (CAT) were evaluated. *Results:* The standardized grape suspension up-regulated both SOD and CAT activity of bladder muscle and mucosa in control animals. There were few differences in the grape suspension treated animals after ischemia, and in general the activities decreased following I/R. *Conclusions:* Increases of SOD and CAT activity in control animals as a result of grape suspension suggest a greater antioxidant capacity. This increase in the antioxidant defense system may explain the increased protection of grape suspension in the face of ischemia and I/R. However, the activities of both enzyme systems decreased in the smooth muscle subjected to I/R showing that reperfusion damages these systems probably via oxidation damage to the enzymes themselves. (Mol Cell Biochem **296:** 11–16, 2007)

Key words: bladder, ischemia, reperfusion, grape

Introduction

Ischemia/reperfusion (I/R) plays an important role in the etiology of the contractile dysfunctions of the bladder induced by partial bladder outlet obstruction (PBOO) in animal models, and in obstructive dysfunction in men [1]. In addition to obstructive dysfunction, I/R is also major etiological factor to postmenopausal female bladder dysfunction [2], as well as bladder dysfunction due to aging [3].

In our recent studies, giving oral grape suspensions to rabbits provided protection against physiological and biochemical damage from both PBOO and bilateral ischemia

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and ischemia followed by reperfusion. Grape suspensions provided excellent protection to the physiological response to field stimulation [4]. Along with the demonstration that grape suspensions also prevented a reduction in nerve density within bladder smooth muscle, our conclusion was that the grapes maintained the structural integrity of neural membranes following bilateral I/R [4]. Because of the known high antioxidant characteristics of grapes [5], we propose that free radicals have an important role in the damage during I/R and that the grape suspension prevents injury by virtue of its antioxidant properties.

The endogenous anti-oxidative defense system includes compounds such as superoxide dismutase and catalase and has an important role in protecting the bladder against free radical damage [6]. There is a balance between the endogenous anti-oxidative defense system and oxidative stress. In the bladder following bilateral I/R the oxidative stress is greater than the endogenous anti-oxidative defense system can handle [7, 8]. Interestingly, grape extracts have been reported to up-regulate anti-oxidative enzyme activity [9].

Based on the protective effects of grape suspensions in our model of bilateral ischemia followed by reperfusion [4], this study evaluates the effects of grape suspension on the activities of both superoxide dismutase and catalase.

Materials and methods

Animals

A total of 24 male New Zealand White rabbits were separated into 6 groups of 4 rabbits each. Rabbits in groups 1–3 were treated by gavage with aqueous grape suspensions consisting of 10 ml aqueous grape suspension twice daily (20 mg/ml standardized grape powder obtained from the California Table Grape Commission). The rabbits in groups 4–6 received sugar-water vehicle 10 ml sugar-water vehicle by gavage (10 mg sucrose + 10 mg fructose/ml). This volume of grape suspension was calculated to equal approximately one serving of fresh grapes for a man.

Rabbits in all groups received their respective treatment twice daily for 3 weeks. After 3 weeks, all rabbits were sedated with ketamine-xylazine. Each rabbit in groups 1 and 4 were subjected to bilateral ischemia by clamping the vesical arteries with micro-vascular clamps for 2 h (ischemia only groups). Rabbits in groups 2 and 5 were subjected to bilateral ischemia for 2 h after which the clamps were removed and the rabbits allowed to recover for 1 week. Rabbits of group 3 and 6 were controls and were not subjected to ischemia or reperfusion. Treatment was continued through-out the one week reperfusion period.

The choice of 2 h ischemia and 1 week reperfusion was based on previous studies showing that these timings produced significant dysfunctions in the rabbit bladder which were significantly reduced by 3 weeks pretreatment by the grape suspension [4]. Likewise, the period of 3 weeks pretreatment was also based on this timing showing significant protection against the contractile and metabolic dysfunctions mediated by both bilateral ischemia and partial outlet obstruction [4, 5].

In the ischemia model of groups 1 and 4, each bladder was excised immediately following the 2-h ischemia and placed in an oxygenated physiological buffer containing glucose at 37 °C for 2 h, in order to re-generate cellular ATP. Previous studies have demonstrated that at 2 h the maximal concentrations of ATP and creatine phosphate were generated.

For all bladders, the bladder base and body were separated at the level of the ureteral orifaces and for the bladder body, the muscle was separated from the mucosa by blunt dissection and analyzed separately. Previous studies demonstrated that the muscle and mucosa are metabolically very different from each other and must be analyzed separately [10, 11].

SOD analyses

SOD (total) activity was determined by the method of Flohe and Otting., using a Cytochrome C Reduction Test [12]. In this model, oxygen free radicals are generated by xanthine oxidase reactions with ferricytochrome C. SOD activity is calculated via the degree of inhibition of this reaction and recorded as the change in optical density (mOD) at 550 nm (using a Hitachi spectrophotometer) per mg protein.

Specifically, bladder tissue was homogenized in a 50 mM phosphate buffer, pH 7.8 at a concentration of 200 mg/mL. The homogenate was centrifuged at 2500 rpm for 10 min. The pellet was eliminated and the supernate was used for the following assay: 2 ml of Solution A (0.76 mg Xanthine in 10 mL 0.001 NaOH, added to 50 mgs Cytochrome C + 3.7 mgs EDTA in 100 ml 50 mM phosphate buffer), at 25 °C, was incubated with 50 μl of the tissue sample or Cu-Zn SOD standards in a 3 ml cuvette. 200 μL of Solution B (5.63 μl Xantnine oxidase in 1 ml 0.1 mM EDTA) was used to start the reaction. After mixing, the absorbance change indicating cytochrome C reduction was measured in a spectrophotometer at 550 nm for 2 min. The change in absorbance with time over the first two minutes for all preparations was linear. The quantitative change in Cu-Zn SOD during this two minutes period was utilized in the figures shown.

Quantitative comparisons were made by calculating the concentration of enzyme (protein) that inhibits the reaction by 25% (IC_{25}). Although the IC_{50} is used more widely, several of the preparations did not reach 50% inhibition. For comparative purposes, the reaction curve of pure Cu-Zn SOD is also given.

Purified Cu-Zn SOD (purchased from Sigma Chemical Co.) was prepared at 25 ng/ml and diluted 1:1 down to 0.39 ng/mL for a standard curve. Specificity was demonstrated by heating both preparations for 10 min at 90 °C and demonstrating that this eliminated all activity.

Catalase analysis

Following the method of Abei [13] the reaction was initiated by adding H_2O_2 in 50 mM phosphate buffer (pH 7.0) in the presence of tissue extract.. The degradation of H_2O_2 was monitored at 240 nm and 25°C, and an extinction coefficient of 43.6 M^{-1} cm^{-1} was used to calculate units of activity. The enzyme activity is expressed in units per mg protein (1 UNIT = 1 mol of H_2O_2 degraded for 1 min). The slope of enzyme reaction was utilized to demonstrate CAT activity in unit of mOD/minute. The slope of the curve was graphed using the best-fit curve using Sigmaplot, and Sigmastat was used for statistical analyses.

Statistical analysis

Data are expressed as the mean ± SEM. Comparisons of groups were performed using analysis of variance, followed by the Student Newman-Keul test for multiple comparisons with $p < 0.05$ considered significant.

Results

Figure 1 shows a typical curve for purified SOD activity with the calculation of the concentration showing 25% inhibition of the curve (IC_{25}). Figures 2A and B show the effect of the grape suspension on SOD activities (IC_{25}) of bladder muscle and mucosa under control, ischemia, and I/R conditions, respectively. It should be noted that the lower the IC_{25} is, the higher the enzyme activity.

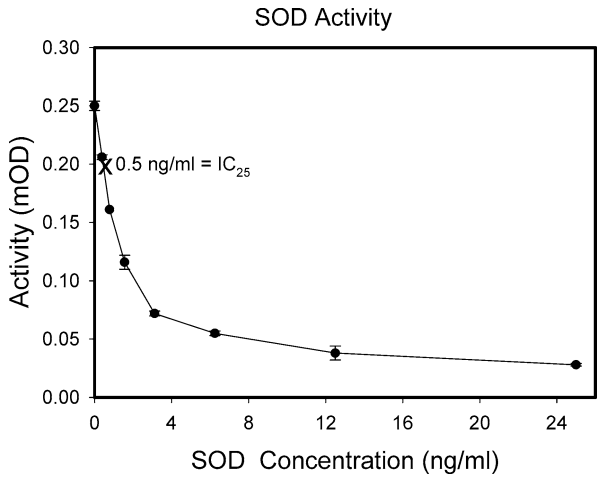

Fig. 1. Shows typical curve of purified SOD activity showing the IC_{25}. (25% of concentration that reacted)

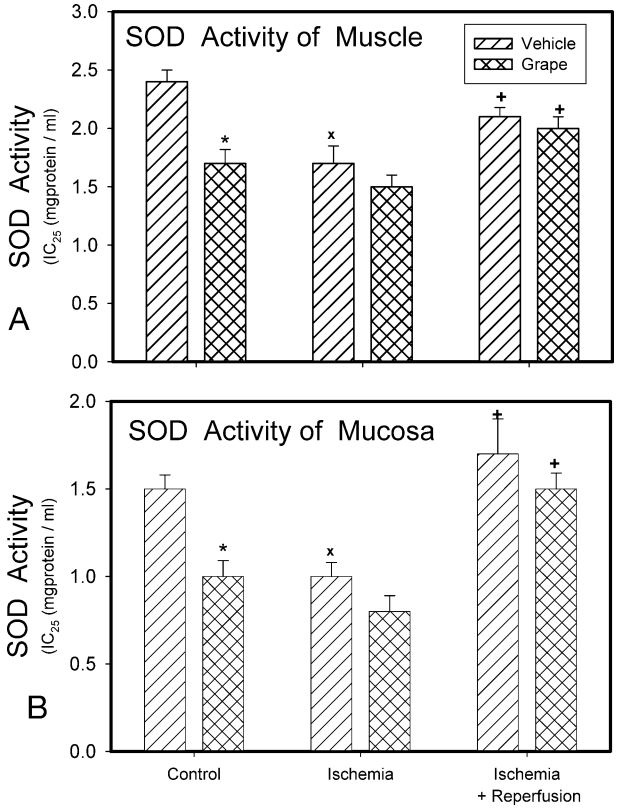

Fig. 2. Shows IC_{25} in bladder muscle (A) and mucosa (B). Each bar is the mean ± SEM for $N = 4$. *Significantly different from vehicle group; ×significantly different from control; and +significantly different from ischemia, $p < 0.05$.

The SOD activity of the control mucosa is higher than control muscle. The SOD activity of the control muscle and mucosa was significantly higher in grape-treated rabbits than in the vehicle-treated rabbits.

Ischemia resulted in a significant increases in SOD activity in the vehicle-treated rabbits (muscle and mucosa) but only a mild increase in SOD activity in the grape-treated rabbits. Reperfusion resulted in significant decreases in SOD activity when compared to the ischemia alone groups for both muscle and mucosa (Fig. 2A and B).

Figure 3 shows typical curves for catalase activity. The higher slope in mOD units/minute, the higher the CAT activity. The data were quantitated by comparing the slope of the curve over the first two minutes. Figures 4A and B show the effect of the grape suspension on comparative CAT activities of bladder muscle and mucosa under control, ischemia, and I/R conditions, respectively.

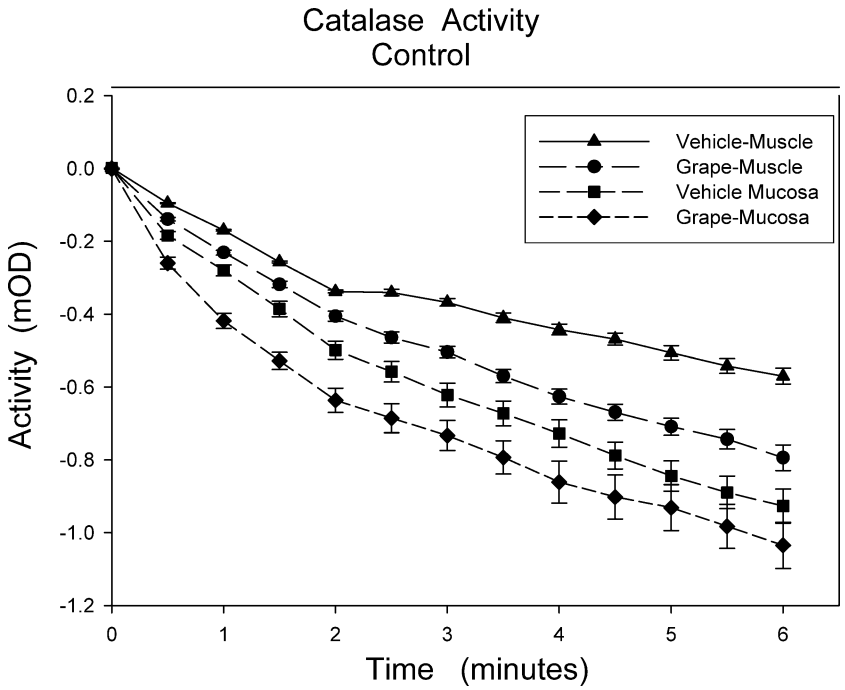

Fig. 3. Shows representative reaction curves for catalase activity.

Similar to SOD, the CAT activities in the control muscle and mucosa were significantly higher in the grape-treated rabbit bladders than in the vehicle-treated rabbit bladders. Ischemia alone resulted in increased activities in the vehicle-treated bladders in the muscle and mucosa. I/R resulted in significant increases in CAT activities in the vehicle and grape treated bladder muscle, but no change in the mucosa, when compared to ischemia alone (Fig. 4A and B).

Discussion

It is clear that feeding rabbits a grape suspension resulted in a significant increase in the activities of both SOD and CAT in both control bladder muscle and mucosa, thus providing initial increased protection against cellular and subcellular damage caused by ischemia and I/R. These biochemical results are consistent with our previous study demonstrating the protective effects on the contractile responses of this same grape suspension in both models of bladder outlet obstruction and bilateral ischemia/reperfusion [4, 5]. We had suggested that neuronal damage observed in these pathological models might be due to the release of free radicals by the smooth muscle [4, 12]. This suggestion was based on our previous studies which showed that field stimulated contractions were preserved by pre-treatment with grape suspensions; which in turn was consistent with observed protection of intrinsic neuron integrity. These results are entirely consistent with our current studies demonstrating that the intial activities of both SOD and CAT were up-regulated by the grape suspension.

Interestingly ischemia itself also resulted in up-regulation of the activities of both SOD and CAT. This is probably a result of the bladder's response to the decreased oxygenation. Other studies have also observed increased SOD and CAT activities after periods of ischemia [14, 15]. I/R however, in general resulted in decreased SOD and CAT activities, especially in the smooth muscle compartments. This would probably be the result from oxidative (free radical) damage to the enzymes themselves.

SOD and CAT are the cell's chief defenses against activated oxygen free radicals. Cytoplasmic SOD contains copper and zinc, which is known to have protective effects to I/R injury [16]. It converts superoxide to peroxide ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), which is then further broken down by CAT to oxygen and water. The intermediate peroxide is itself a dangerous molecule and could cause damage to the bladder. These radical detoxifying systems may be up-regulated during oxidative injuries and thus counterbalance the consequences of oxidative stress [17].

The beneficial effects of grape extracts have been shown in other model systems. With a strong antioxidant function, grape extracts can directly scavenge reactive oxygen species (ROS) and thus preserve the endogenous antioxidant detoxifying capacity whenever encountering oxidative stress [18]. Second, grape extract was reported to inhibit ROS production and release from leukocytes during oxidative damage [19].

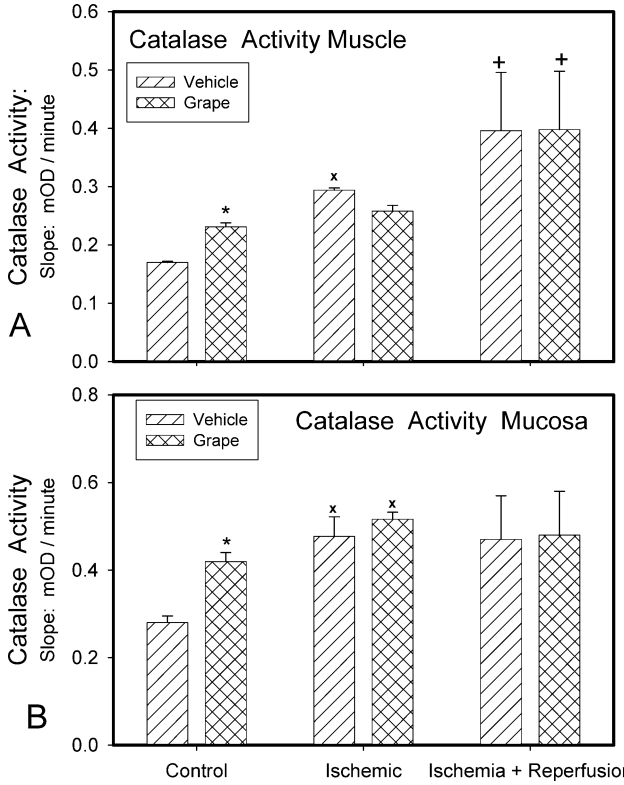

Fig. 4. Shows quantitative catalase activity (as the initial mean slope) in bladder muscle (A) and in bladder mucosa (B). Each bar is the mean ± SEM for $N = 4$. $^{\times}$Significantly different from vehicle group; *significantly different from vehicle group; $^{\times}$significantly different from control; and $^{+}$significantly different from ischemia, $p < 0.05$.

Third, additional studies demonstrated that the activities of SOD and CAT are increased by grape extracts; Balu *et al*. demonstrated that grape seed extract enhanced the antioxidant status (SOD and CAT activities) and decreased the incidence of free radical-induced lipid peroxidation in the central nervous system of aged rats [9]; Iwasaki *et al*. found that Proanthocyanidin, a grape-seed polyphenol, was shown to have a protective effects on the gastric mucosa by means of increased prostaglandin and increased superoxide dismutase activities in the gastric mucosa [20]. Fourth, Lu *et al*. also emphasized the protective ability of grape might be directly related to the concept that grape extract protects genes from oxidative damage and thus preserves the gene expression of endogenous antioxidant enzymes [19]. Furthermore, Ray and Bachi *et al*. reported that grape extract demonstrates a DNA repair ability [21], which might enhance the recovery of antioxidant defense system during the 1-week reperfusion period. Collectively, it is our opinion that the effects of grape on SOD and CAT activity whenever encountering oxidative stress are multidimensional. It would be interesting to study the protective effects of grape on the gene expression, transcription and translation of SOD, and CAT. Whenever encountering ischemia, anaerobic metabolism dominates [22]. Our study design was to provide the cells with an equal amount of substrate in each group. Both the vehicle and the grape suspension can be converted to pyruvate that could be utilized by the cells as an energy substrate during anaerobic metabolism. By maintaining the viability of the tissue, this may allow the cells to generate elevated SOD and CAT activities. Other studies have also shown that ischemia stimulates SOD and CAT activities in anaerobic exercise [23]. In addition, pyruvate itself has been shown to be beneficial in a model of hepatic I/R [24]. Based on our results, it is likely that grape suspension was beneficial as a substrate for pyruvate metabolism in addition to its direct antioxidant effects.

Our results are relevant to human bladder dysfunction. I/R and its resultant oxidative damage is noted in obstructive bladder dysfunction in men [1]; and in ovariectomized rabbit bladders via decreased blood flow resulting in frank hypoxia [25, 26]. Furthermore, increased oxidative stress are reported to be associated to various bladder diseases, such as postmenopausal incontinence in women [2], bladder dysfunction due to aging effect [3], interstitial cystitis [27], diabetic cystopathy [28], radiation cystitis [29], and bladder cancer [30]. The present study demonstrates that antioxidant defense system (SOD, CAT) was up-regulated by grape suspension under control and ischemic conditions; and perhaps further investigation of the effects of grapes on those diseases might be beneficial to the treatment to those patients.

Acknowledgments

Supported in part by funds from the California Table Grape Commission, the Office of Research and Development Medical Research Service, Department of Veteran's Affairs, and NIH grant RO-1-DK067114.

We would like to thank the California Table Grape Commission for supplying the Standardized Grape Preparation.

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