Molecular and Cellular Biochemistry 78: 151–159 (1987)

Original Article

Effects of N-(2-mercaptopropionyl)glycine on ischemic-reperfused dog kidney *in vivo* and membrane preparation *in vitro*

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Received 24 June 1987; accepted 28 September 1987

Key words: catalase, Fe, free radical, hydrogen peroxide, lipid peroxidation, malondialdehyde, mercaptopropionylglycine, microsome, Na, K, ATPase, renal ischemia, sulfhydryl, superoxide dismutase, thiol

Abstract

The results of our experiments demonstrated that one hour of ischemia followed by one hour of reflow in the kidney caused a reduction in (Na^+K^+)ATPase activity and microsomal sulfhydryl content as well as an increase in microsomal lipid peroxidation. Renal venous malondialdehyde concentration was increased soon after reperfusion of the ischemic kidney. All these changes were rectified by an infusion of 0.123 mmol N-(2-mercaptopropionyl)glycine/kg over a 70 min period. On the other hand, an *in vitro* addition of 0.01–0.5 mM N-(2-mercaptopropionyl)glycine to a membrane preparation in the presence of H_2O_2 and Fe^{3+} did not prevent but rather potentiated the free radical effect on the enzyme activity. However, addition of superoxide dismutase alone or with catalase together with 2-MPG were effective in preventing the enzyme depression induced by H_2O_2. The results therefore indicate that free radical generation participates in the evolution of ischemia/reperfusion cell injury and thiol-reducing agents may be beneficial in alleviating the cell damage *in vivo*.

Introduction

A number of studies have been published in recent years implicating the involvement of free radical generation in the ischemia/reperfusion injury of various organs. It has been postulated that during this injury reactive oxygen intermediates are produced by activated polymorphonuclear leucocytes or through the xanthine oxidase reaction, prostaglandin synthesis or mitochondrial electron transport chain ([1–8] for reviews). Despite the fact that acute renal shutdown is a terminal event often encountered in clinical medicine, there have been relatively few studies dealing with problems of cessation of renal perfusion ([9–11] for reviews).

Our previous work demonstrated that isolated membrane preparation containing partially purified (Na^+K^+)ATPase was damaged by free radical reactions induced by H_2O_2. The enzyme activity, sulfhydryl content and ouabain binding were suppressed by free radical formation, whereas lipid peroxidation was enhanced. The effects on membrane protein and membrane lipid were partially uncoupled by the use of thiols and lipid anti-oxidants [12, 13]. The outer medulla of the kidney, where thick ascending limbs of Henle are localized, and the inner cortex, where S3 segments of proximal tubules are found, are the two areas most susceptible to ischemic damage in the kidney [9, 10]. Furthermore, high concentrations of (Na^+K^+)ATPase are present in these

*Dr. Kato was on leave from the Department of Medicine (Prof. M. Nagano), Jikei University-Aoto Hospital, Tokyo, and was supported by the Uehara Foundation.

areas [14]. For these reasons we have examined metabolic changes linked to free radical pathology in the outer medullar region of ischemic/reperfused dog kidney.

N-(2-mercaptopropionyl)glycine (2-MPG) (Thiola) has been used in Japan since 1970 for the treatment of hepatitis, cysteinuria, cataracts and rheumatoid arthritis ([15] for review). Its structure is similar to penicillamine and hence it exerts an anti-inflammatory action. It can be administered orally, and its thiol reducing characteristics may be used as a free radical scavenger. However, until very recently 2-MPG has not been tried as an agent to alleviate deleterious effects of free radicals during the development of ischemia/reperfusion tissue injury [16, 17]. Furthermore, acute renal failure which follows hypoperfusion is a serious clinical problem [9, 10]. Therefore, we studied effects of 2-MPG in our experiment using kidney reperfusion *in vivo*. These results were compared with its *in vitro* effects, since we observed in our previous experiments that dithiothreitol, another thiol reducer, did not always protect the membrane preparation from free radical effects *in vitro* [12].

Experimental procedures

Surgical procedure

Mongrel dogs, 17–20 kg, were anesthetized by an intravenous injection of sodium pentobarbital, 26 mg/kg. The ECG, femoral arterial pressure, rectal temperature and urinary output were recorded throughout the experimental period. The dogs were ventilated via an endotracheal tube with a mixture of oxygen, nitrous oxide and penthrane. The femoral vein was cannulated and Ringer-lactate solution was infused at a rate of 10–20 ml/kg · h during the experiment. A second catheter was inserted into the renal vein via the inferior vena cava. The abdomen was opened and a Foley catheter was inserted into the bladder. The renal artery of one kidney was occluded by a small clip and the abdomen was closed. After an ischemic period of 1 h, the clip was released. Following a reflow period of 1 h, during which time blood samples were withdrawn from the renal vein, both kidneys were removed for the analysis [12]. N-(2-mercaptopropionyl)-glycine (Sigma) was dissolved in 0.9% saline solution, its pH was adjusted to 7.4 with HCl, and a total dose of 20 mg/kg (0.123 mmol/kg) was infused over a period of 70 min, starting 30 min prior to the beginning of reperfusion [16]. This infusion protocol was adopted in order to raise the plasma concentration of 2-MPG sufficiently high prior to the kidney reperfusion period.

Preparation of microsomal fraction

Tissue from the outer medulla was obtained by dissecting transverse sections with a scalpel. The dark red outer medulla was incised along the light grey inner medulla [18]. Approximately 2 g of tissue were obtained from one kidney. The tissue was homogenized in 10 vol of histidine-sucrose with a Teflone-glass homogenizer. The homogenate was centrifuged at 6000 × g for 15 min. The sediment was resuspended by homogenization and centrifuged once more at 6000 × g for 15 min. The combined supernatant from the two centrifugations was spun at 48000 × g for 30 min [19, 20]. The resulting pellet was resuspended by gentle homogenization in histidine-sucrose to a concentration of approximately 3 mg/ml. All operations were carried out at 4 °C.

In vitro *experiment with partially purified* (Na^+K^+)*ATPase preparation*

The microsomal fraction was incubated for 30 min at 20 °C in a mixture composed of 0.4 mg sodium dodecylsulfate/ml, 3 mM ATP, 2 mM EDTA, and 50 mM imidazole, pH 7.5 [18]. The protein concentration was adjusted to 1.4 mg/ml before this incubation. Discontinuous density gradients of 25 ml were made in 33 ml tubes of the Beckman rotor SW 28. The gradient consisted of three successive layers of sucrose; 12.5 ml of 29.4%, 7.5 ml of 15% and 5 ml of 10% (w/v) sucrose in 2 mM EDTA and 50 mM imidazole, pH 7.5. Portions of the sample, 8 ml, were layered on each of the gradients in the tubes of the SW 28 rotor and were centrifuged at

27000 rpm for 3 h [18]. After centrifugation the pellets were resuspended by homogenization in 100 mM Tris/HCl, pH 7.5, to a protein concentration of about 1 mg/ml and used immediately for experiments.

Free radical generation

The membrane preparation was incubated in a mixture of 0.1 mM $FeCl_3$, 1 mM ADP, 0.5 mM H_2O_2 and 100 mM Tris/HCl, pH 7.5, at 37 °C for 30 min in the presence or in the absence of 0.01–0.5 mM 2-MPG [12, 16]. In another series of experiments, 5 or 10 units superoxide dismutase/ml, 50 units catalase/ml, or both were added into the incubation mixture.

(Na^+K^+)ATPase activity assay

The enzyme activities were measured by using 10 μg (protein) of the membrane fraction. The medium was composed of 3 mM $MgCl_2$, 10 mM KCl, 100 mM NaCl, 3 mM ATP, and 50 mM histidine, pH 7.5 in the presence or in the absence of 1 mM ouabain. Preincubation was carried out at 37 °C for 10 min and then the reaction was started by an addition of ATP. Liberated inorganic phosphate was determined using molybdate [21].

Protein determination

This was performed by the method of Peterson [22]. The samples were solubilized with 0.15% deoxycholate, precipitated with 72% trichloroacetic acid, and treated with a mixture of copper-tartrate-carbonate, NaOH and sodium dodecylsulfate; Folin-Ciocalteu phenol reagent was then added and the developed colour was read at 750 nm by a spectrophotometer.

Sulfhydryl groups

The protein samples were precipitated with acetone containing 0.1 N HCl. The sulfhydryl groups were reacted with mercury orange (Sigma) in 0.1 M phosphate buffer, pH 7.0. Following washing with acetone, the colour was eluted with acidic acetone and read at 470 nm. The results were calculated by using an extinction coefficient of 18330/M · cm [23].

Malondialdehyde determination

Suspensions containing 200 μg of microsomal protein were heated at 90 °C for 60 min in a mixture composed of sodium dodecylsulfate, acetic acid and thiobarbituric acid. The developed colour was extracted with n-butanol-pyridine (15:1, v/v) and read at 532 nm. The results were calculated by using standard solutions of 1,1,3,3-tetraethoxy propane [24]. For malondialdehyde in the serum (0.3 ml), the procedure was modified slightly (heating period of 30 min, extraction with n-butanol-methanol, 5:1).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The (Na^+K^+)ATPase preparation (30 μg) was treated with 2% sodium dodecyl sulfate, 1 mM EDTA and 0.1% 2-mercaptoethanol in 10 mM sodium phosphate buffer, pH 7.0, heated (70 °C, 5 min) and then applied to the 3.5% polyacrylamide gel in a 6 mm × 100 mm tube. Electrophoresis was carried out at 5–7 v/cm gel for 4 h, following Weber and Osborne's procedure [25]. The gel was stained with 0.05% Coomassie brilliant blue, destained and scanned by a densitometer (Gilford) at a wave length of 543 nm. The molecular weight was estimated by using protein standards with a molecular weight range of 29000 to 205000 (Sigma).

Statistical analysis

The significance of differences was calculated by the Student's t test and analysis of variance.

Results

The unilateral ischemia/reflow procedure resulted in decreases, in the microsomal fraction, in

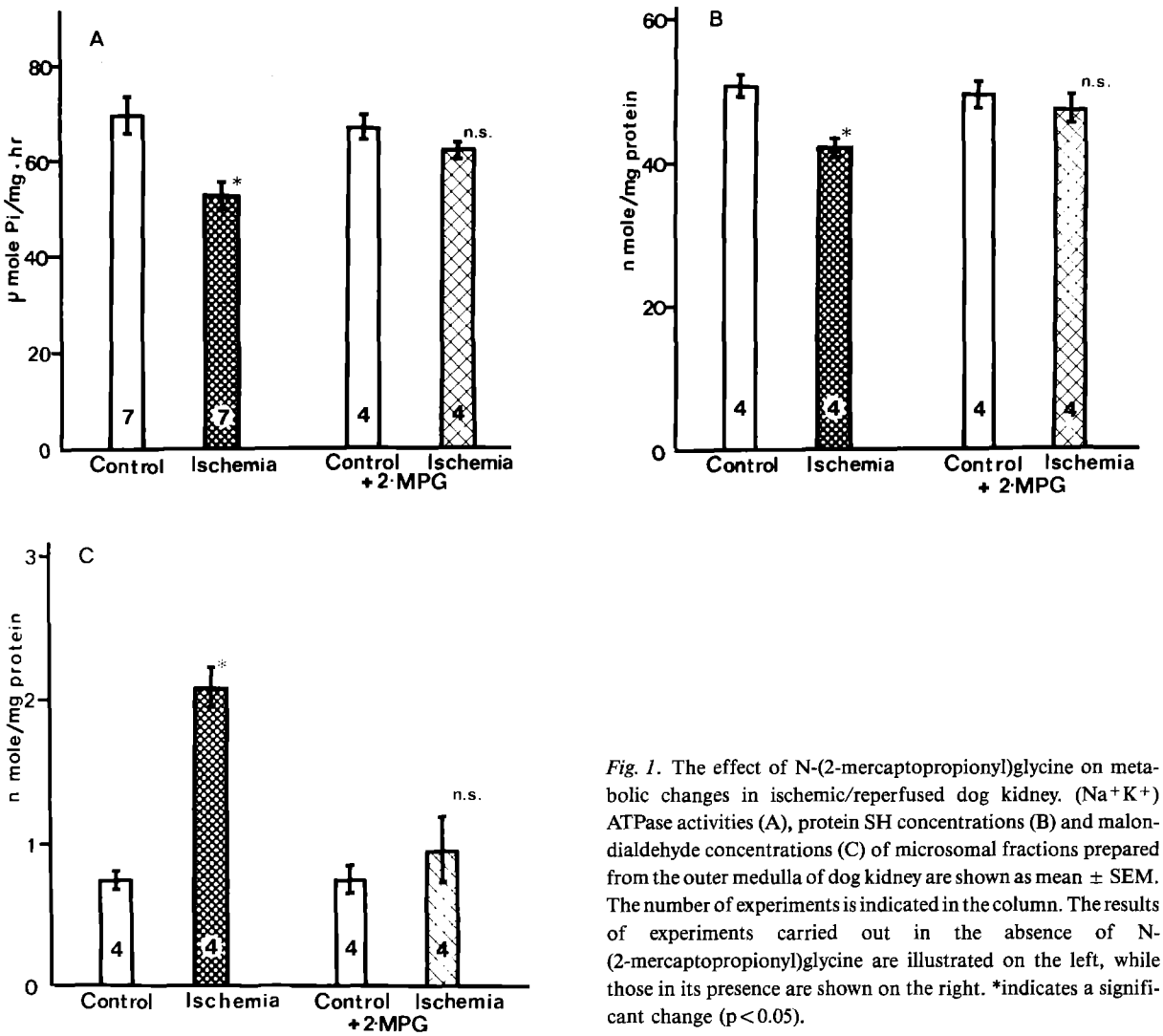


Fig. 1. The effect of N-(2-mercaptopropionyl)glycine on metabolic changes in ischemic/reperfused dog kidney. (Na^+K^+) ATPase activities (A), protein SH concentrations (B) and malondialdehyde concentrations (C) of microsomal fractions prepared from the outer medulla of dog kidney are shown as mean ± SEM. The number of experiments is indicated in the column. The results of experiments carried out in the absence of N-(2-mercaptopropionyl)glycine are illustrated on the left, while those in its presence are shown on the right. *indicates a significant change ($p < 0.05$).

(Na^+K^+)ATPase activity and sulfhydryl content as well as an increase in lipid peroxidation (Fig. 1a, b, c). Infusion of 0.123 mmole 2-MPG/kg over 70 min in the dog prevented these metabolic alterations, i.e., ischemia/reperfusion did not produce significant decreases in (Na^+K^+)ATPase activity and microsomal SH content, and lipid peroxidation was not augmented (Fig. 1). The analysis of partially purified (Na^+K^+)ATPase of the ischemic kidney medulla by gel electrophoresis showed that the majority of protein in the membrane fraction was (Na^+K^+)ATPase and that reperfusion did not affect a relative proportion of alpha and beta subunits (Fig. 2)

A concentration of malondialdehyde in the renal vein was not significantly changed in 2 min following the commencement of reperfusion. A sudden outflow of malondialdehyde was observed thereafter, reaching 150% of the control value at the 10th min, 166% at the 20th min and 132% at 40th min of reflow. This increased output of malondialdehyde from the ischemic/reperfused kidney was also abolished by the infusion of 2-MPG (Fig. 3). Although the total renal blood flow recovers almost to preischemic levels, the homogeneity of renal perfusion is significantly altered following ischemia [26, 27]. Therefore, it is not possible to accurately describe a relationship between the regional hypoper-

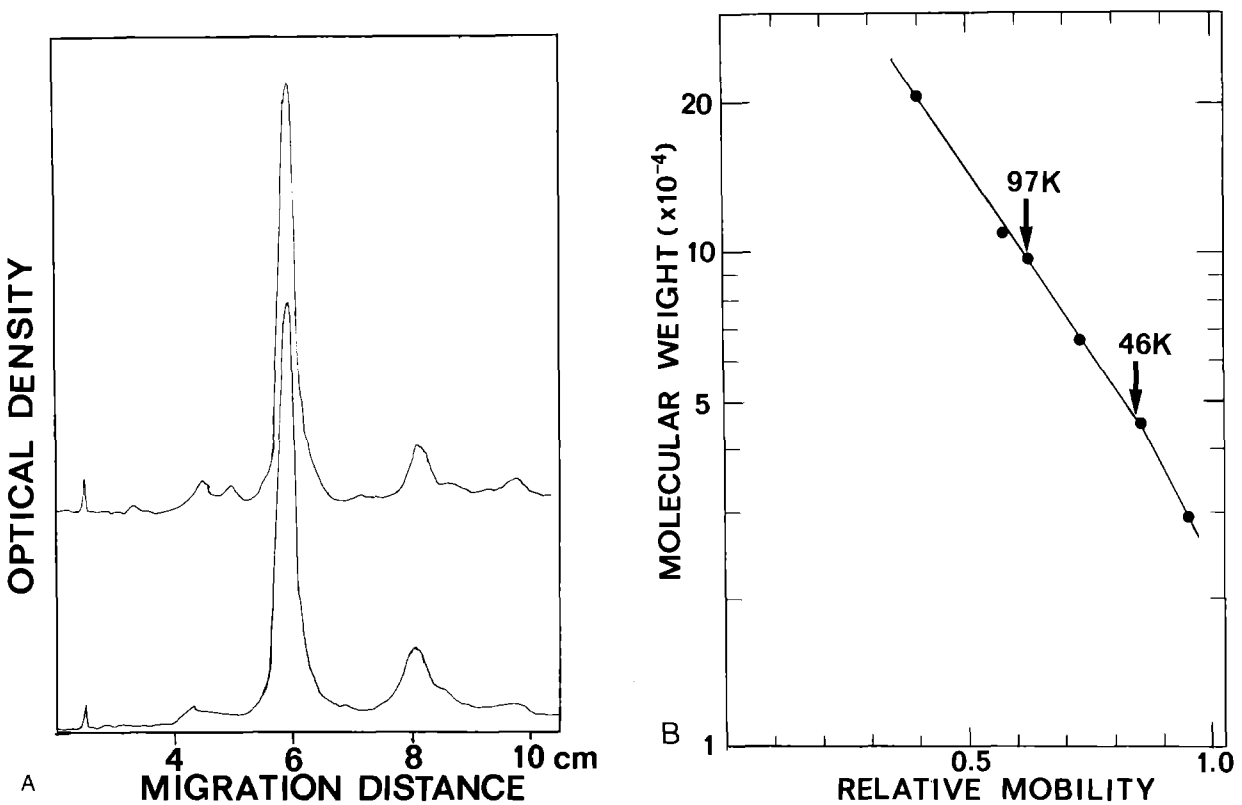


Fig. 2. Densitogram of polyacrylamide gel of partially purified (Na^+K^+)ATPase preparation isolated from the dog kidney. (a) Membrane preparations containing (Na^+K^+)ATPase was isolated from the outer medulla of ischemic/reperfused dog kidney, and sodium dodecylsulfate polyacrylamide gel electrophoresis was carried out as described in the Methods section. The gel was stained with Coomassie blue and scanned by a densitometer. The upper trace is from the ischemic kidney and the lower trace is from the contralateral kidney. (b) shows the positions of molecular weight standards.

fusion and the observed changes in malondialdehyde concentration of the renal effluent.

The scavenging action of 2-MPG was tested by isolating a membrane fraction from the outer medulla of normal dog kidney. Free radical generation was achieved by incubating the membrane fraction in the presence of H_2O_2, $FeCl_3$ and ADP. (Na^+K^+) ATPase activity was suppressed by this maneuver in a dose- and time-dependent manner [12, 13]. Addition of 2-MPG was not beneficial but rather potentiated the suppression of (Na^+K^+)ATPase activity (Fig. 4). Changes in enzyme subunits, as consequences of the free radical reaction, could not be detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (not shown). In another series of experiments 2-MPG similarly augmented H_2O_2-induced enzyme depression of the basolateral membrane preparation (Fig. 5a, b). Interestingly, however, the free radical effect on (Na^+K^+)ATPase was reversed, when a low concentration of an enzyme-radical scavenger, such as superoxide dismutase or catalase was present. The addition of 2-MPG in these cases dramatically improved the H_2O_2-induced suppression of (Na^+K^+)ATPase activity (Figs. 5a, b).

Discussion

The results of our experiments demonstrated that one hour of ischemia followed by one hour of reflow in the kidney caused a reduction in (Na^+K^+) ATPase activity and microsomal sulfhydryl content as well as an increase in microsomal lipid peroxida-

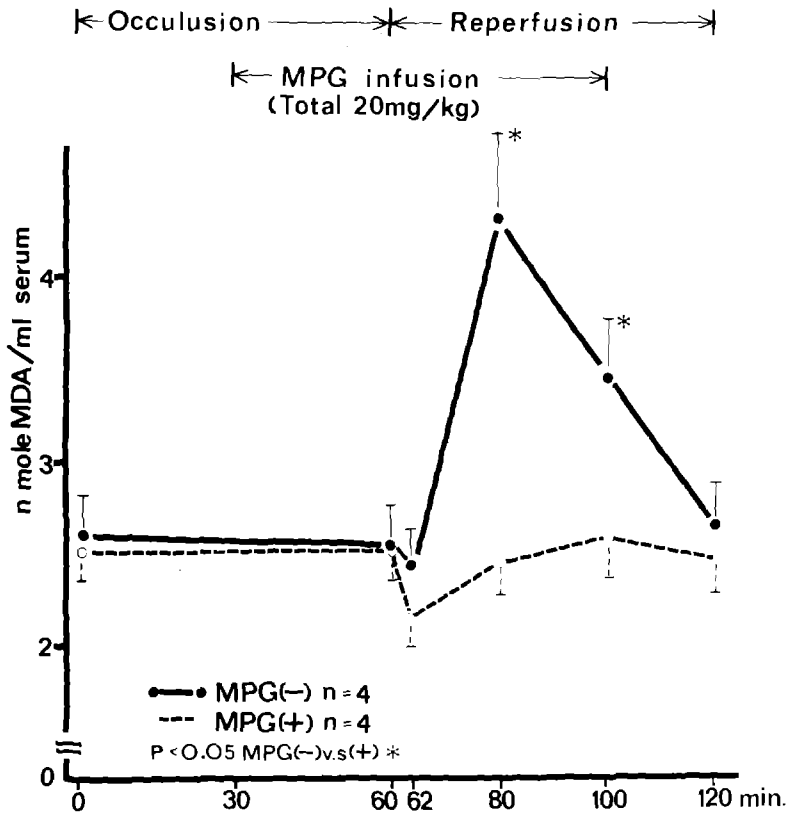
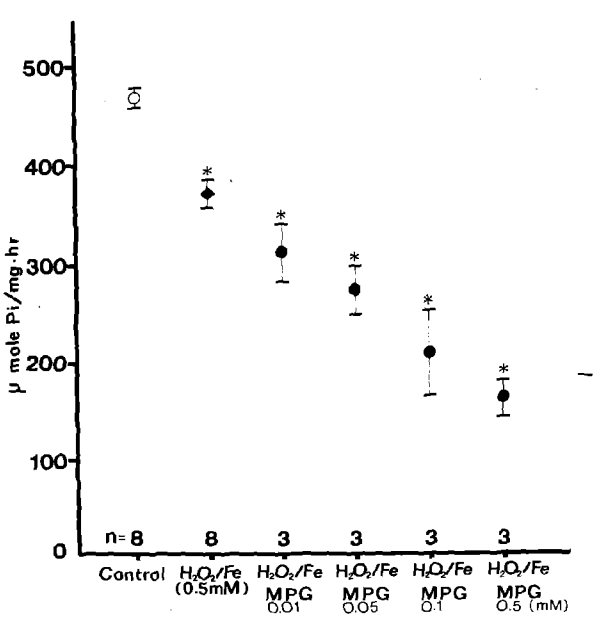


Fig. 3. The effect of N-(2-mercaptopropionyl)glycine on the malondialdehyde concentration of renal vein in the ischemic-reperfused dog kidney. Unilateral renal arterial occlusion was applied for 60 min, followed by removal of occlusion. N-(2-mercaptopropionyl)glycine (MPG) (20 mg/kg) was infused over a 70 min period starting on the 30th min. The values are expressed as mean ± SEM.



tion. Renal venous malondialdehyde concentration was increased 20 min after reperfusion of the ischemic kidney. All these changes were prevented by an infusion of 0.123 mmol 2-MPG/kg over a 70 min period. On the other hand, an *in vitro* addition of 0.01–0.5 mM 2-MPG to a membrane preparation in the presence of H_2O_2 and Fe^{3+} did not correct but rather potentiated the free radical effect on the enzyme activity. However, addition of a low concentration of superoxide dismutase alone or with catalase

Fig. 4. Potentiation of H_2O_2-induced inhibition of (Na^+K^+)ATPase by N-(2-mercaptopropionyl)glycine. Partially purified (Na^+K^+)ATPase was isolated from the outer medulla of dog kidneys and incubated with 0.5 mM H_2O_2, 0.1 mM $FeCl_3$ and 0.01–0.5 mM N-(2-mercaptopropionyl)glycine (MPG). The enzyme activity is expressed as mean ± SEM (μmol Pi/mg protein · h). *indicates a significant change ($p < 0.05$) compared to control.

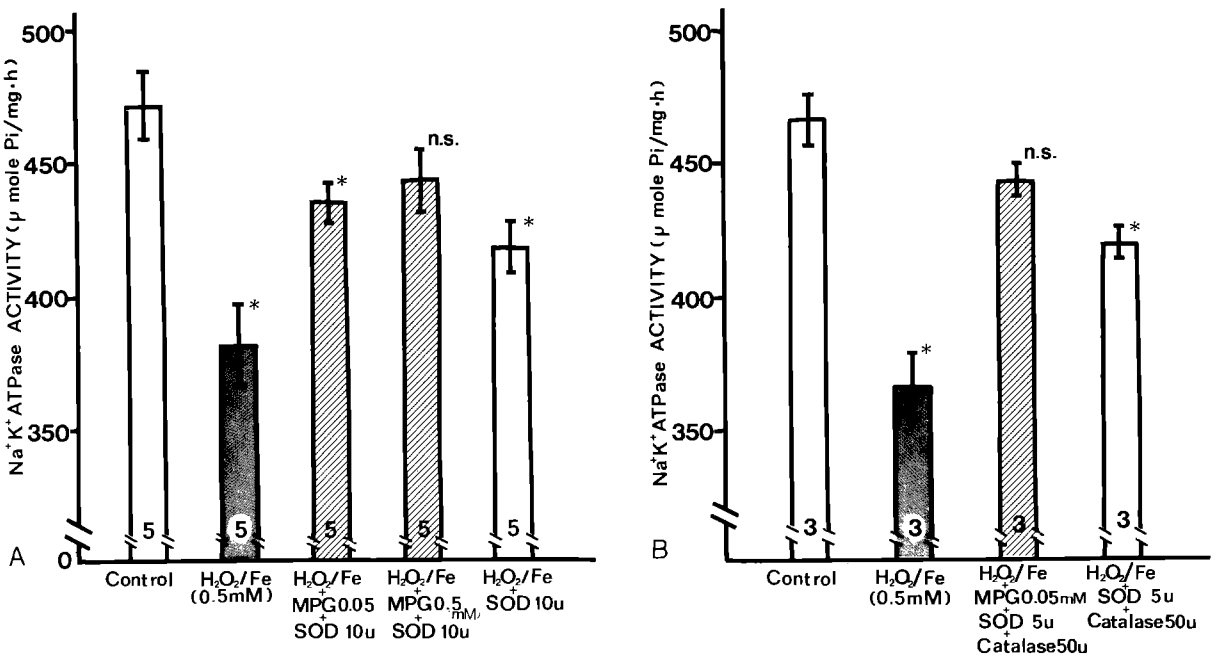


Fig. 5. Effects of scavengers *in vitro* on H_2O_2-induced inhibition of partially purified (Na^+K^+)ATPase. (Na^+K^+)ATPase activity is expressed as mean ± SEM (μmol Pi/mg protein · h). *indicates a significant change ($p < 0.05$) as compared to control. The concentrations were; H_2O_2 (0.5 mM), $FeCl_3$ (0.1 mM), catalase (50 units/ml), superoxide dismutase (SOD) (5 or 10 units/ml) and N-(2-mercaptopropionyl)glycine (MPG) (0.05 or 0.5 mM).

together with 2-MPG were effective in preventing the enzyme depression induced by H_2O_2.

Previous reports indicated that 2-MPG was beneficial in protecting ischemic damage of mitochondrial respiration and in preventing H_2O_2 induced perturbation of calcium homeostasis of isolated hepatocytes [28, 29]. It was reported recently that 2-MPG, at a dose similar to that used in our study, reduced infarct size and improved systolic wall thickening in ischemic/reperfusion injury of myocardium [16, 17]. Our study using a kidney ischemia/reperfusion model agreed with these findings in that 2-MPG, used *in vivo*, prevented certain metabolic changes in ischemic/reperfused kidney microsomes. Although the exact mechanism responsible for this beneficial effect is not known, it is likely that 2-MPG acts as a thiol-reducing agent, since available evidence is in favour of the view that oxygen free radicals oxidize not only membrane lipids but also membrane proteins [7, 8]. A number of amino acid residues, in addition to cysteine residues, are vulnerable to oxidative attack, and sulfhydryl reducing agents may be able to protect against such damage. However, the exact chemical nature of electron donors is unknown. Evidence in support of the hypothesis that free radicals play a critical role in the development of ischemia/reperfusion injury of the heart, brain and intestine is mostly circumstantial [1–8]. In a recent study, Paller *et al.* observed that ethane production, as an indicator of lipid peroxidation, was increased following renal reperfusion and that this increase was prevented by allopurinol or superoxide dismutase [31]. Earlier these investigators reported that superoxide dismutase, allopurinol or dimethylthiourea protected renal function after ischemia/reperfusion [32]. 2-MPG can readily cross the cell membrane and can accumulate in the mitochondria [15], and the thiol-disulfide redox state is critical in modulating various functions of mitochondria, sarcoplasmic reticulum and plasma membrane [30]. Thus, it is most likely that 2-MPG exerted its action as a free radical scavenger in the reperfused dog kidney.

Our study demonstrates that 2-MPG, in a concentration range between 0.01 mM and 0.5 mM, potentiated *in vitro* the free radical-induced changes of

membrane-bound (Na^+K^+)ATPase preparation. Our previous study dealing with the metabolic action of free radicals in a similar membrane model showed sulfhydryl reagents, such as dithiothreitol, enhanced free radical effects. The dithiothreitol effect was biphasic, in that with low concentrations of dithiothreitol (<1 mM) it augmented free radical effects, whereas at higher concentrations (>100 mM) it ameliorated free radical-induced enzyme depression [12, 13]. It was postulated that low concentrations of thiol reducing agents act as catalysts for oxidation/reduction reactions, facilitating free radical formation. Similar concentration-dependent inhibitory and stimulatory effects of thiol compounds towards lipid peroxidation have been observed previously, and they were attributed to the trace metal ions catalyzing the autoxidation of sulfhydryl groups [33].

Alternative interpretations for the disagreement between results of our *in vivo* and *in vitro* experiments with 2-MPG may be possible. It has been known that ischemia/reperfusion injury causes accumulation of polymorphonuclear leucocytes and phagocytes [2, 3, 5]. They are activated by complements and form reactive oxygen intermediates [3]. 2-MPG may have scavenged these free radicals in the intracellular space, thus preventing parenchymal cell damage. Mitsos *et al.* observed that 2-MPG enhanced the protective effect of neutrophil depletion, and suggested that intramyocardial and extramyocardial oxygen free radicals contributed to reperfusion induced myocardial injury [16]. Secondly, 2-MPG may have acted on the mitochondrial thiols [28]. Mitochondrial function is maintained, energy production is sustained, and thus sufficient ATP is produced to support cellular activities, including (Na^+K^+)ATPase. The critical importance of intracellular levels of ATP leading to free radical-induced cell injury was demonstrated recently both by Schraufstatter *et al.* and Altschuld *et al.* [34, 35]. Likewise, we have shown that loss of viability of isolated cardiomyocytes caused by oxidants was prevented by sulfhydryl agents [13].

In conclusion, our study demonstrates that depression of (Na^+K^+)ATPase activitiy, reduction of sulfhydryl content and formation of malondialdehyde in the microsomal fraction as consequences of the unilateral renal ischemia/reperfusion procedure were prevented by the administration of 2-MPG. The results suggest that free radical generation participates in the evolution of ischemia/reperfusion cell injury and thiol-reducing agents alleviate the cell damage.

Acknowledgements

The excellent technical assistance by M. Bosc-Davie, C. Kako and R. Seymour is gratefully acknowledged. Grant support was given by J. P. Bickell Foundation, Heart & Stroke Foundation of Ontario and Medical Research Council.

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