Effect of oxygen derived free radicals and glycine on sodium-potassium adenosine triphosphatase in the basolateral membrane of the kidney in ischemia-reperfusion

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ABSTRACT

Objectives: The aim of the present study was to examine the effect of exposing rats to ischemia-reperfusion while breathing 100% oxygen or room air, to find the effect of glycine on renal sodium-potassium adenosine triphosphatase (Na+-K+ATPase) and endogenous antioxidant enzymes, superoxide dismutase and catalase, also to ascertain the effect of ischemia-reperfusion on renal nitric oxide and lipid peroxides.

Methods: This study was carried out at King Saud University, Riyadh, Kingdom of Saudi Arabia, over a period of 11 months, February to December 2001. All previous measurements were carried out on the renal homogenate after 60 minutes ischemia, then after reperfusion while animals breathed room air or 100% oxygen and also after glycine treatment.

Results: The activity of Na+-K+ATPase, catalase and superoxide dismutase concentration was decreased significantly in the ischemic rats compared to the control, a further decrease was found after 20 minutes of reflow while breathing room air. Breathing 100% oxygen resulted in a significant decrease in catalase and Na+-K+ATPase activity and concentration of superoxide dismutase, glycine caused insignificant change of these enzymes after ischemia-index of lipid peroxidation and nitric oxide they were significantly elevated following reperfusion while rats breathed room air and further elevation was noticed after breathing 100% oxygen. However, potassium and creatinine did not change in all study groups, showed significant decrease after ischemia and ischemia-reperfusion may be due to marked Na+ loss in urine and lack of Na+ reabsorption. The inhibition of superoxide dismutase and catalase can be explained by increased reactive oxygen species during reperfusion and hyperoxia, also due to nitric oxide production and lipid peroxidation as shown by high malondialdehyde. Lack of Na+K+ATPase can be contributed to loss of antioxidant enzymes, nitric oxide production, and high reactive oxygen species.

Conclusion: Hyperoxia in ischemia-reperfusion induces severe damage to cellular defence mechanisms and enhances reactive oxygen species injury. Glycine, as antioxidant, is involved in kidney protection from massive injury induced by ischemia-reperfusion, protects renal antioxidant enzymes and Na+-K+ATPase, normalizes malondialdehyde, and nitric oxide levels. This data further supports the possible role of glycine therapy as an adjunct in the treatment of renal failure.

Saudi Med J 2002; Vol. 23 (11): 1380-1385

Reactive oxygen species (ROS) can be formed from several sources as mitochondrial cytochrome oxidases, xanthine oxidases, neutrophils and transitional metals. Under normal conditions, 95% of molecular oxygen undergoes reduction through addition of 4 electrons in mitochondrial

cytochrome oxidase system to form water, the remaining molecular oxygen leaks from this pathway and undergoes reduction to produce superoxide anion (O_2^-) hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radical (OH).[1] The latter can attack and damage the living cells, it can attack membrane lipids and set of lipid peroxidation which disrupt its function, decreases fluidity, damages the membrane receptors and enzymes.[2] In aerobic cells, there is continuous generation of ROS that have high reactivity, and lead to development of important defense mechanisms to prevent damage caused by these ROS. There are enzymatic and nonenzymatic defense mechanisms. The enzymatic defense mechanisms include SOD it catalyzes the dismutation of O_2^- to H_2O_2 and O_2 at a rate 10,000 times faster than spontaneous dismutation. As a result, no O_2^- is available to react with H_2O_2 to form the OH radical through iron-catalyzed reactions. Catalase and selenium-containing glutathione peroxidase is examples of enzymatic antioxidants.[2] A number of non-enzymatic endogenous antioxidant mechanism also exist within normal cells such as vitamin E, vitamin C, glutathione and sulfhydryl containing compounds. Vitamin E and C react with free radicals to form radicals which are less reactive than the radicals they reacted with.[3] There is increasing evidence from animal studies that severe damage can occur in heart, intestine and stomach during reperfusion period following a period of ischemia. The most important hypotheses explaining reperfusion damage are calcium overload (Calcium paradox) and ROS (oxygen paradox). Calcium paradox is based on that if calcium is completely removed from extracellular space then reintroduced, the result is cellular damage, enzyme release and muscle contracture.[4] Oxygen paradox originates from that oxygen deprivation in ischemia leads to release of enzymes, reintroduction of oxygen to such tissues results in severe damage and release of larger amounts of enzymes.[4] Kidney injury by ischemia and reperfusion manifest a variety of functional defects, prominent among which is an impairment of tubular reabsorption of sodium and water. The proximal tubule and the outer medullary thick ascending limb have been demonstrated to suffer the most from severe injury after an ischemic insult.[5] It can be hypothesized that changes in the expression of major renal sodium transporters after renal ischemia-reperfusion injury may play a critical role in the impairment of tubular sodium and water reabsorption in experimental acute renal failure as well as in the impairment of urinary concentration seen in post ischemic period.[6] Kidney Na+-K+ pump is the driving force for renal Na+ reabsorption and is critically implicated in the control of extracellular volume and blood pressure. In the kidney, the resultant electrochemical gradient for sodium supports the luminal sodium linked transport of many solutes such as amino acids, glucose and phosphate in renal tubules.[7] Sodium adenosine triphosphatase Pase is situated in the basolateral membrane of proximal tubules, it has been suggested that during ischemic injury this Na+-K+ATPase is partly redistributed to the apical plasma membrane in this segment.[7] Recent researches state that Na+-K+ATPase is irreversibly inactivated by exposure to radiation or enzyme generated ROS, due to sulfhydryl oxidation and changes in enzyme membrane environment resulting from loss of amino group, DNA damage and lipid peroxidation caused by generation of ROS and NO in hypoxia-reperfusion injury.[7] Glycine amino acid protects against hepatic and renal toxicity by heavy metals as it reduces the oxidative stress. Preliminary studies suggest that glycine may be effective in management of acute cerebral ischemia, endotoxemia and acute central nervous system injuries.[8] The aim of this study was to investigate whether there are changes in the activity of major renal sodium transporter Na+-K+ ATPase, SOD and catalase antioxidant activity, renal NO and malondialdehyde as an index of lipid peroxidation in rats with experimental acute renal failure induced by temporary ischemia-reperfusion while breathing room air or 100% oxygen to find the role of oxygen tension on these enzymes and on kidney functions, since it was assumed that the formation of ROS is prevented by lack of oxygen during ischemia. Also to investigate whether changes in Na+-K+ ATPase are associated with alterations in renal function. The time is now ripe to study this aspect and the possibility of its modification by the potential antioxidant effect of glycine.

Methods. This study included 50 male wistar rats weighing 200-250 gm, housed under standard laboratory conditions with free access to food and water ad libitum. They were randomly divided into the following groups comprising 10 rats each. Group one (Sham operated group) in which rats exposed to sham operation both kidneys served as controls and both renal arteries were intact. Group 2 (ischemic group) in which rats exposed to right nephrectomy and left renal artery occlusion for 60-minutes, The rats were anesthetized with halothane and placed on a heated table, both kidneys were exposed through flank incisions, mobilized, the left renal artery was occluded with vascular clip for one hour, total ischemia was confirmed by observing blanching of kidney surface. Group 3 (reperfused group) in which rats after ischemia period were reperfused by removal of clamp and reflow allowed for 20-minutes. Group 4 (oxygenated group) in which animals exposed to the same protocol of ischemia then reperfusion while the animals breathed 100% oxygen instead of room air. Group 5 rats received intraperitoneal glycine injection in a dose of 100 mg/

kgm bw/day,[8] 6 times a week for 4-weeks, then subjected to ischemia-reperfusion. A blood sample was taken from renal veins of normal rats, then from ischemic group and reperfused groups. In all groups kidneys were removed, capsule was peeled, kidney frozen at -20°.

The following parameters were estimated in all groups. 1. Renal function tests - blood samples from renal vein collected to measure serum Na+,K+ and creatinine.[9] 2. Na+-K+ ATPase activity of renal tubular basolateral membrane measured as inorganic phosphate resulting from ATP hydrolysis, (method of Jorgensen and Skou.[10] Kidney removed, chilled in ice cold isotonic saline, after capsule removal, the renal medulla, papillae and cortex will be blotted on filter paper and weighed, homogenized in a 10/l (V/W) solution containing sucrose, EDTA, histidine buffer and sodium deoxycholate at pH 6.8 and homogenate centrifuged in cell density ultracentrifuge to separate basolateral membranes and are used to measure Na+-K+ ATPase activity first by estimation of total protein in kidney tissue homogenate,[11] then inorganic phosphorus estimation in the homogenate by calorimeter.[10] The reactions were carried out in presence and in absence of Kcl, Na+-K+ ATPase activity is defined as the difference between inorganic phosphate liberated in presence and absence of Kcl, and expressed in Umole inorganic phosporus released per milligram protein/minute. 3. Nitric oxide metabolites in the renal tissue.[12] The final products of NO in vivo are nitrite and nitrate, The sum of both is the best index of NO production by kidneys. Cayman chemical nitrite/nitrate assay kit was used. Tissue homogenized in lead sulfide, ultracentrifuge samples to get supernatant at 100,000 xg for 15 minutes. Griess reagent to convert nitrite ino deep purple azo compound, then colorimetric measurement of absorption. 4. Measurement of renal MDA levels as an index of lipid peroxidation by spectrophotometer and results are expressed as MDA nmoles/L. (The tissue hamogenate incubated with N-ethyl maleimide and acetylsalicylic acid then reaction terminated by 2.3% perchloric acid and thiobarbituric acid[13] 5. Measurement of renal SOD as an index of antioxidant activity by chemiluminescence.[14] The kidney were hamogenized in ice cold 50 m M K-phosphate buffer containing one mM phenyl methyl sulfonyl flouride (Sigma), a protease inhibitor and centrifuged at 1000 g for 10 min at 4°C, supernatant was centrifuged for 30 min, hemoglobin free supernatant is used to measure SOD. 6. Catalase enzyme activity was measured depending on H_2O_2 decomposition which, was followed spectrophotometrically at 240 nm in 50 mMK-phosphate buffer (pH 7.05) with 12.5 mM H_2O_2 5-20 ul of 1:10 dilution of supernatant were used for one ml of reaction mixture. The specific activity of catalase was expressed as number of umole H_2O_2 decomposed/mg renal protein/min.[15]

Statistics. The results are expressed as mean ±SD of 10 rats in each group. Appropriate statistical analysis of variance was calculated by student's t-test for unpaired data. The level of statistical significance was taken as p <0.05(t >2.1)

Results. The results of that study showed that acute renal ischemia produced significant decrease in renal tissue Na+-K+ ATPase, which also significantly decreased by reperfusion while breathing room air or 100% oxygen. Acute renal ischemia (group 2) produced significant decrease in renal tissue Na+-K+ ATPase compared to control (t=5.29*), also significant decrease in its level was shown in group 3 and 4 compared to control (namely reperfusion while breathing room air and 100% oxygen) (t=10.6*, 5.46*), group 3 and 4 also showed significant decrease in Na+-K+ ATPase activity as compared to the ischemic group 2 (tı=2.8*, 4.6*). Group 4 (glycine treated rats) showed insignificant change in Na+-K+ ATPase as compared to control group I (t=0.035). **Table 1**. The level of MDA as an index of lipid peroxidation in renal tissue was in significantly changed in ischemic group 2 as compared to the control (=0.14), but it was significantly increased in reperfusion group 3 and 4 as compared to the control (t=2.12*, 3.1*), it also showed significant increase in reperfusion groups as compared to ischemic group 2 (tı=14.4*, 2.7*), glycine treated rats had insignificant MDA level with ischemia-reperfusion as compared to control (t=0.003) **Table 1**. The level of antioxidant enzyme SOD showed significant decrease in ischemic (group 2) and ischemia-reperfusion groups (3 and 4) as compared to control (t=10.33*, 23.4*, 29.5*), it was also significantly decreased in reperfusion groups (3 and 4) as compared to control group (tı=17.3*, 9.27 *). Glycine treated rats (group 5) have SOD levels insignificantly changed as compared to control (t=1.8) **Table 1**. Renal catalase activity was significantly decreased in ischemic group 2, ischemia- reperfusion group 3 and 4 as compared to the control (t=17.4*, 39.02*, 30.89*), also it was significantly decreased in group 3 and 4 (reperfusion groups) as compared to ischemic group 2 (t=13*, 3.2*), but it was insignificantly changed in group 5 as compared to control (t=0.14) **Table 1**. Renal tissue nitric oxide was significantly increased in group 2, 3, and 4 as compared to control (t=3.08* , 6.25*, 3.2), it was also significantly low in reperfusion groups (3 and 4) as compared to ischemic group 2 (tı =3.8*, 4.1*), glycine treated rats (group 5) had insignificant change in renal level of NO as compared to control group I (t=0.3) **(Table 1)**. Serum sodium was significantly low in ischemia (group 2) and in ischemia-reperfusion (3 and 4) as compared to control (t=2.6*, 3.1*), but it was insignificantly changed in group V(glycine treated) as compared to

Table 1 - Renal Na-K ATPase activity (umole inorganic phosphorus/mg protein/min), renal superoxide dismutase (u/mg protein), catalase activity (umol H_2O_2 decomposed/mg protein/min), renal malondialdehyde (nmol/dl), renal nitric oxide (umol/L), serum creatinine (mg/dl) in all study groups.

Parameter	Mean ± SD	Group one (Control) n=10	Group 2 (Ischemic) n=10	Group 3 (Ischemia+ reperfusion) n=10	Group 4 (Ischemia + Reperf+100% oxygen) n=10	Group 5 Glycine treated n=10
Renal NA-K ATPase	Mean ± SD t t₁	84.97 ± 28.5	48.2 ± 17.96 5.29*	29.6 ± 10.6 10.6* 2.8*	35.39 ± 3.34 5.46* 4.6*	85.3 ± 7.08 0.035
Renal SOD	Mean ± SD t t₁	318 ± 9.48	280.8 ± 6.3 10.33*	228.8 ± 7.37 23.4* 17.3*	194.5 ± 9.26 29.5* 9.27*	325.3 ± 6.56 1.8
Renal catalase activity	Mean ± SD t t₁	8.57 ± 0.28	5.55 ± 0.479 17.4*	3.34 ± 0.3 39.02* 13*	2.73 ± 0.53 30.89* 3.2*	8.54 ± 0.347 0.21
Renal NO	Mean ±SD t t₁	52 ± 8.88	62.5 ± 6.3 3.08*	72 ± 4.83 6.25* 3.8*	72.5 ± 4.2 3.2* 4.1*	53.2 ± 8.6 0.3
Renal MDA	Mean ± SD	2.65 ± 0.287	3.06 ± 0.47 0.14	8.82 ± 1.4 2.15* 14.4*	2.66 ± 0.27 3.1* 2.7*	12.4 ± 3.9 0.003
Serum NA	Mean ± SD t t₁	3.8 ± 0.52	1.67 ± 0.13 2.6*	1.5 ± 1.33 5*	1.3 ± 0.44 13.1*	4.06 ± 0.368 1.3
Serum K	Mean ± SD t t₁	1.57 ± 0.125	1.64 ± 0.195 1.6	1.6 ± 0.18 0.44	1.73 ± 0.14 2.07	1.53 ± 0.17 0.66
Serum creatinine	Mean ± SD t t₁	043 ± 0.04	0.4 ± 0.049 1.5	0.412 ± 0.059 0.9	0.364 ± 0.03 1.8	0.415 ± 0.07 0.75

n - number, t - study group versus control, t₁ - ischemia-reperfusion group versus ischemic group, Na-K ATpase - sodium potassium adenosine triphosphatase, SOD - superoxide dismutase, NO - nitric oxide, MDA - malondialdehyde, Na - sodium, K - potassium H_2O_2 - hydrogen peroxide, SD - standard deviation

control (t=1.3). Concerning serum levels of K and creatinine showed insignificant change in all study groups as compared to control also insignificantly changed in reperfusion groups as compared to ischemic group.

Discussion. There is increasing evidence accumulated over the last decade indicating that ROS play a crucial role in kidney damage following ischemia-reperfusion. During ischemia, the substrates from which ROS are generated as hypoxanthine are increased, and enzymes involved in ROS production are formed. The ability of the cell to scavenge these radicals is decreased due to diminished antioxidants. Not only ROS resulted from ischemia-reperfusion but also NO which may form a cytotoxic metabolite peroxynitrite which is capable of causing lipid peroxidation and DNA damage.[2] The significant reduction of SOD level and catalase activity by ischemia and further decrease after reflow in our study could be explained by the notion that ischemia induces damage to renal tissues and enzymes, the restoration of oxygen supply during reperfusion may exacerbate ischemic renal damage due to production of superoxide radicals and cascades of oxidative reactions may be initiated and lead to formation of reduction products as H_2O_2, and OH, these products elicit widespread damage to lipids, mitochondria, DNA and enzymes.[16] Reperfusion inactivates SOD and catalase enzymes by reduction products as H_2O_2. large amounts of superoxide radicals can be formed with introduction of oxygen during reflow period, this inhibits SOD and catalase so allows accumulation of H_2O_2 and superoxide radical with further inhibition of SOD and catalase.[16] Hyperoxia induced by breathing 100% O_2 during reperfusion caused a profound decrease in SOD and catalase, indicating that hyperoxia of ischemic tissue induces severe damage to cellular defense mechanisms and enhances ROS injury.[16] Direct measurement of ROS is very difficult due to their extremely short lives, most evidence of their action comes from indirect studies such as demonstration of lipid peroxidation and by the rise in peroxidative by products as MDA.[17]

In the present study the level of renal MDA was insignificantly changed after ischemia and was significantly high in ischemia-reperfusion and hyperoxia. This is in accordance with the findings of Paller[17] who stated that renal 60 min ischemia did not increase renal contents of lipid peroxides, whereas ischemia and reflow resulted in a large increase of renal MDA levels due to formation of nitric oxide metabolites, H_2O_2, and OH which elicit widespread damage and lipid peroxidation.[18] The finding that ischemia-reperfusion induces a rise of nitric oxide metabolites in this study is in agreement with Eisei Noiri et al[18] who stated that induction of inducible nitric oxide synthase inhibition by L-nitro arginine methyl ester resulted in amelioration of renal dysfunction, reduced nitric oxide metabolites formation, reduced lipid peroxidation and DNA damage . Administration of Ebselen a scavenger of peroxynitrite before reperfusion period resulted in amelioration of lipid peroxidation and acute renal failure so improved renal function and prevented oxidative DNA damage. This could explain the lack of SOD and catalase activity with rise of lipid peroxide MDA in ischemia-reperfusion and hyperoxia where the levels of NO metabolites are significantly high. In agreement with that is the finding of Jose et al[19] who stated that renal ischemia induces an increase in glomerular NO synthesis subsequent to an activation of endothelial NO synthase.

In the present work we have demonstrated that Na+-K+ ATPase was severely reduced after renal ischemia-reperfusion injury. Glycine treatment significantly prevented the ischemic-induced down regulation of in Na+-K+ ATPase. The results suggest that decreased levels of sodium transport due to Na+-K+ ATPase lack along the basolateral membrane of the nephron in post ischemic kidneys may play a critical role in impairment of tubular sodium reabsorption and contribute to increased sodium and water excretion during the recovery phases of ischemia-induced renal failure.[20] This is consistent with previous findings of a reduced level of Na+-K+ ATPase mRNA in the basolateral membrane of proximal tubules by ischemia, this suggest that the proximal tubule is susceptible to ischemia and reperfusion injury, and that the proximal tubule reabsorption of filtered sodium, bicarbonate and phosphate may be severely compromised in postischemic kidney due to significant reduction in expression of proximal tubule sodium transporters. Lipid peroxidation, increased NO and MDA may alter enzymatic plasma membrane fluidity and alter amino groups leading to altered transport and enzyme properties of Na+-K+ ATPase.[20] Inhibition of renal Na+-K+ ATPase could disrupt the normal concentration gradient for cellular sodium and thus affect many transport processes dependent on that gradient.[19] Kukreja et al[21] had stated that sarcolemmal

Na+-K+ ATPase activity is markedly inhibited by H_2O_2, OH, and by NH2cl in ischemia reperfusion injury of the myocardium, These radicals disrupt sarcolemmal function and destroy Quabain binding sites. Also the finding that Na+-K+ ATPase is reduced following ischemia-reperfusion together with a rise of NO metabolites is consistent with the finding of Mingyu and Franklyn[22] who found that NO reduces molecular activity of Na-KATpase in opossum kidney cells, No activates protein kinase C which in turn inhibits Na+-K+ ATPase. Renal tissue injury by ischemia-reperfusion model can stimulate polymorphonuclear leukocytes (PMNs) which are ROS generating system, ROS promote neutrophil chemotaxis and adherence to vascular endothelium which may be followed by diapedesis and migration to the site of tissue injury. Activated PMNs are characterized by a respiratory-burst which involves release of lipid and oxygen metabolites.[23] The activated PMNs induce lipid peroxidation and inhibition of endogenous protective renal cytosolic enzymes such as SOD and glutathione peroxidase. Such an environment would be expected to contribute to Na+-K+ ATPase inhibition, Proteolytic enzymes such as collagenase and elastase released by PMN could also contribute to progressive inhibition of Na+-K+ ATPase.[23] Because Na+-K+ ATPase is embedded in plasmalemma with subunit containing several transmembrane segments, protein-lipid interaction may be crucial for proper enzyme function. Lipid peroxidation and auto oxidation following ischemia-reperfusion produces oxyradicals that may oxidize amino acids directly or indirectly resulting in alterations of protein structure and function. lipid peroxides as MDA may oxidize essential sulfhydryl groups of the Na+-K+ ATPase enzyme directly.[23] It is reasonable to assume that antioxidant activity will be directly related to kidney function, so there is diminished Na+-K+ ATPase activity in basolateral membrane of the kidney together with diminished SOD and catalase levels and low serum sodium due to increased Na excretion in urine or diminished tubular absorption of Na. Normal creatine levels in serum may reflect normal glomerular filtration rate. This means that 60-minutes of ischemia did not alter kidney functions or produce severe deterioration of renal function.[23] Shifrasel et al[16] had found that ischemia then 90 reperfusion under room air restore antioxidant enzyme activity but not kidney functions, and that no correlation could be found between antioxidant enzyme levels and kidney function. Sergey et al[24] proved that there is accumulating circumstantial evidence that endothelial cell dysfunction contributes to no-reflow phenomenon in reperfused-post ischemic kidneys. Oxidative stress and ischemia compromised the integrity of endothelial lining and caused no-reflow phenomenon as shown by invasive intravital microscopy of blood flow in peritubular capillaries,

and this could contribute to lack of antioxidant SOD and catalase, Na+-K+ ATPase and high MDA in ischemia-reperfusion models in our study.

Lastly, the results of the present study stated that glycine administration prevented ischemia-reperfusion kidney damage. It prevents the reduction in SOD, catalase and Na-KATpase activity and prevents the rise in NO production and lipid peroxidation. This is in accordance with previous studies which confirmed that the nonenzymatic scavenger intracellular reduced glutathione by itself or through its ability to produce glycine is a major antioxidant and scavenger of ROS during postischemic renal injury. These findings also confirm the notion that glycine is a strong antioxidant, protects the kidney from tissue damage by ischemia-reperfusion, and maintain antioxidant enzymes activity.[8] This is in agreement with the findings of Mauriz et al[25] who stated that glycine enters in composition of glutathione antioxidant, necessary to build collagen and connective tissue also remove waste products from tissues. Glycine prevents the rise of NO in hemorrhagic shock in rats, activates SOD, catalase, glutathione peroxidase, nuclear factor Kappa B and prevents oxidative stress and NO synthase expression, it converts ROS to their original stable state and discontinuing their damaging behavior.

In conclusion, based on the results observed in this study, it could be concluded that reperfusion after ischemia resulted in lipid peroxidation and No production which cause decrease of Na+-K+ ATPase activity, SOD levels and catalase activity. Hyperoxia induced by breathing 100% O_2 exaggerates ischemia-reperfusion renal injury and inhibition of renal enzymes, and that amino acid glycine has a protective antioxidant effect on ischemia-reperfusion kidney damage. This supports the possible role of glycine therapy in treatment of renal failure. These results may indicate that ROS are involved in postischemic renal damage and that the same mechanism is induced by hyperoxia, which render the kidney vulnerable for massive injury.

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