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Nitric oxide kinetics during hypoxia in proximal tubules: Effects of acidosis and glycine

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Nitric oxide kinetics during hypoxia in proximal tubules: effects of glycine and acidosis. In the present study, we directly monitored nitric oxide (NO) with an amperometric NO-sensor in suspensions of rat proximal tubules. Hypoxia-stimulated NO generation was characterized by an initial rise and a subsequent sustained increase which preceded cell membrane damage as assessed by lactic dehydrogenase (LDH) release. In contrast, the NO concentration remained unmeasurable in normoxic controls. Nitro-L-arginine-methyl ester (L-NAME) prevented the hypoxia-induced increase in NO in a dose dependent manner in parallel with incremental cytoprotection. The hypoxia-induced elevation in NO and the associated membrane injury were both markedly prevented by extracellular acidosis (pH 6.95). *In vitro* proximal tubular nitric oxide synthase (NOS) activity (^{3}H-arginine to ^{3}H-citrulline assay) was pH dependent with optimum activity at pH 8.0 and greatly reduced activity at acidic pH even in the presence of calcium and co-factors. However, glycine, a well recognized cytoprotective agent, did not attenuate the NO concentration during hypoxia. The present study therefore provides direct evidence that NO is generated by rat proximal tubules during hypoxia and demonstrates that the protective effect of low pH against hypoxic rat tubular injury is associated with an inhibition of this NO production.

Nitric oxide (NO) is a recently recognized messenger molecule mediating diverse functions including vasodilation, neurotransmission, antimicrobial, tumoricidal and immunological activities [1]. Alterations in NO synthesis have been incriminated in several pathophysiological conditions, including arterial hypertension and progression of renal failure [2], septic shock [3], diabetic renal disease [4], various inflammatory, autoimmune and degenerative disorders [5] and neuronal destruction in vascular stroke and other neurodegenerative conditions [6].

Both constitutive and inducible isoforms of NO synthases have been identified in the kidney, specifically in macula densa cells, inner medullary collecting duct, and proximal tubular cells [7, 8]. In the kidney physiological amounts of NO have an important role in renal hemodynamic regulation and sodium and water excretion [9].

The use of freshly isolated rat proximal tubules in suspension allows the study of tubular epithelial cells in the absence of other sources of NO, such as endothelial cells and neutrophils. Using this model, we have previously provided indirect evidence that NOS activity is increased significantly during hypoxia [10]. In that study plasma membrane damage, as assessed by LDH release into the medium, was prevented by both a NO synthase inhibitor (L-NAME) and a NO scavenger (hemoglobin). Additionally, a further increase in hypoxic injury was observed when the NO synthase substrate, L-arginine, was added to hypoxic tubules. However, like other studies, the evidence of excess NO generation was indirect and it was not possible to establish a temporal relationship between NO production and cell injury. The recent development of a NO-selective electrode [11] prompted us in the present study to monitor continuously the NO concentration in freshly isolated rat proximal tubules during normoxia and hypoxia. The further objective of the study was to establish a temporal relationship between NO levels and cell membrane injury, and to study the effects of incremental doses of L-NAME. Lastly, the effect of the recognized cytoprotective agents, namely low pH and the amino acid glycine, on NO kinetics during hypoxia was investigated.

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Methods

Proximal tubule isolation

Renal proximal tubules were isolated by methods similar to those previously described by this laboratory [12]. Briefly, male Sprague-Dawley rats (150 to 300 g) were anesthetized with sodium pentobarbital (60 mg/kg body wt i.p.), the aorta was cannulated with polyethylene tubing (PE 100) and kidneys perfused with 60 ml of cold heparinized (4000 U) oxygenated solution (Solution A) containing (in mM): NaCl 112, $NaHCO_3$ 25, KCl 5, $CaCl_2$ 1.6, NaH_2PO_4 2.0, $MgSO_4$ 1.2, glucose 5, Hepes 2.5, mannitol 10, glutamine 1, sodium butyrate 1 and sodium lactate 1, buffered to pH 7.4. Subsequently, the kidneys were perfused with 30 ml of Solution A containing 15 mg of collagenase (type D, Boehringer Mannheim Biochemical, Indianapolis, IN, USA) and 15 mg of hyaluronidase (Sigma Chemical Co, St. Louis, MO, USA). After perfusion, the renal cortices were dissected, minced and digested in 55 ml of oxygenated Solution A containing 40 mg of collagenase and 10 mg of hyaluronidase at 37°C under 95% O_2/5% CO_2 in a shaking water bath. The digestion was halted at 15, 20, 25, 30 minutes to remove the suspended digested tubules which were placed in 30 ml Solution A containing 1 gram of fatty acid free bovine serum albumin (ICN). Meanwhile, the larger particles were returned to the water bath for further digestion. At

30 minutes of digestion, all tissue was placed in the albumin supplemented Solution A for 10 minutes. The digested tissue was then washed and suspended in 45% isotonic percoll and layered on top of 100% percoll. The tissue was centrifuged at 15,000 g for 10 minutes to separate the proximal tubules. The lowest band, which was composed primarily (95%) of proximal tubules, was removed and washed three times with Solution A. The tubules were subsequently incubated in a cold oxygenated solution (Solution B) containing (in mM): NaCl 106, $NaHCO_3$ 18, KCl 5, $CaCl_2$ 1, NaH_2PO_4 2, $MgSO_4$ 1, glucose 5, Hepes 2.5, glutamine 2, sodium butyrate 10 and sodium lactate 4 buffered to pH 7.1. Six milliter aliquots (containing 1.0 to 1.5 mg protein/ml) were placed in ice-cold siliconized Erlenmeyer flasks and superfused with 95% O_2/5% CO_2 for five minutes. Thereafter, the flasks were capped and kept at room temperature for five minutes and then placed in a shaking water bath at 37°C for 10 minutes; during this time the pH of the suspension increased to 7.4. After this "recovery" period, experiments were initiated.

Normoxia/hypoxia protocol

After the equilibration period isolated tubules were divided into an experimental group and a time control group. The pO_2 in the time control group was kept throughout the experiment in the 300 to 400 mm Hg range. The experimental group was made hypoxic (pO_2 averaged 20 to 40 mm Hg) by gassing with 95% N_2/5% CO_2 for five minutes. The duration of hypoxia was 15 minutes except when specified. The effects of the following substances were evaluated: superoxide dismutase (Sigma); N-nitro-L-arginine methyl ester (Calbiochem, CA, USA), a NO synthase inhibitor; N-nitro-D-arginine methyl ester (Calbiochem) as a control because the NO synthase inhibition is stereospecific; glycine (Sigma); low pH (6.95) effect was studied by reducing $NaHCO_3$ to 7.5 mM in the suspension buffer (Solution B).

At the end of the hypoxia period, 1 ml of tubule suspension was sampled from controls and hypoxic tubules for measurement of LDH. Increased LDH release during hypoxia is a marker of cell membrane damage. LDH activity was measured in the supernatant and pellet according to Bergmeyer [13]. LDH activities were converted to percent release by dividing supernatant activity by total activity.

Nitric oxide measurement

NO measurement studies were conducted separately from the cell injury experiments. Before the experiment, 12 ml of tubule suspension was aliquoted to ice-cold siliconized Erlenmeyer flasks and recovered as described above. The incubation medium was supplemented with 200 U/ml of superoxide dismutase (SOD; Sigma) for a 10 minutes preincubation period as described by others [14, 15]. SOD has access only to membrane diffusible, extracellular NO. The mechanism of SOD involves the dismutation of superoxide with consequent protection of NO against oxidative inactivation by superoxide to peroxynitrite [1]. This makes the detection of free NO possible. SOD in the dose used (200 U/ml) to scavenge extracellular superoxide in this study did not effect LDH release during hypoxia (SOD + H 33.4 ± 1.5% vs. H 36.7 ± 3%; P = NS; N = 4). Hence all experiments were conducted supplemented with SOD. NO measurements were made with a commercially available NO meter (Iso-NO, World Precision Instruments, Inc., Sarasota, FL, USA) based on a published electrode design [14, 15]. The probe polarographically measures the concentration of NO gas in aqueous solutions. The NO concentration is displayed as redox current. The probe cannot detect peroxynitrite or any other free radical [15]. The sensor probe housing is covered with a gas permeable membrane. Similar to the Clarke-type electrode for oxygen, NO diffusing through the membrane is oxidized at the working platinum electrode, resulting in an electrical current. The current flow is proportional to the rate of diffusion through the membrane, which is, in turn, proportional to NO concentration at the membrane's outer surface. Calibration of the electrode was performed daily prior to the experiments. For calibration, $NaNO_2$ (0.05 mM) was used as a generator of NO in a KI, H_2SO_4 and K_2SO_4 mixture, based on the following equation:

$$2NaNO_2 + 2KI + 2H_2SO_4 + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + 4K_2SO_4$$

The titration method always detected NO standards with good linearity (r = 0.94, $P < 0.001$; N = 16). For experiments, the tubule suspension was gassed for five minutes with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2 and the sensor probe was inserted vertically into the proximal tubule suspension, so that the tip of the electrode was dipped approximately 1 cm into the solution. The flask was capped. All measurements were performed at 37°C in a shaking water bath.

Nitric oxide synthase activity determination

Nitric oxide synthase activity was assessed by measurement of the conversion of L-arginine to L-citrulline using a method described earlier [10]. The tubule pellet from either normoxic or 15-minute hypoxic tubules was reconstituted in 5 ml of cold homogenizing buffer (50 mM Hepes, pH 7.5, 0.5 mM EDTA, 1 mM dithiothreitol and 10 mg% phenylmethylsulfonyl fluoride) and sonicated for two minutes (30 seconds on/30 seconds off) on ice. The homogenate was then centrifuged at 20,000 g for one hour at 4°C and the supernatant passed over a Dowex AG50WX-8 (Na^+ form) column to remove endogenous L-arginine. Protein content was determined using the Bio-Rad protein reagent with bovine serum albumin as the standard. The nitric oxide synthase reaction mixture (400 μl) consisted of 320 μl enzyme extract (1 to 2 mg prot/ml), 100 μM ^{3}H-L-arginine (1 μCi/ml), 1 mM NADPH and 2 mM $CaCl_2$. The reaction was carried out at 37°C and stopped at 40 and 80 minutes by removing 200 μl of the reaction mixture and adding it to 2 ml of 20 mM Hepes, pH 5.5, 2 mM EDTA and 2 mM EGTA. The mixture was then passed over a 1 ml Dowex AG50WX-8 (Na^+ form) column and citrulline was eluted with an additional 2 ml of water. Radioactivity in the eluate was measured by liquid scintillation counting. When the eluate was analyzed by thin layer chromatography (silica gel 60; chloroform:methanol: ammonium hydroxide:water, 1:4:2:1), greater than 70% of the radioactivity comigrated with authentic citrulline. NO synthase activity was measured in both normoxic and 15-minute hypoxic tubules. The assay was also carried out in the presence or absence of N-nitro-L-arginine-methyl ester (L-NAME, 5 mM), glycine (2 mM) and varying pH. Results are expressed as nmol of ^{3}H-citrulline produced per mg protein in 80 minutes.

Video imaging fluorescence microscopy (VIFM)

Quantitative fluorescence video microscopy was performed on a customized VIFM system which has been described in detail

elsewhere [16]. Briefly, a Nikon epifluorescence microscope is interfaced to a PC computer and images are processed using image processing software (Image I/Fluor, Universal Imaging Corp., West Chester, PA, USA), which allows for multiple excitation ratio imaging. A microperfusion chamber was mounted onto the stage of the microscope. The design and configuration of the perfusion chamber has been described previously [16]. It allows for continuous perfusion of gassed buffer and meticulous control of the environment in which the tubules are visualized during microscopy. The perfusion buffer contained (in mM): NaCl 113, $NaHCO_3$ 18, KCl 5, $CaCl_2$ 1, Na_2HPO_4 2, $MgSO_4$ 1, glucose 5, glutamine 1, sodium butyrate 1, sodium lactate 1. For low pH studies, the concentration of $NaHCO_3$ was reduced to 7.5 mM.

Intracellular pH measurements

Intracellular pH measurements were made using the dual-excitation ratioable pH fluorophore SNAFL-2 and VIFM. SNAFL-2 fluoresces at 620 nm and the ratio of fluorescence intensities when excited at 500 nm and 550 nm is proportional to the intracellular pH. Intracellular pH calibration curves were performed by adding 5 μg/ml nigericin to PT in a high K^+ buffer (in mM): 120 KCl, 30 NaCl, 0.5 $MgSO_4$, 1 $CaCl_2$, 1 $NaHPO_4$, 5 glucose, 10 HEPES, 10 PIPES [17]. The high K^+ buffer was adjusted with 1 or 10 N NaOH to varying pHs to generate a calibration curve for each experiment.

Statistical analyses

All values are reported as means ± SE. Data were analyzed by analysis of variance (ANOVA) and the Student-Newman-Keuls multiple comparison test.

Results

Effect of hypoxia on NO production, NOS activity and LDH release in rat proximal tubules

Basal levels of NO in the incubation medium containing intact rat proximal tubules were consistently unrecordable during normoxia (Fig. 1A). However, hypoxia resulted in a rapid rise of NO in the incubation medium, followed by a sustained elevation (Fig. 1A). The level of NO was maximal by 7.5 minutes of hypoxia (Fig. 1A) and preceded cell injury as assessed by LDH release (Fig. 1B). Since the rate of loss of NO in this system is not known, the NO levels measured may represent an underestimate of the actual NO produced by the tubule suspension. In addition, since the NO probe was inserted after five minutes of gassing, it is possible that the NO concentration had already increased by this time, but was not immediately detected due to the scavenging effect of oxygen introduced during the uncapping and immersion of the probe; in this case, the rise in NO that was recorded during the first 2.5 minutes after insertion of the probe would probably reflect the exhaustion of this oxygen. The likelihood of an earlier increase in NO generation is strengthened by the finding that cytosolic calcium is already significantly increased by two minutes of hypoxia in this suspension [16]; this would be expected to immediately stimulate constitutive NOS.

Figure 2 shows that the NO concentration can be lowered by reoxgenation or the addition of hemoglobin (20 μM) at the end of hypoxic period.

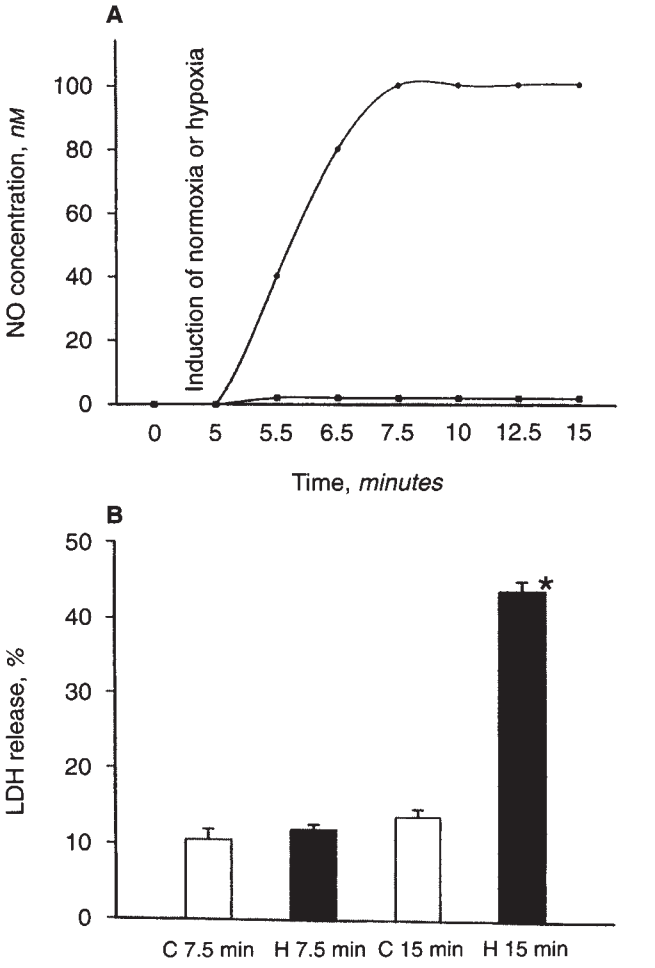

Fig. 1. A. *Representative recording of NO concentration in the incubation medium conditioned by freshly harvested isolated rat proximal tubules during normoxia (■) and hypoxia (●).* Experiments were repeated 5 times with similar results. **B.** Effect of 7.5 minutes and 15 minutes of normoxia (C) and hypoxia (H) on LDH release (*$P < 0.0001$).

Effect of L-NAME and D-NAME on hypoxia-induced NO production, NOS activity and LDH release

NO generation during hypoxia was inhibited in a dose dependent manner by L-NAME, a NO synthase inhibitor (Figs. 3 and 4). Furthermore, the dose dependent reduction in NO by L-NAME was associated with dose dependent cytoprotection at 15 minutes of hypoxia (Fig. 4). In contrast, D-NAME, which does not inhibit NO synthase, had no effect on NO generation or hypoxia-induced cell injury (Figs. 3 and 4). NOS activity in the normoxic tubules was significantly inhibited by 5 mM of L-NAME (6.2 ± 2.4 vs. 2.4 ± 0.1, $P < 0.001$), as reported previously [10].

Effect of low pH and glycine on hypoxia-induced NO production, NOS activity and LDH release

The effects on NO production during hypoxia of two other known cytoprotective maneuvers were also examined, specifically, the effects of either lowering pH of the medium or the addition of 2 mM glycine. Both of these measures resulted in complete

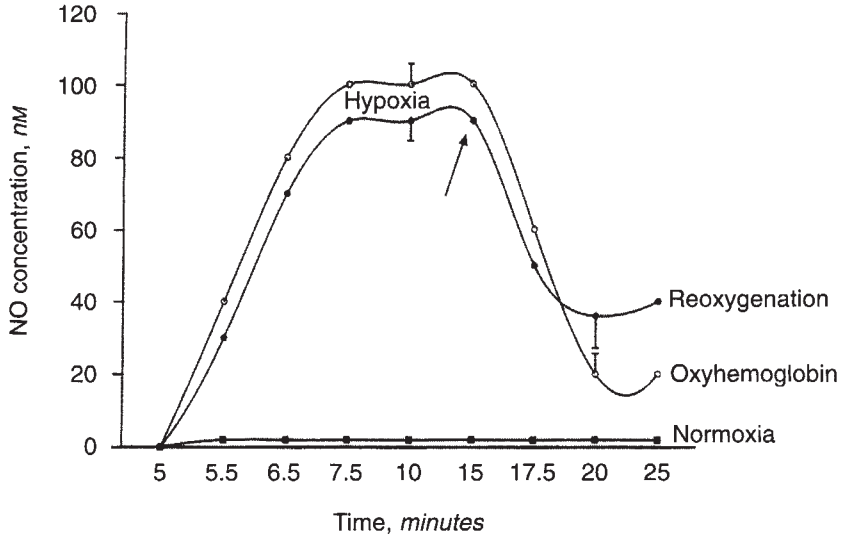

Fig. 2. *Effects of reoxygenation and oxyhemoglobin on NO kinetics following hypoxia-induced NO generation (N = 3).*

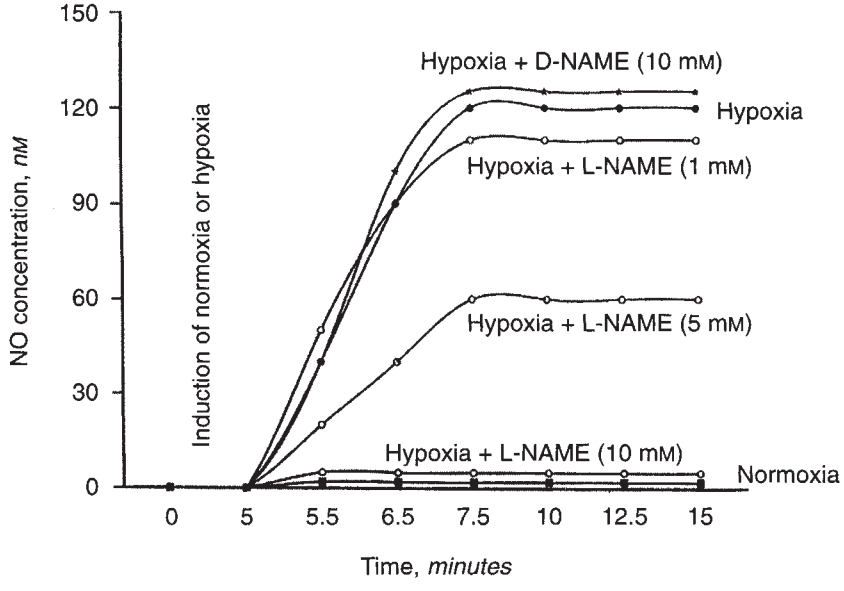

Fig. 3. *Representative recording of NO concentration in the incubation medium conditioned by isolated rat proximal tubules during normoxia, hypoxia with and without L-NAME and D-NAME.* Experiments were repeated 3 times with similar results.

protection of the tubules against hypoxic injury (Fig. 4B). However, glycine had no effect on NO production (Fig. 5). Furthermore, addition of 2 mM glycine to the tubular extract assay had no effect on NOS activity (5.89 ± 0.8 vs. 5.5 ± 0.63, P = NS).

When the proximal tubule suspension containing the usual 18 mM $NaHCO_3$ was used to maintain extracellular pH at 7.4, proximal tubule intracellular pH as determined by video imaging was 7.35 ± 0.07 (N = 6) and did not change during hypoxia. However, when tubules were incubated in the low pH solution containing 7.5 mM $NaHCO_3$ (pH 6.95), proximal tubule intracellular pH dropped to 7.03 ± 0.09 (N = 6) and remained at this pH during hypoxic perfusion (intracellular pH, 7.03 ± 0.04, N = 6). In contrast to the results obtained with glycine, lowering of the extracellular and intracellular pH was associated with almost complete inhibition of hypoxia-induced NO generation (Figs. 4 and 5). Moreover, *in vitro* NOS activity (^{3}H-arginine to ^{3}H-citrulline assay) was also pH dependent with optimum activity at pH 8.0 and almost no activity at 6.95 even in the presence of calcium and co-factors (Fig. 6).

Discussion

NO is synthesized by the enzyme NOS using L-arginine and oxygen as substrates and NADPH, tetrahydrobiopterin and flavins as cofactors. Two biochemically different isoforms of NO synthase have been identified, namely the constitutive and inducible forms. The direct detection of NO hitherto was very difficult because it is highly unstable with a half-life of 3 to 5 seconds in aqueous solutions [18]. NO reacts with oxygen and generates stable metabolites, NO_2/NO_3, which have been used as an index of NO production by various investigators [1]. However, such measurements lack the accuracy of continuous, real time, direct assessment. The present study provides the first direct observation that hypoxia elevates NO concentrations in proximal tubules. However, the increase in NO during hypoxia could contribute to or

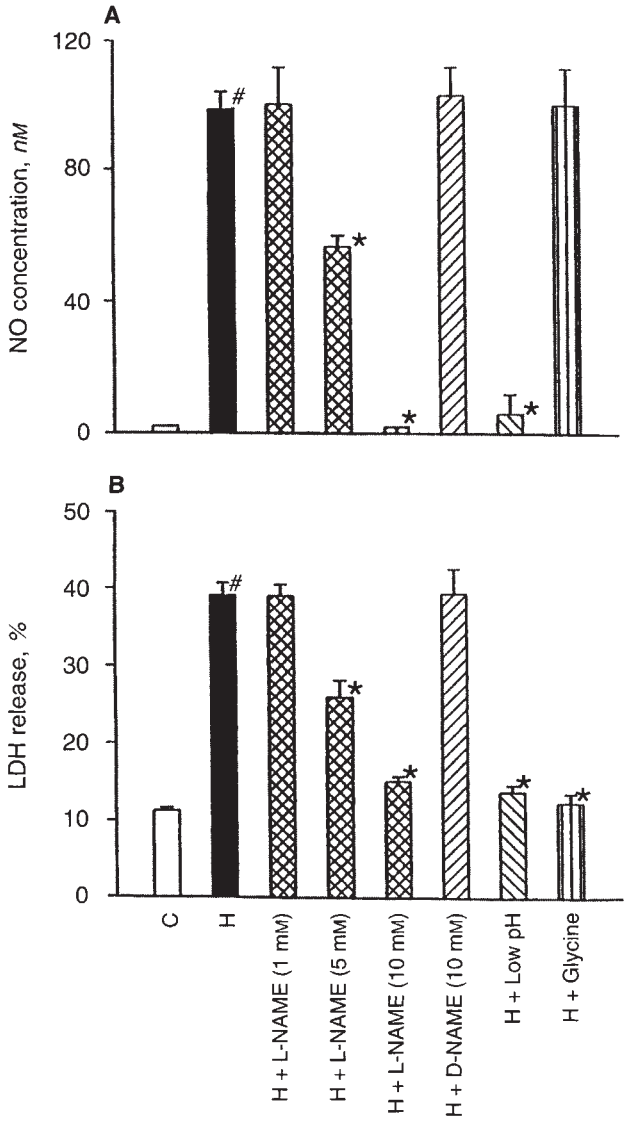

Fig. 4. **A.** *Peak NO levels (N = 3; 10 min) and* **B.** *LDH release (15 min) during different experimental settings.* Results are means ± SEM. Symbol # shows level of significance between controls (C) and hypoxia (H), while * represents the level of significance between H and other experimental settings.

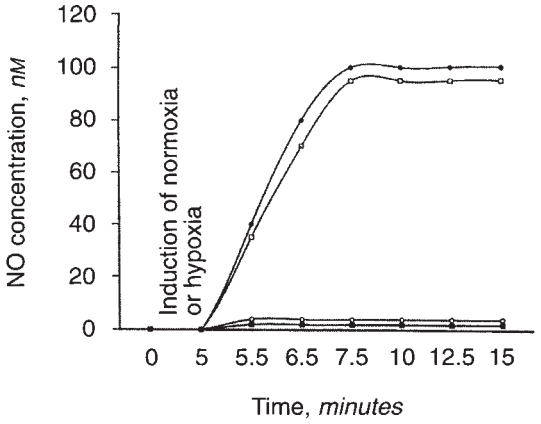

Fig. 5. *Representative recording of NO concentration in the incubation medium conditioned by isolated rat proximal tubules during normoxia (■), hypoxia (●) with and without low pH buffer (○) and 2 mM glycine (□).* Experiments were repeated 3 times with similar results.

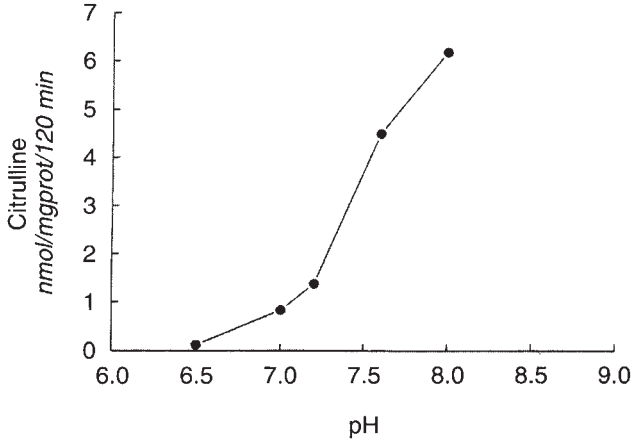

Fig. 6. *Effects of pH on rat proximal tubular NOS activity as assessed by ^{3}H-arginine to ^{3}H-citrulline conversion assay.*

merely be a consequence of cell injury. Direct continuous NO measurements are therefore essential to establish a causative relationship between hypoxia-induced increase in NO and cell injury as was possible in the present study. The results demonstrated increased NO levels during hypoxia prior to membrane damage, a finding compatible with, but not proving, a cause and effect relationship.

As shown in Figure 2 the effects of reoxygenation and oxyhemoglobin on NO kinetics were similar and consistent with their scavenging effects [1, 10]. Important results emerged from the functional studies using L-NAME. The results demonstrated a dose dependent effect of L-NAME to lower NO levels with parallel protection against hypoxia-induced membrane damage. This effect of L-NAME could not be reproduced by D-NAME, which does not inhibit NOS. The high dose of L-NAME required to totally inhibit NO production could be explained by the agent's charge neutrality and diminished potency at physiological pH, which is secondary to its low pKa [19, 20]. One consequence of these characteristics of L-NAME is that it would not be expected to be transported on the y^+ amino acid transport system. This is the system largely responsible for the cellular uptake of positively charged amino acids such as L-arginine, L-ornithine and L-lysine [20]. In this regard, it has been shown that L-nitro-arginine, the parent compound of L-NAME, does not compete with L-arginine for transport. Nevertheless, while these characteristics of L-NAME could explain the relative high dose requirement, the stereospecificity of L-NAME supports its effect to inhibit NOS.

It has been shown that low extracellular pH provides protection against hypoxia-induced injury [22], but the mechanism of cytoprotection is unknown. Since NOS is pH dependent and is therefore less active at an acidic pH [23], the possibility existed that the protective effect of low pH during hypoxia is mediated in part by blockade of NO release. In the present study, low pH almost completely prevented the increase in NO during hypoxia

and this resulted in a degree of cytoprotection comparable to that produced by 10 mM L-NAME. Moreover, *in vitro* proximal tubular NOS activity as assessed by ^{3}H-arginine to ^{3}H-citrulline assay was strongly inhibited at acidic pH despite the presence of calcium and cofactors. The degree of extracellular acidosis (pH 6.95) used in our studies maintained intracellular pH around 7.0. Both *in vivo* and *in vitro* data strongly favor the effect of low pH on the V_{max} of NOS [23]. Nevertheless, the importance of NOS inhibition in the mechanism of protection against hypoxia by low pH must be critically weighed in light of the multiplicity of potential effects of low pH on cell function.

Furthermore, in our *in vitro* experiments, the pH of the tubule suspension is preset at 6.95, resulting in reduced NOS activity and cytoprotection. In contrast, the *in vivo* situation is different in that ischemia or hypoperfusion may induce an early rise in cytosolic calcium, which at physiological pH may stimulate NOS activity, as well as other calcium-dependent enzymes. Based on our results, one could postulate that the known acidotic response to *in vivo* ischemia may serve as a compensatory mechanism to counteract potentially detrimental enzyme activation.

In addition to low pH, glycine has been shown to provide protection against hypoxia-induced membrane damage in proximal tubules [24]. Studies were therefore performed to examine whether this protective effect of glycine is associated with lower NO levels. The results demonstrated that glycine protection against oxygen deprivation injury was not accompanied by inhibition of NO release. This finding favors the view that glycine promotes plasma membrane stability during hypoxia [25, 26]. Thus glycine may act on potential targets of NO rather than affecting its generation or metabolism. In this regard further studies will be necessary to define the cellular targets of NO toxicity.

In conclusion, the present study for the first time provides direct evidence of NO release from rat proximal tubules during hypoxia. This was characterized by a prompt initial rise and subsequent sustained elevation of NO. The hypoxia-induced increase in NO concentration was demonstrated to precede cell injury, as assessed by LDH release, and was inhibited in a dose dependent manner by L-NAME but not D-NAME. Furthermore, inhibition of NO generation by low pH was associated with marked cytoprotection against hypoxia-induced cell membrane injury in the proximal tubules. However, the cytoprotective effect of glycine was shown to be independent of NO production.

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