Effect of hypoxia on proximal tubules isolated from nitric oxide synthase knockout mice

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Effect of hypoxia on proximal tubules isolated from nitric oxide synthase knockout mice. Nitric oxide (NO) has been shown to be a mediator of hypoxic injury in rat renal proximal tubules (PT). However, the role of NO in hypoxic injury to mouse PT has not been examined. The aim of the present study was to determine the effect of knockout of nitric oxide synthase (NOS) isoforms on hypoxic injury in mouse PT. Mouse PTs were isolated by collagenase digestion and Percoll centrifugation. The nonselective NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME, 10 mM), but not its inactive stereoisomer D-NAME, protected against hypoxic injury as assessed by LDH release. Carboxy-imidazolineoxyl N-oxide (carboxy-PTIO, 100 μM), a stable NO scavenger, also afforded cytoprotection against hypoxic injury. To determine the role of the different NOS isoforms in the hypoxic injury, we examined the effect of hypoxia on PT isolated from knockout mice in which either the inducible NOS (iNOS), endothelial NOS (eNOS) or neuronal NOS (nNOS) gene was lacking. PT isolated from iNOS knockout mice were resistant to hypoxic injury compared to wild-type controls. In contrast, PT isolated from both nNOS and eNOS knockout mice were not protected against hypoxic injury. In conclusion, the present study demonstrates that NO is a mediator of hypoxic PT injury in the mouse and that knockout of the iNOS gene is cytoprotective against this hypoxic PT injury.

Nitric oxide (NO) is a messenger molecule mediating diverse functions including vasodilation, neurotransmission, antimicrobial, antitumorigenic and immunological activities [1]. The formation of NO from L-arginine in mammalian cells is catalyzed by a family of isoenzymes, called NO synthases (NOS) [2]. Thus far, three distinct NOS isoforms have been isolated, purified and cloned from neuronal tissue (nNOS), endothelial cells (eNOS) and macrophage/vascular smooth muscle cells (macNOS/vs-mNOS) [2–5]. All three isoforms of NOS are expressed in the kidney. The macula densa is the principal site of nNOS gene expression in the rat kidney [6]; this isoform has also been observed to a lesser degree in collecting duct segments of the nephron, glomeruli and renal vasculature [7]. nNOS has also been detected by Northern analysis in cultured human kidney cortical epithelial cells grown under conditions selective for cells with characteristics of proximal tubular cells [8]. In the rat, eNOS mRNA has been detected by reverse transcription-polymerase chain reaction (RT-PCR) in both the renal vasculature and glomerulus as well as in several nephron segments including the PT [9]. In the mouse, eNOS protein was detectable by Western blot in whole kidney extracts, and by immunohistochemical analysis eNOS was shown to be localized in the PT [10]. Both RT-PCR and in situ hybridization studies have demonstrated that iNOS mRNA is tonically and differentially expressed along the rat nephron [11–13]. Induction of iNOS by cytokines has been observed in primary cultures of rat PT [14] and Mayeux et al have measured increased NOS activity induced by LPS in freshly isolated rat PT [15].

In two recent studies, iNOS was reported to be induced during renal ischemia/reperfusion in rats and mice [16, 17]. In the studies of Noiri et al, treatment of the rats with antisense oligonucleotides to iNOS prevented both the induction of iNOS measured in whole kidney protein extracts and the increase in nitrite produced by isolated PT [16]. This antisense treatment resulted in functional protection assessed by blood urea nitrogen (BUN) and serum creatinine. In studies by Chiao et al, prevention of iNOS induction by alpha-melanocyte-stimulating hormone during ischemia/reperfusion was also associated with functional protection [17]. In that study, iNOS protein was shown to be enriched during ischemia in the outer medulla, which is the major site of histological damage in this model of ischemic acute renal failure and is comprised largely of PT.

We have previously examined freshly isolated renal PT from the rat and have shown that NO is an important mediator of hypoxia-induced PT injury in this in vitro model, which allows us to examine effects of hypoxia on PT independent of potentially influencing factors from other nephron segments or the vasculature [18]. The present study was designed to examine whether NO is also involved in hypoxic injury to mouse PT. In addition, we also sought to determine which isoform is responsible for the hypoxic injury observed in PT. We therefore examined the response to hypoxia of PT isolated from knockout mice that lack the gene for the specific NOS isoforms.

METHODS

Knockout mice

iNOS knockout mice (129SV/C57B6) [19] were generously provided by Drs. John Mudgett (Merck Research Laboratories, Rahway, NJ, USA) and Carl Nathan (Cornell University, New
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York, NY, USA). eNOS [20] and nNOS knockout mice (129SV/C57B6) [21, 22] were kind gifts from Dr. Paul Huang (Harvard Medical School, Boston, MA, USA). The genotype of each of these knockout mice has been characterized previously by these investigators [19–21].

### Mouse PT isolation and hypoxia

Mouse PT were freshly isolated by the methods previously described by this laboratory for rats with slight modification [23]. Male 129SV (Jackson Laboratories, Bar Harbor, ME, USA) weighing 20 to 25 g were used as wild-type controls. Kidneys were removed and the renal cortices were dissected, minced and digested in oxygenated Solution A (in mM: NaCl 112, NaHCO3 18, KCl 5, CaCl2 1.6, NaH2PO4 2.0, MgSO4 1.2, glucose 5, Hepes 2.5, mannitol 10, glutamine 1, sodium butyrate 1, alanine 2 and sodium lactate 1, buffered to pH 7.4), plus 40 mg of collagenase type IV and 10 mg of hyaluronidase at 37°C under 95% O2/5% CO2 for 3 hours. LDH release was measured to evaluate cell damage as previously described [24].

### Lactate dehydrogenase measurement

LDH release was measured to evaluate cell damage as previously described [23, 24]. The percentage LDH released from tubules was calculated by determining the ratio of LDH in the supernatant compared to that in the lysed tubule pellet plus the supernatant.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the aorta of wild-type and iNOS knockout mice treated with either lipopolysaccharide (LPS; 20 mg/kg, i.p. for 8 hr; Sigma, St. Louis, MO, USA) or vehicle (normal saline) using TRIZOL Reagent (Life Technologies). Total RNA was reverse transcribed using murine leukemia virus reverse transcriptase and random hexamers. The resulting cDNA was amplified using 12.5 μM of each primer in a final volume of 40 μl. Primers used for amplification were forward, 5′-GCA TGT CAT GAG CAA CAA-3′ and reverse, 5′-GCT TCT GGT CGA TGT CAT GAG CAA-3′ designed to yield a 222-bp fragment [14].

**Fig. 1.** Freshly isolated mouse proximal tubules (PT) were exposed to hypoxia for 5, 15 and 30 minutes. Cell membrane damage was assessed by lactate dehydrogenase (LDH) release (N = 3).

### Statistical analysis

Values are expressed as mean ± se. Multiple group comparisons were done using the analysis of variance (ANOVA) with post-test according to Newman-Keuls. A P value of less than 0.05 was considered statistically significant.

### RESULTS

### Hypoxia-induced tubular injury

Freshly isolated mouse PTs were exposed to hypoxia for various periods of time. As shown in Figure 1, hypoxia resulted in cell membrane damage in a time dependent manner, as assessed by LDH release. A significant increase of LDH release occurred after 15 minutes of hypoxia and lasted up to 30 minutes as compared to normoxic controls.

### Role of nitric oxide in the hypoxic-induced tubular injury

To examine the roles of NO in hypoxic PT injury, freshly isolated wild-type mouse PT were incubated with either a non-selective NOS inhibitor, L-NAME (10 mM) or a stable NO scavenger, carboxy-PTIO (100 μM) prior to the onset of hypoxia. In the knockout mouse experiments, PT were isolated from wild-type and knockout mice in parallel and exposed to either normoxia or hypoxia in an identical manner. At the end of the hypoxic period, 1 ml of tubular suspension was sampled for measurement of lactate dehydrogenase (LDH) release.

94°C, 58°C, and 72°C, respectively, for three minutes each for 35 cycles, followed by an extension at 72°C for seven minutes (Thermocycler; Perkin-Elmer Cetus). MgCl2 concentration was 2.5 mM/sample. The PCR product was separated on a 1.2% agarose gel containing ethidium bromide, and DNA bands were visualized with an ultraviolet transilluminator. RT-PCR of GADPH showed equivalent amounts of RNA in each lane.

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Effects of hypoxia on tubular injury of knockout mice

As assessed by LDH release, PT isolated from iNOS knockout mice were resistant to hypoxia-induced cell membrane damage. LDH release was 12% in wild-type normoxia, 41% in wild-type hypoxia, \( P < 0.01 \); 12% in iNOS knockout mice + normoxia and 18% in iNOS knockout mice + hypoxia, \( P = \text{NS, } N = 6 \) (Fig. 3). In contrast, PT isolated from both nNOS and eNOS knockout mice were not protected against hypoxic injury. LDH release was 12% in wild-type + normoxia, 30% in wild-type + hypoxia, \( P < 0.01 \); 13% in nNOS knockout mice + normoxia and 27% in nNOS knockout mice + hypoxia, \( P < 0.01 \); 12% in eNOS knockout + normoxia and 34% in eNOS knockout + hypoxia, \( P < 0.01 \) (Figs. 4 and 5).

Determination of inducible nitric oxide synthase gene deletion

The mRNA and protein of NOS isoforms in all three knockout mice have been previously examined to confirm the successful deletion of the specific NOS genes [19–22]. However, to confirm that there was no genetic modification due to either continuous breeding or environmental changes, we performed a RT-PCR analysis of mouse aortic RNA to verify iNOS gene deletion in iNOS knockout mice. RNA was obtained from control and LPS stimulated wild type and iNOS knockout mice. As shown in Figure 6, a single PCR product that matched the predicted size of iNOS (222 bp) was observed in wild-type but not in iNOS knockout mice, indicating a slight basal expression in the wild-type mice. LPS treatment substantially increased iNOS message in the wild-type mice. In contrast, iNOS message was undetectable in aorta from iNOS knockout mice and LPS treatment had no effect.

DISCUSSION

We have previously shown NO to be an important mediator of rat hypoxic PT damage [18]. The present study demonstrates that NO is also a mediator of hypoxic cell injury in isolated mouse PT. The nonselective NOS inhibitor, L-NAME, was shown to be
protective against hypoxia-induced damage, whereas its inactive stereoisomer, D-NAME, had no effect. In addition, protection was also afforded by treatment of the PT with an NO scavenger, carboxy-PTIO.

To determine the role of the different NOS isoforms during hypoxic PT injury, we studied the effect of hypoxia on PT isolated from mice lacking either the nNOS, eNOS or iNOS gene. The results demonstrated that hypoxia produced significant membrane damage in PT from both nNOS and eNOS knockout mice, to a similar degree to that observed in wild type control mice. In contrast, PT isolated from iNOS knockout mice were highly resistant to hypoxia.

The mechanism of the resistance to hypoxia in iNOS knockout mice is unknown, however, there are various possibilities. Because of the short exposure of the PT to hypoxia in these experiments (15 min), and the observation that iNOS induction most likely takes much longer, it is possible that iNOS was already present in the PT. The basal amount of iNOS may be in such a low abundance in PT that it is undetectable using presently available antibodies. In this regard, studies of Mohaupt et al [12] and Ahn et al [11] indicate that iNOS mRNA is tonically expressed in the PT, and these investigators have speculated that basal NO production by iNOS may be important in normal renal physiology. Specifically, these investigators demonstrated two isoforms of iNOS to be expressed basally in the rat kidney. One form was highly homologous to the macrophage iNOS (macNOS) and another isoform distinct from the macroNOS was found to be identical to the vascular smooth muscle iNOS (vsmNOS). The isoform found to be expressed basally in the PT was the macNOS. In in vivo studies, by targeting macNOS with specific antisense nucleotides, Noiri et al were able to dramatically attenuate the functional and histological changes induced in a model of renal ischemia in the rat [16]. Interestingly, antisense to the vsmNOS worsened the injury in this ischemic model. Our current findings are supportive of those of Noiri et al and suggest that iNOS expressed by PT cells is directly damaging to these cells during hypoxia. On the other hand, one cannot rule out the possibility that other factors are involved in the resistance of the iNOS knockout PT to hypoxia.

Recent studies with recombinant iNOS protein indicate that iNOS is twice as active in the presence of Ca$^{2+}$ as in its absence [25]. Other studies have also demonstrated the Ca$^{2+}$ dependency of iNOS activity [15, 26, 27]. Thus, it is possible that iNOS activity during hypoxia is to some extent Ca$^{2+}$-dependent. In this regard we have demonstrated that 10 minutes of hypoxia induces a prelethal increase in free cytosolic Ca$^{2+}$ in proximal tubules [28].

It is known that in vivo S3 proximal tubules are more poised for hypoxic damage than S1 and S2 tubules. Chiao et al could only demonstrate induction of iNOS protein in the outer medulla of the kidney (site of S3 proximal tubules) and not in the cortex (site of S1 and S2 proximal tubules) [17]. However, Noiri et al detected NO by measurement of nitrite production in cortical tubules [16]. Our results are in agreement with the latter study as our proximal tubule preparation consists of cortical tubules.

Absence of the iNOS gene afforded greater protection against hypoxic injury in the wild-type mice PT than did either L-NAME or the stable NO scavenger carboxy-PTIO. A possible reason for this is that NOS inhibitors and NO scavengers may not optimally block or scavenge NO. They can also have poor membrane penetration and lack of selectivity for a particular isoform. For these reasons we believe that the results in the knockout mice are more important than the L-NAME and carboxy-PTIO studies.

As NO is formed from L-arginine and molecular oxygen, it is possible that hypoxia could substrate limit NO production. However, it has been demonstrated that although $^3$H-citrulline production is reduced at a pO$_2$ of 28 mm Hg compared to 130 mm Hg, there is still significant $^3$H-citrulline production at the lower pO$_2$ [29]. Thus, even this lesser amount of NO, formed in the...
REFERENCES