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Research Article

Gene products of all three distinct nitric oxide synthases are present in the mammalian kidney. This mosaic topography of nitric oxide synthase (NOS) isoforms probably reflects distinct functional role played by each enzyme. While nitric oxide (NO) is cytotoxic to isolated renal tubules, inhibition of NO production in vivo invariably results in the aggravation of renal dysfunction in various models of acute renal failure. We reasoned that the existing ambiguity on the role of nitric oxide in acute renal failure is in part due to the lack of selective NOS inhibitors. Phosphorothioated derivatives of antisense oligodeoxynucleotides targeting a conserved sequence within the open reading frame of the cDNA encoding the inducible NOS (iNOS) were designed to produce a selective knock-down of this enzyme. In vivo use of these antisense constructs attenuated acute renal failure in rats subjected to renal ischemia. This effect was due, at least in part, to the rescue of tubular epithelium from lethal injury. Application of antisense constructs did not affect endothelial NOS, as evidenced by a spared NO release after the infusion of bradykinin during in vivo monitoring with an NO-selective microelectrode. In conclusion, the data provide direct evidence for the cytotoxic effects of NO produced via iNOS in the course of ischemic acute renal failure, and offer a novel method to selectively prevent the induction [...]

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In Vivo Targeting of Inducible NO Synthase with Oligodeoxynucleotides Protects Rat Kidney Against Ischemia

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Abstract

Gene products of all three distinct nitric oxide synthases are present in the mammalian kidney. This mosaic topography of nitric oxide synthase (NOS) isoforms probably reflects distinct functional role played by each enzyme. While nitric oxide (NO) is cytotoxic to isolated renal tubules, inhibition of NO production in vivo invariably results in the aggravation of renal dysfunction in various models of acute renal failure. We reasoned that the existing ambiguity on the role of nitric oxide in acute renal failure is in part due to the lack of selective NOS inhibitors. Phosphorothioated derivatives of antisense oligodeoxynucleotides targeting a conserved sequence within the open reading frame of the cDNA encoding the inducible NOS (iNOS) were designed to produce a selective knock-down of this enzyme. In vivo use of these antisense constructs attenuated acute renal failure in rats subjected to renal ischemia. This effect was due, at least in part, to the rescue of tubular epithelium from lethal injury. Application of antisense constructs did not affect endothelial NOS, as evidenced by a spared NO release after the infusion of bradykinin during in vivo monitoring with an NO-selective microelectrode. In conclusion, the data provide direct evidence for the cytotoxic effects of NO produced via iNOS in the course of ischemic acute renal failure, and offer a novel method to selectively prevent the induction of this enzyme. (*J. Clin. Invest.* 1996. 97:2377–2383.) Key words: ischemia • antisense oligonucleotides • nitric oxide • cytotoxicity • NO-selective microelectrode

Introduction

Products of all three distinct nitric oxide synthase (NOS)¹ genes are represented in the mammalian kidney: endothelial constitutive NOS is expressed in the tubular epithelium, in ad-

dition to its localization to the vascular endothelium; neuronal isoform is found in the macula densa cells and epithelium of Bowman's capsule; whereas inducible NOS is present in the proximal tubule, glomerulus, medullary thick ascending limb, inner medullary collecting duct and has virtually unlimited representation in various pathologic conditions (1–4). This mosaic topography of all known isoforms of NOS, probably, reflects distinct functional role played by each isoform, depending on the requirements and conditions of the microenvironment in specific segments of the nephron. Under the circumstances, application of nonselective inhibitors of NOS would produce uninterpretable results on the involvement and role of individual isoforms.

Indeed, there is growing ambiguity as to the role of nitric oxide (NO) and its metabolic product peroxynitrite (5–7) (ONOO⁻) in the pathophysiology of acute renal failure. NO and ONOO⁻ have been shown to mediate hypoxia-reperfusion injury in different organs including the kidney (8), while the inhibitors of NOS exhibit antiinflammatory properties (9, 10). Paradoxically, NOS inhibitors, N^G-nitro L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine (L-NMMA), in in vitro experiments, protected renal tubular epithelium against hypoxic injury (8), whereas they invariably aggravated renal dysfunction in different in vivo models of acute renal failure (11–13). Therefore, the major challenge is to reconcile these experimentally solid, but controversial observations. One of the possible explanations for such discrepant in vitro and in vivo effects of NOS inhibitors is in a poor selectivity of the available antagonists (14, 15). Investigation of the functional role of each isoform awaits highly selective inhibitors. We elected to utilize the antisense oligodeoxynucleotide (AS-ODN) (16) strategy for a selective knock-down of inducible NOS (iNOS). Antisense oligodeoxynucleotides targeting a conserved sequence within the open reading frame of the cDNA encoding iNOS were designed to produce selective knock-down of this enzyme. In vivo use of these antisense constructs attenuated acute renal failure in rats subjected to renal ischemia. This effect was due, at least in part, to the rescue of tubular epithelial cells from injury induced by a product of iNOS.

Methods

Design and synthesis of oligodeoxynucleotides. The phosphorothioated oligodeoxynucleotides were synthesized in an automated solid-phase DNA synthesizer (Applied Biosystems, Foster City, CA). Since at least two isoforms of iNOS are expressed in the proximal nephron of cytokine-stressed rat kidneys, vascular smooth muscle-specific and macrophage-specific (1), we targeted a conserved sequence within the open reading frame (ORF) of their cDNAs, nucleotides 1829–1809, which are homologous in mouse and rat macrophage, as well as

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1. Abbreviations used in this paper: AS-ODN, antisense oligodeoxynucleotide; BUN, blood urea nitrogen; iNOS, inducible NOS; NOS, nitric oxide synthase; ORF, open reading frame.

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rat kidney-specific iNOS's (GenBank accession No. M84373) (1, 17, 18) AS-ODN-ORF: (5'-3') CTT CAG AGT CTG CCC ATT GCT.

To account for any possible nonspecific effects of oligodeoxynucleotides, several control sequences were used:

(a) Antisense oligodeoxynucleotides directed toward the initiation codon of the vascular smooth muscle isoform (AS-ODN-VSM), nucleotides 6-26 (GenBank accession No. D14051): 5'-3' GCA AGC CAT GTC TGT GAC TTT GTG; (b) Sense and scrambled oligodeoxynucleotides: sense primer (5'-3') AGC AAT GGG CAG ACT CTG AAG, and scrambled primer (5'-3') TCT CAG TGA GCC CTC ATT CTG.

The designed sequences showed no homology with other known mammalian sequences deposited in GenBank database, as screened using Blast program (19). Oligonucleotides were purified, dried, resuspended in 10 mM Tris/1 mM EDTA, pH 8.0, and quantified spectrophotometrically. These constructs were injected into rats subjected to renal ischemia, a classical model of acute renal failure which is associated with the induction of iNOS (1).

Surgical techniques. Male Sprague-Dawley rats weighing 110–130 grams were allowed rat chow and water ad libitum for 5 d before experiments. After an overnight fast, animals were anesthetized with ketamine HCl and xylazine HCl (11.6 and 0.77 mg/100 grams body weight, respectively). Bilateral renal pedicle cross-clamping for 45 min was performed as previously described (20). 24 h after reperfusion, blood was drawn for blood urea nitrogen (BUN) and creatinine, at which time the animals were killed, kidneys flushed with normal saline, and fixed with 4% paraformaldehyde (followed by embedding in Tissue-Tek and frozen sectioning) for immunocytochemistry and with 10% formalin (followed by paraffin-embedding) for morphological scoring. Pathological findings were analyzed in a blind fashion according to the previously reported criteria (21) which encompass both the degree of lethal injury to the epithelium and tubulo-obstructive manifestations. The second ischemic kidney was harvested for preparation of isolated cortical tubules and for Western analysis of iNOS (see below). Oligonucleotides were injected intracardially at the concentration 1 mg/kg and L-NAME (Bachem California, Torrance, CA) was injected intraperitoneally at the concentration 75 mg/kg 10 h before the surgery.

Nitrite production by renal cortical tubules. Harvested ischemic or control kidneys were placed in ice-cold DMEM, cortical tissue was dissected and minced. The fraction of cortical tubules was obtained after successive passages through the nylon mesh filters 150 and 75 μ m, as previously detailed (8). The collected tubular fraction was resuspended in DMEM without phenol red and incubated for 6 h at 37 °C in the atmosphere of 95% O₂ + 5% CO₂. At the end of incubation, the supernatant was collected and the concentration of nitrite was determined according to the Griess technique (22). The amount of protein in each sample was determined using bicinchoninic acid protein assay reagent (23).

In vivo NO monitoring. Anesthetized rats (100 mg/kg intraperitoneal injection of Inactin-Byk) were fixed on a heating table, a venous line was placed, and the left kidney was accessed via a flank incision. The kidney was immobilized in a holding cup, and a precalibrated NO-selective reinforced microelectrode with a tip diameter of 30 μ m (24), designed for in vivo experiments (Intra Medical, Nagoya, Japan), was driven using a micromanipulator (Zeiss-Eppendorff) to impale the renal cortex. After the acquisition of baseline measurements, bradykinin (25 nmol/100 grams) was injected intravenously (25). 5–10 min later, depending on the duration of the response, L-NAME (50 μ mol/100 grams) was injected intravenously. Electrode current was digitally transformed using a two-channel recording system Duo-18 (World Precision Instruments, Sarasota, FL) and stored in a PC microcomputer. Calibration of the electrode was performed using NO donor S-nitroso-N-acetylpenicillamine (SNAP; Molecular Probes, Eugene, OR), according to the previously described technique (24).

Expression of iNOS in the kidney. Harvested kidneys were homogenized in liquid nitrogen, the lysates were electrophoresed on 5% SDS-polyacrylamide gel, and Western blotting was performed with

monoclonal antibodies to iNOS, according to the manufacturer's instructions (Transduction Labs, Lexington, Kentucky). Immunohistochemical staining of frozen 8- μ m sections was performed using indirect immunofluorescence technique. The thawed cryosections were treated with 0.1% Triton X-100 for 20 min, and the nonspecific binding sites were blocked with 0.1% bovine serum albumin (BSA). Sections were incubated for 60 min with monoclonal antibodies (0.5 μ g/ml each) against iNOS or endothelial isoform of NOS (eNOS) (Transduction Labs), followed by the rhodamine-conjugated anti-mouse IgG. Control sections were subjected to second antibody only. Mounted preparations were examined under an inverted fluorescence microscope. Images were similarly processed using Image-1 software (Universal Imaging, West Chester, PA).

Morphologic evaluation of kidneys. Formalin-fixed sections were stained with hematoxylin-eosin and scored in a blind fashion for the severity of acute renal failure, based on the well established criteria (21).

Results

To optimize the experimental conditions for in vivo delivery of systemically injected oligodeoxynucleotides, the time-course of their accumulation in the proximal nephron was examined. In experiments with 3'-biotinylated AS-ODN derivatives, we found that maximal labeling of proximal tubular epithelium occurred 4–8 h after systemic injection (Fig. 1). According to the above time-course, the rats were treated with control and experimental ODN constructs ca. 8 h before cross-clamping of renal arteries. As shown in Fig. 2 A, administration of AS-ODN-ORF resulted in a dramatic functional protection of kidneys from acute ischemia. The concentration of plasma BUN and creatinine in this group was not different from sham-operated animals and significantly lower than in experimental animals receiving AS-ODN-VSM, as well as sense (S) and scram-

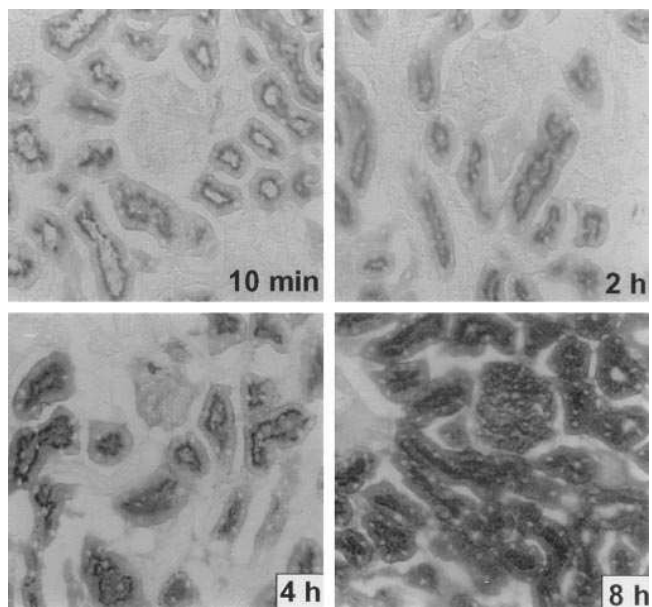


Figure 1. The time-course of accumulation of 3'-biotinylated phosphorothioated ODN in the kidney. Oligodeoxynucleotides were injected intraarterially at the concentration 1 mg/kg, and rats were killed at the indicated times. Kidney sections were prepared, as detailed in Methods, and stained with streptavidin conjugated with alkaline phosphatase. Diffuse cytoplasmic staining becomes conspicuous at 4 h after injection and increases in intensity at 8 h. $\times 190$.

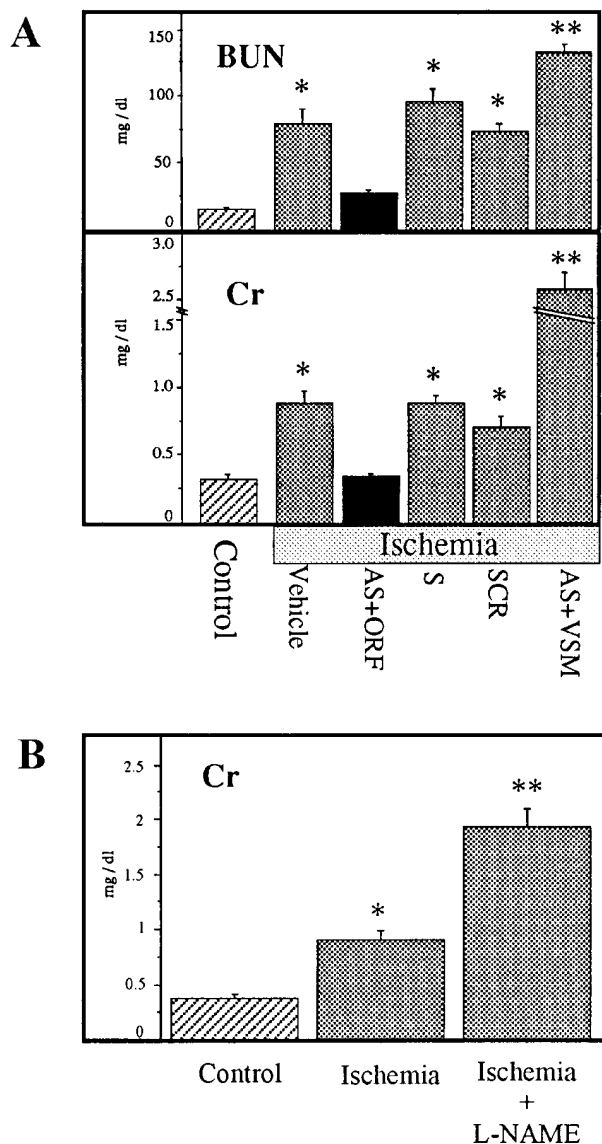


Figure 2. Serum BUN and creatinine in rats subjected to 45 min of renal ischemia after pretreatment with phosphorothioate oligonucleotides (A) or L-NAME (B). The data represent the results obtained from 6–9 animals 24 h after reperfusion. In A animals received intracardiac injection of 1 mg/kg body weight oligodeoxynucleotides, except for the group treated with AS-VSM which received 0.5 mg/kg injections. AS-ORF, antisense phosphorothiated (S) oligodeoxynucleotides directed toward the open reading frame sequence of iNOS; S, sense S-oligomers; Scr, scrambled S-oligomers; AS-VSM, antisense S-oligomers to the initiation codon of the vascular smooth muscle isoform of iNOS. B summarizes serum creatinine concentration in rats subjected to the same degree of renal ischemia-reperfusion and treated with L-NAME (75 mg/kg body weight). * $P < 0.05$ compared with the sham-operated control; ** $P < 0.05$ vs. ischemic group receiving vehicle.

bled (Scr) constructs. In sharp contrast with the results obtained after selective knock-down of iNOS, the use of a non-selective inhibitor of NOS, L-NAME, had no therapeutic effect (Fig. 2 B), illustrating the validity of our zero-hypothesis on the functional diversity of NO produced at different sites and by different NOS isoforms.

To verify the efficacy of AS-ODN-ORF treatment, nitrite production by freshly-isolated cortical tubules obtained from control and ischemic kidneys was determined in vitro. As evident from Fig. 3 A, ischemic kidneys show almost sixfold increase in nitrite production. Pretreatment with AS-ODN-ORF virtually abolished this increase in nitrite production. Furthermore, the expression of iNOS in AS-ODN-ORF-pretreated ischemic kidney homogenates was significantly attenuated compared to ischemic control (Fig. 3 B), again confirming the efficacy of AS-ODN-ORF treatment. Immunohistochemical data (Fig. 4) complemented these findings. While immunodetectable iNOS was conspicuously enhanced in ischemic kidneys (Fig. 4 C), antisense constructs partially prevented this induction (Fig. 4 E). Of note, staining with antibodies to eNOS did not reveal significant differences between the experimental groups (Fig. 4, B, D, and F).

To obtain additional insights into the selectivity of the AS-ODN-ORF towards iNOS, in vivo monitoring of NO release from control and ischemic kidneys was performed using an NO-selective electrochemical technique (24). 24 h after cross-clamping, the left kidney of experimental or control animals was immobilized (to prevent breathing artifacts) and impaled with the precalibrated electrode working in the amperometric mode (Fig. 5 A). As shown in Fig. 5, B–D depicting representative NO-electrode tracings, intravenous administration of bradykinin (*single arrowhead*) resulted in a substantial increase in NO release, as judged by the amplitude of the current, in control (B) and AS-ODN-ORF-pretreated rats subjected to renal ischemia (D). In contrast, bradykinin had only a marginal effect in nontreated ischemic rats (C). These observations on the blunted NO release in renal ischemia corroborate the previously reported findings in norepinephrine-induced renal failure which showed the absence of vasorelaxation in response to endothelium-dependent vasodilators (3). Fig. 6 represents the summary of baseline currents detected with NO-selective electrode and the amplitude of responses elicited by bradykinin infusion. It is evident that the baseline current recorded from ischemic kidneys was higher than that detected in control and AS-ODN-ORF-treated ischemic kidneys (A). Furthermore, although profoundly suppressed in nontreated ischemic kidneys, the bradykinin-induced elevation in NO release was spared in AS-ODN-ORF-treated animals (B). These data further substantiate our conclusion that the applied antisense construct resulted in a selective knock-down of iNOS.

To elucidate the mechanism of protection afforded by AS-ODN-ORF, histological scoring of injury to the nephron was performed (criteria used were reported previously) (21). As summarized in Fig. 7, the major registered effects of AS-ODN-ORF were in prevention of tubular necrosis, diminution of the loss of the brush border membrane, and reduction in cast formation. Since both the loss of the brush border and cast formation reflect the degree of tubular cell injury, it can be implied that the AS-ODN-ORF protection of renal function against ischemia results from prevention of sublethal and lethal injury to renal tubular epithelium.

Discussion

Cytotoxicity of NO and its metabolite ONOO⁻ has been demonstrated in diverse cell types (5, 6) and has been implicated in the development of atherosclerosis (7), DNA damage and mutation in human lymphoblastoid cells (26), lipid peroxidation

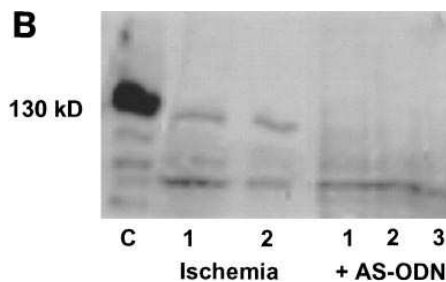
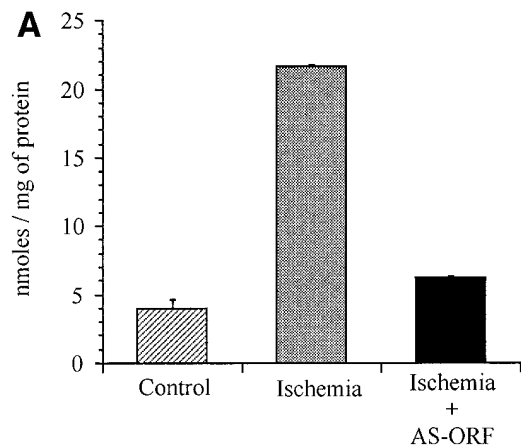


Figure 3. Validation of the blockade of iNOS induction with the antisense construct. (A) Nitrite production by freshly-isolated renal cortical tubules obtained from control animals, and rats subjected to renal ischemia-reperfusion treated with vehicle or phosphorothiotated AS-ORF oligomers (1 mg/kg body weight). (B) Western analysis of iNOS expression in kidney homogenates obtained from control ischemic (samples 1 and 2) and pretreated ischemic groups (samples 1–3). Macrophage NOS-positive control is shown in lane c.

(27), and cytotoxicity of the human immunodeficiency virus type 1 coat protein in primary cortical cultures (28). Diverse viral infectants (bornea disease virus and rabies virus in rats, herpes simplex in mice) cause encephalitis via induction of NO

synthesis (29). Furthermore, NO appears to be responsible for brain and heart ischemic damage after cerebral artery occlusion (30) and after myocardial infarction (31, 32), respectively. Most recently, NO cytotoxicity has been described in renal tu-

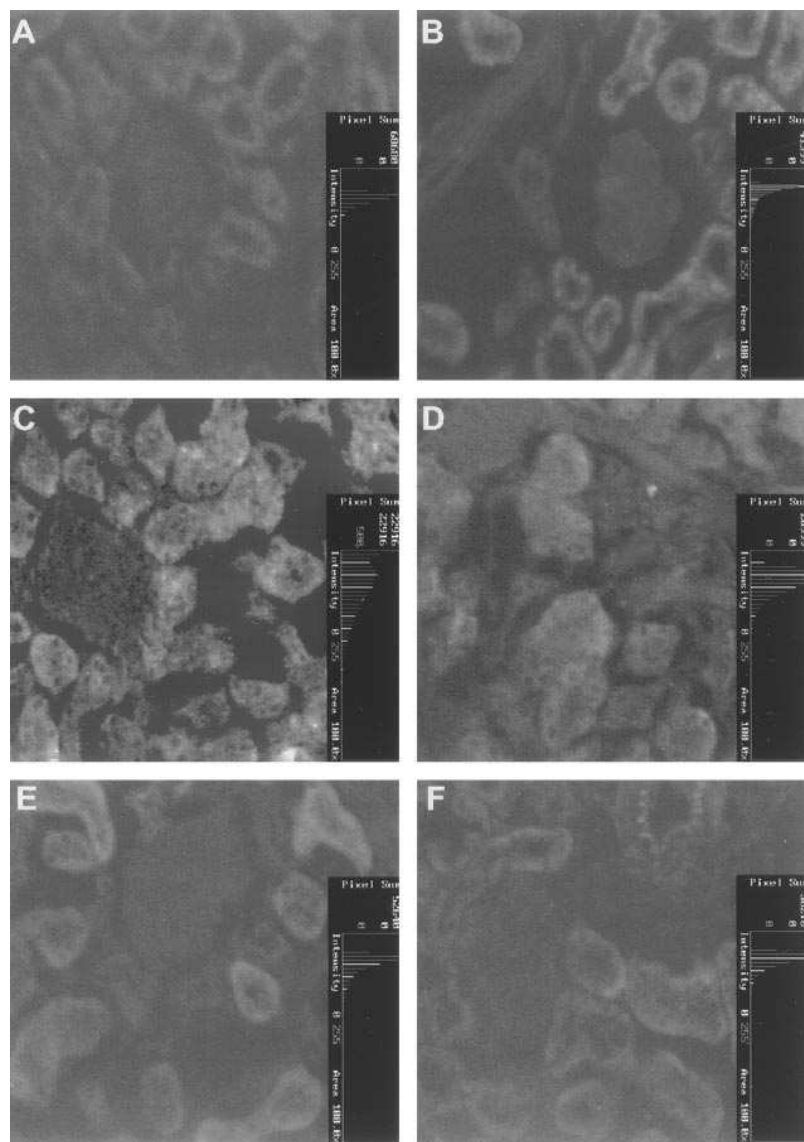


Figure 4. Immunohistochemical detection of iNOS and eNOS. Immunohistochemical staining of kidneys from control and experimental groups with antibodies directed against the inducible (A, C, and E) and endothelial (B, D, and F) nitric oxide synthases. Note the increased intensity of staining with monoclonal antibodies in ischemic kidneys (C) compared with AS-ORF oligodeoxynucleotides-pretreated ischemic kidneys (E). Staining with monoclonal antibodies to eNOS showed comparable patterns and intensities in all groups (see intensity histograms in the right corner of each panel). (A and B) control kidneys; (C and D) ischemic kidneys; (E and F) ischemic kidneys with AS-ODN-ORF. $\times 200$.

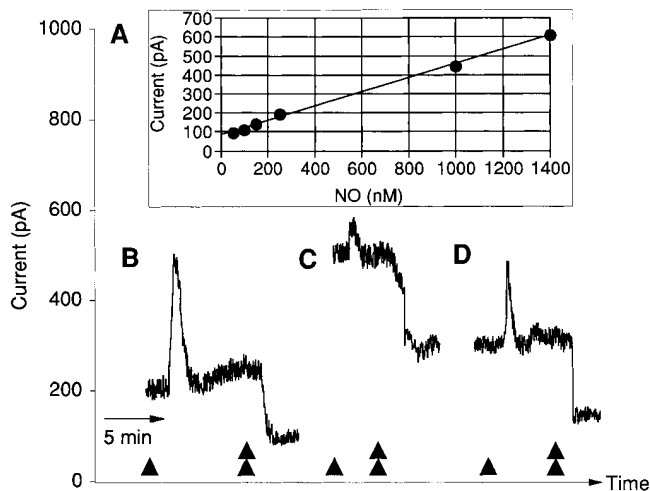


Figure 5. In vivo (NO) monitoring within the renal cortex using an NO-selective electrode. Animals were instrumented and kidneys immobilized as detailed in Methods. Using a micromanipulator, an NO microelectrode (tip diameter, 30 μm) was inserted ~ 1 mm deep into the renal cortex. Acquisition of baseline currents was followed by the consecutive i.v. infusions of bradykinin (arrowhead) and L-NAME (double arrowheads). A representative calibration curve is depicted in A. (B–D) show actual recordings obtained from sham-operated, nontreated ischemic, and treated ischemic rats, respectively. Time-scale is shown as an arrow (5 min).

bular epithelial cells (8). Hence, there is a plethora of observations that causally relate NO to cytotoxicity.

It can be argued that the available inhibitors of NOS are not selective enough and/or sufficiently specific (14, 15). Different types of NOS may differently affect cell viability, as has recently been demonstrated in mice deficient in neuronal NOS (33). Therefore, if alternative sources of NO exert reciprocal effects, the use of nonselective NOS inhibitors may produce ambiguous results. This is, probably, the case with the results obtained in in vivo and in vitro models of acute renal failure (8, 11–13). To circumvent these problems, we elected to use the antisense oligodeoxynucleotide strategy. We have previously investigated the effects of antisense S-ODN to inducible nitric oxide synthase on the viability of African green monkey kidney cells, BSC-1, under resting conditions and after exposure to oxidant stress, and demonstrated their cytoprotective effect (34). These in vitro findings prompted us to investigate the effect of antisense constructs on the course of ischemic acute renal failure in vivo. Only through a selective knock-down of iNOS has it become possible to demonstrate the functional disparity of NOS's in the kidney: targeted inhibition of iNOS expression attenuated ischemia-induced dysfunction, while a nonselective inhibitor L-NAME was ineffective, and even resulted in the deterioration of renal function compared to nontreated animals. These observations reinforce the recent findings of submaximal eNOS stimulation early in the course of norepinephrine-induced acute renal failure and profound vasoconstriction accompanying L-NAME infusion (3). Collectively, these observations disclose the reciprocity in NOS's functions, implying that the degree of injury is linked to the iNOS, while the restoration of renal function after noxious stimuli depends on eNOS. When the function of all isoforms of NOS is blocked, as with a nonselective inhibitor, the deleteri-

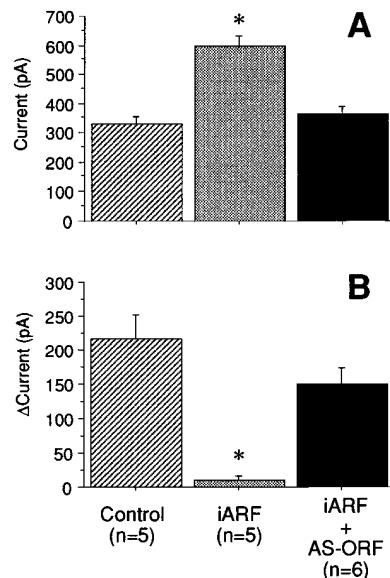


Figure 6. Bradykinin-induced increase in microelectrode currents over the baseline as detected with in vivo [NO] monitoring. (A) Baseline electrical currents recorded from the cortex of control, ischemic, and treated ischemic kidneys. (B) Bradykinin-induced increments in electrical current detected with NO-selective microelectrode in the cortex of control, ischemic, and treated ischemic kidneys. (*n*, number of rats in each group). **P* < 0.05 vs. control and treated group.

ous consequences of inhibiting eNOS invariably prevail over the possible benefits of inhibiting iNOS.

The data presented above provide direct evidence for the cytotoxic effects of NO produced via iNOS, and offer a novel method to selectively prevent the induction of this enzyme. The application of this approach in rats with renal ischemia resulted in a dramatic attenuation of epithelial cell damage, the established hallmarks of the syndrome of acute renal failure.

While in vitro use of antisense oligodeoxynucleotides has been successfully utilized in the past (16, 35), there are but a few examples of their efficacy as therapeutic agents in vivo (36–38). Intraperitoneal injection in mice of antisense phosphorothioate ODN hybridizing to the AUG translation initiation codon of mRNA encoding murine protein kinase C- α results in a dose-dependent reduction of the targeted mRNA in the liver with the IC-50 value of 30–50 mg/kg (39). Antisense phosphorothioate constructs to galanin can be successfully introduced through the proximal end of a transected sciatic nerve to the dorsal root ganglia (40). Transcatheter delivery of *c-myc* antisense ODN results in the reduction of neointimal formation in a model of coronary artery balloon injury (41). In our case, the need to target the proximal tubular epithelium, the site of preferential injury in acute ischemia-reperfusion model, has certain advantages for applying antisense ODN strategy. The substantial accumulation of systemically injected phosphorothioate ODNs by the kidney, and especially by the proximal tubular epithelium, has been demonstrated previously in mice and rats (42–44), thus drastically reducing (at least 10-fold) the required concentration of ODN to ~ 1 mg/kg body weight.

The blockade of the inducible enzyme iNOS carries an additional potential benefit, allowing for the reparative processes to take place in the postischemic period. In this respect, it has

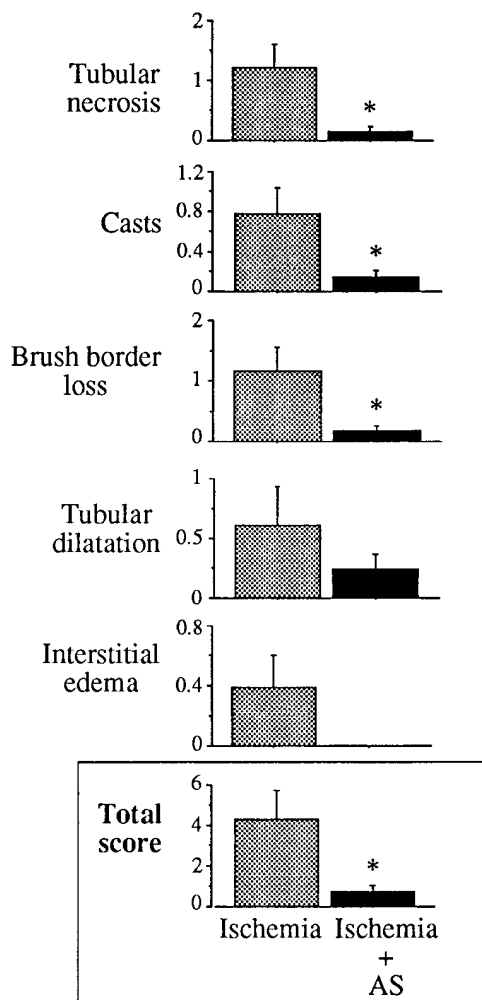


Figure 7. Pathological scores of intensity of acute renal failure in ischemic kidneys treated with AS-ODN-ORF. Scoring was performed in a blind fashion on $n = 9$ kidneys for control ischemia and $n = 11$ kidneys for the antisense ODN-treated group. * $P < 0.05$ vs. control ischemia.

been demonstrated that NO attenuates protein and DNA synthesis (45), the effects which may hamper reparative processes in the injured epithelial cells. Hence, the dramatic functional and structural preservation of ischemic kidneys treated with antisense oligomers to iNOS, as demonstrated above, may be the consequence of both the reduced cytotoxicity and the enhanced regenerative processes. The successful use of antisense oligonucleotide strategy targeting the iNOS expression in alleviating ischemic renal damage represents the first attempt at this approach in the treatment of acute renal failure and, possibly, other pathological conditions accompanied by the induction of this gene.

Acknowledgments

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