The Role of Nitric Oxide in Renal Transplantation

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This review discusses the concept that nitric oxide synthase (NOS) may orchestrate both the inflammatory response to the renal allograft and anti-inflammatory defense in the graft itself. NO is produced by endothelial, epithelial, as well as inflammatory cells. In the setting of transplantation, the endothelium is the first lining to be subjected to the early response to injury. In turn, activated endothelial cells facilitate leukocyte recruitment, immune-mediated injury, and angiogenesis. On activation by inflammatory stimuli, endothelial cells up-regulate multiple vasoactive substances, oxygen radicals, cytokines, chemokines, and growth factors. Therefore, endothelial integrity, especially the expression of protecting vasoactive agents, such as NO, may be a key factor in resistance or sensitivity to transplantation-mediated injury. Thus, evaluating the mechanisms by which NO is involved in either protecting or injuring the transplanted allogeneic kidney is important for our understanding of renal allograft rejection. This review focuses on the role of NO in the inflammatory endothelial-leukocyte interactions, which are implicated in acute and chronic rejection of the transplanted kidney.

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kidney transplantation has improved survival and quality of life for patients with end-stage renal failure. On transplantation and during graft rejection, protection by endothelium-derived nitric oxide (NO) appears to be defective because vasoconstriction, inflammation, thrombosis, and intimal proliferation are common features in graft vasculopathy.1,3 Acute phenomena, before the inflammatory response and endothelial dysfunction, are leukocyte-endothelial interactions including the expression of cell adhesion molecules,4 and release of chemokines,5,6 cytokines,7 and growth factors.8 The cell adhesion molecule–mediated process of leukocyte recruitment often results in endothelial cell dysfunction, manifested as impaired endothelium-dependent vasorelaxation in arterioles, excess fluid filtration in capillaries, and enhanced protein extravasation in venules.9,11

Hence, the condition of the endothelium and its release or deficit of vasodilating agents, such as NO, have been implicated in a variety of vascular disorders such as ischemia/reperfusion injury,12 vasculitis in acute allograft dysfunction,10 as well as hypertension,13 angiogenesis,14 and arteriosclerosis3 in chronic allograft dysfunction. This review discusses these aspects for the transplanted kidney as observed in the subsequent phases of ischemia/reperfusion, acute rejection, and chronic vasculopathy.

NO SYNTHASE IN THE TRANSPLANTED KIDNEY

Role of Endothelial NO Synthase

Contribution of the different NO synthase (NOS) isoforms can be distinguished either by selective pharmacologic inhibition or by knockout models. The protective properties of NO derived from constitutive NOS are well established in renal transplantation.12,15-19 Inhibition of NO production by all NOS isoforms, with the L-arginine analogues N(G)-nitro-L-arginine methyl ester and L-NNA, decreased renal allograft survival15,20 either by aggravation of the allo-immune response or by graft ischemia. Aortic allografts deficient in endothelial NOS (eNOS) were associated histologically with marked graft allo-arteriosclerosis, compared with grafts from inducible NOS (iNOS)-deficient mice.21 Functionally, genetic deficiency of eNOS expression was correlated with hypertension in human recipients.22 Thus, NO production by NOS is essential for maintaining graft function.

Role of iNOS

The role of iNOS in the kidney graft is both advantageous and disadvantageous.23 Several in vitro and in vivo investigations have shown that selective inhibition of NO production by iNOS could prevent NO-mediated renal transplant injury. Tubules isolated from iNOS knockout mice, or treated with anti-sense iNOS, were resistant to hypoxic and ischemia/reperfusion injury, in contrast to tubules from eNOS knockout mice.24,25

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Selective iNOS blockade by iminoethyl-lysine and butylhexahydro-azepin-imine protected the transplanted kidney from tubulointerstitial macrophage infiltration and injury. The deleterious effect of iNOS also has been seen in acute rejection of lung and heart. However, long-term iNOS inhibition, as well as targeted deletion of the iNOS gene in heart mouse recipients, was shown to correlate with graft allo-arteriosclerosis. Moreover, transduction with iNOS by using an adenoviral vector completely suppressed the development of myointimal hyperplasia in chronic cardiac and aorta allograft rejection. Interestingly, Minamino and Pinsky showed that tracheal transplants into iNOS-deficient recipients selectively showed reduced intima proliferation leading to graft occlusion. In contrast, allografts donated from iNOS(-/-) knock-out mice transplanted into WT allograft recipients were not protected from rejection, suggesting that recipient-derived iNOS expressed by graft-infiltrating leukocytes modulated and promoted rejection.

NO Availability: Production Versus Degradation

NO availability can be reduced by a decrease in NO production and/or increase in NO degradation. Less NO is produced when eNOS activity is reduced, either owing to a deficiency of the NOS substrate L-arginine or the NOS cofactor tetrahydrobiopterin (BH₄). NOS may be depleted from L-arginine owing to competitive inhibition by endogenous asymmetric and symmetric dimethylarginine, which has been found to be increased in chronic renal failure. BH₄ depletion can occur on ischemia/reperfusion owing to degradation by oxygen radicals.

However, the predominant cause of impaired NO bioavailability might be increased degradation of NO by superoxide (O₂⁻), directly or indirectly by inactivating BH₄, rather than impaired formation of NO. In the context of transplantation, this pathologic imbalance between NO and O₂⁻ is critically involved in ischemia/reperfusion-associated endothelial injury and leukocyte recruitment, providing a key component for rejection. Thus, we postulate that NO-mediated effects, either beneficial or detrimental, are dependent on the relative availability of NO versus vasoconstrictors. By definition, NO availability depends on net NO promoting versus NO degrading conditions in the microenvironment, as well as the site of production, hence, the microenvironment itself.

Collectively these findings lead to 3 important conclusions about NOS in transplant rejection:

1. NO produced by eNOS is protecting the allograft: on unselective NOS inhibition, the pro-inflammatory and vasoconstriction effects by inhibiting the eNOS isoform probably offset the beneficial effect of inhibiting iNOS, which has been associated with macrophage cytotoxicity;
2. NO produced by iNOS is an intriguing modulator of vascular rejection, depending on temporal and spatial patterns. (a) The early detrimental features of iNOS oppose the late protective potential of iNOS. The latter acts mainly by suppressing inflammatory cell recruitment and neointimal smooth muscle cell accumulation. (b) Whether iNOS acts as a beneficial NO-producing enzyme depends on sufficient cofactor availability. Detrimental effects of iNOS are related to peroxynitrite formation on insufficient cofactor and/or antioxidant capacity in the microenvironment;
3. Recipient-derived iNOS, expressed by graft-infiltrating leukocytes, exerts the dominant influence on rejection outcome rather than the potentially beneficial donor-derived iNOS, expressed by graft resident parenchymal cells.

ISCHEMIA/REPERFUSION
Oxidative Stress: Free Radical Formation

Ischemia and subsequent reperfusion are inevitable events in organ explantation and implantation. However, ischemia/reperfusion injury in organ transplantation is a major cause of delayed graft function. Ischemia, and in particular reoxygenation during reperfusion, disturb several metabolic systems, thereby inducing massive generation of reactive oxygen-derived species (ROS). Free radical production secondary to ischemia (ie, reductive stress) and reperfusion (ie, oxidative stress) primarily is a direct consequence of adenosine triphosphate depletion. Adenosine triphosphate degradation into adenosine diphosphate, adenosine monophosphate, adenosine, and, ultimately, hypoxanthine, creates a substrate that will be oxidized by xanthine oxidase on reoxygenation, leading to the formation of superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂). When O₂⁻ reacts
with H₂O₂, catalyzed by free iron, which has been accumulating during the preceding period of ischemia, hydroxyl radical (OH⁻/H₂O₂) is formed. This enhanced generation of ROS will lead to destruction of biomolecules in membranes (lipid peroxidation), in enzymes (protein oxidation), and DNA (strand breaks and cross-links to other molecules) in endothelium, and underlying parenchymal tissue in the reperfused kidney. However, recently we found that intrarenal infusion of O₂/H₂O₂ by hypoxanthine plus xanthine oxidase causes a marked increase in fractional sodium excretion without a decrease in glomerular filtration rate, mimicking the natriuresis often seen after ischemia/reperfusion. Remarkably, this effect was fully reversible and occurred in the absence of glucosuria or proteinuria. Thus, initially O₂/H₂O₂ has functional effects on sodium transporters before the development of structural changes.

Superoxide Production by NOS: Uncoupling Concept

With low L-arginine substrate or low BH₄ cofactor, degradation of NO by oxygen-derived free radicals was even more pronounced. In fact, in conditions of insufficient L-arginine or BH₄, NOS itself may produce O₂⁻ (instead of NO), a process called NOS uncoupling, as oxidation of the reduced form of nicotinamide-adenine dinucleotide phosphate at the reductase domain and subsequent electron shift are uncoupled from NO synthesis from L-arginine at the oxygenase domain. Sensitivity of NOS isoform dimer stability to L-arginine and BH₄ deficiency in vitro varies markedly, showing that eNOS association depended most on L-arginine, whereas iNOS association depended more on BH₄ binding. Substantial amounts of O₂⁻ are generated by uncoupled eNOS, as we showed in patients with hypercholesterolemia. Uncoupled iNOS recently has been shown to be a peroxynitrite-generating enzyme in vitro and in vivo conditions.

Although (i)NOS uncoupling in the setting of transplantation remains to be investigated further, blockade of BH₄ synthesis (by inhibiting guanosine triphosphate cyclohydrolase) resulted in increased O₂⁻ production with a reciprocal reduction of NO production by eNOS in ischemia/reperfusion injury in coronary arteries. In the transplanted rat kidney, BH₄ deficiency appears to be the underlying condition of iNOS uncoupling because in vivo sepiapterin (ie, BH₄ precursor) injection of the recipient decreased renal O₂⁻ release while enhancing basal NO production (Fig 1). Moreover, this was associated with a reduction of the primary inflammatory response to the graft. Consistently, activated mononuclear infiltrate, pro-
ducing high levels of both $O_2^-$ and NO, were colocalized with 3-nitrotyrosine in early rat and human kidney graft rejection.\textsuperscript{52}

**Antioxidant Capacity of the Renal Transplant**

Under normal conditions, free radicals are scavenged by endogenous antioxidants such as catalase, glutathione peroxidase, and superoxide dismutase.\textsuperscript{40} or exogenous antioxidants such as vitamins C and E.\textsuperscript{53} However, antioxidant capacity of renal tissue was decreased significantly after transplantation,\textsuperscript{54} with lower enzymatic activity of catalase, glutathione peroxidase, and superoxide dismutase.\textsuperscript{55} MacMillan-Crow et al\textsuperscript{56} showed that manganese superoxide dismutase was tyrosine nitrotrated and inactivated during human kidney allograft rejection, leading to increased levels of $O_2^-$ and concomitant increases in peroxynitrite ($ONOO^-$). Interestingly $ONOO^-$, generated from $O_2^-$ and NO in a diffusion-limited reaction with a rate constant of $>6.7 \times 10^9$ mol/L·s\textsuperscript{-1},\textsuperscript{40} would be the most potent biological oxidant to inactivate enzymatic activity of manganese superoxide dismutase as well as to destroy proteins, DNA, and lipids on ischemia/reperfusion. Indeed, intravenous administration of superoxide dismutase before reperfusion increased graft survival.\textsuperscript{56} Moreover, N-acetylcysteine (a potent antioxidant) and phosphoramidon (an endothelin-converting enzyme inhibitor) synergistically attenuated renal ischemia/reperfusion injury with the NO donor nitroprusside by protecting cells against free radical damage.\textsuperscript{55} Thus, ROS may underlie ischemia/reperfusion injury by causing oxidative degradation of NO.

**Site of Oxidative Stress**

In our model of acute renal allograft rejection, selective iNOS inhibition diminished tubulointerstitial injury and nitrotyrosine staining in tubular epithelium and infiltrating cells despite a minor decrease of vascular and glomerular injury. However, in the same transplant model, chronic inhibition of all NOS isoforms increased scores for vascular injury much more than for parenchymal lesions.\textsuperscript{15} The lesions in both compartments, however, were accompanied by severe T-cell and monocyte/macrophage infiltration. Because macrophages account for one half of the infiltrating leukocytes, and macrophage activation markers appeared to be indicative of the severity of (sub)clinical renal allograft rejection,\textsuperscript{57} the invading macrophages probably are a primary site of NADP(H)-dependent oxidative stress in the transplant. In addition, infiltrating monocytes and iNOS expression have been found to be colocalized in the transplanted kidney,\textsuperscript{58} indicating macrophages as the primary site of iNOS production in this model. Thus, activated macrophages produce both NO and $O_2^-$ radicals via activation of iNOS and NADP(H)oxidase, accounting for macrophage cytotoxicity.\textsuperscript{39}

In chronic NOS deficiency, renal cortical $O_2^-$ activity was increased markedly after 3 weeks and associated with renal injury and increased blood pressure. ROS formation was attenuated by the $O_2^-$ scavenger vitamin E.\textsuperscript{53} Application of antioxidants cannot identify the primary source or site of oxidative stress. Although uncoupling of eNOS implies that the vasculature is the source of free radical formation, we recently showed that in chronic NOS inhibition in the kidney the extravascular compartment was the responsible site. By means of a novel lipophylic ROS-sensitive probe we could link $O_2^-$-mediated lipid peroxidation to the tubular epithelium.\textsuperscript{60} Moreover, attenuation of lipid peroxidation to control levels by use of the selective NADPH-antagonist apocynin, implied that in the tubules NADPH oxidase is the source of $O_2^-$. This approach has not yet been applied to models of acute or chronic allograft rejection. Thus, it is unknown to what extent the tubular compartment is contributing the oxidative stress responses in the transplanted kidney.

**ACUTE REJECTION**

Acute rejection can occur in the first days to months posttransplantation and is cell mediated.\textsuperscript{61} Recruitment, adhesion, and extravasation of leukocytes into tissue are critical for normal healthy immune surveillance, as well as inflammatory responses in ischemia/reperfusion injury, vasculitis, and alloimmune responses to the graft. Endothelial cells actively recruit inflammatory cells by producing cytokines and cell adhesion molecules that assist transendothelial migration into the parenchymal compartment. In addition, early endothelial injury will facilitate random as well as antigen-driven inflammation and rejection by increasing graft immunogenicity,\textsuperscript{62} as depicted in Figure 2A. We postulate a central role for NO through nuclear factor κ B (NF-κB) (Fig 2B).
NF-$\kappa$B and NOS

Posttransplantation endothelial injury elicits an inflammatory response by neutrophils, macrophages, platelets, as well as allo-activated T cells. Recruitment of activated T cells and effector cells into the renal allograft has been shown to involve locally expressed chemokines$^{6,63}$ and leukocyte-endothelial adhesion molecules.$^4$ Peritubular capillary vascular cell adhesion molecule-1 is reported to be associated specifically with chronic rejection.$^7,64$ NF-$\kappa$B appears to be the key upstream component of leukocyte recruitment because it is activated by oxygen-derived free radicals and pro-inflammatory cytokines such as tumor necrosis factor-$\alpha$ and interleukin-1. In turn, NF-$\kappa$B facilitates inflammation by transcriptional activation of iNOS, various cytokines (interferon-$\gamma$, tumor necrosis factor-$\alpha$, interleukin-2, and interleukin-6), adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selec- tin), and chemokines monocyte chemotactic protein-1 [MCP-1], macrophage inflammatory protein [MIP]-1$\alpha$ in endothelial and (tubular) epithelial cells.$^65$ The fact that NF-$\kappa$B enhances immunogenicity by up-regulating major histocompatibility complex (MHC)-II expression, reveals NF-$\kappa$B as an important effector mechanism in allograft rejection. Importantly, NO itself is involved in a negative feedback loop inhibiting NF-$\kappa$B.$^66$ This implies that NO could be used to modulate rejection (Fig 2B).

Immunogenicity and NOS

Persistent host alloresponsiveness is reported in kidney allografts despite adequate maintenance of immune suppression. First, the recipient-derived leukocytes create a continuous immunologic stimulus leading to destruction of donor endothelial cells. As such, the donor-derived dendritic cells (ie, passenger leukocytes) are critical for the direct T-cell allorecognition response to the graft endothelial and epithelial cells. Second, in particular, the antigen-specific recall responses by donor alloantigen presentation to self-MHC restricted T cells...
(indirect pathway) are critical for the effector mechanism of rejection. In the context of antigen-specific leukocyte recruitment, it is very interesting that iNOS activation in the recipient is reported to be essential for indirect platelet antigen processing.

Endothelial Permeability, Vascular Endothelial Growth Factor, and NOS

Because mononuclear cells infiltrate the acutely rejecting kidney, and within the kidney foster oxidative stress and a cytokine-enriched milieu, they themselves are implicated in changed permeability of the endothelium. Alteration in the endothelial junctions can be induced by leukocyte-derived thrombin, bradykinin, histamine, vascular endothelial growth factor (VEGF), and inflammatory cytokines. One of the key players that regulate acute changes in endothelial permeability is NO because endothelial cells that were pretreated with NOS inhibitors lose their ability to respond to VEGF. The pro-angiogenesis factor VEGF is produced by endothelial cells, vascular smooth muscle cells, as well as macrophages and T cells. VEGF is activated by various stimuli such as hypoxia, some cytokines, and CD40 (on endothelial cells and monocytes) with CD40 ligand (on activated T cells) binding. Moreover, NO generated by iNOS enhances synthesis of VEGF in vascular smooth muscle cells and macrophages. Recent evidence in bovine retinal microvascular endothelial cells and in umbilical vein endothelial cells revealed that VEGF is critically involved upstream of NF-κB–induced pro-inflammatory genes. VEGF stimulated activated protein-1 (AP-1) and NF-κB activity, respectively, in a concentration- and time-dependent manner.

Thus, on endothelial cell activation a cellular stress response is triggered with up-regulation of VEGF and activation of endothelial NF-κB. The subsequent cytokine-adhesion molecule cascade promotes an initial inflammatory response with infiltration of leukocytes, activation of macrophages, hence, up-regulation of iNOS, and production of diverse pro-inflammatory cytokines. Because NF-κB is involved in the expression of priming cytokines, MHC antigens, as well as iNOS, it may provide positive feedback in immune- and injury-mediated inflammation.

CHRONIC TRANSPLANT VASCULOPATHY

Chronic Inflammation

Graft failure develops over time in most vascularized allografts. The leading risk for chronic allograft nephropathy are acute rejection episodes and donor age. Chronic rejection occurs after months or years and may be injury-mediated rather than being solely driven by a continuing immunologic process. This slowly progressing allograft nephropathy is characterized by vascular obliteration owing to proliferation and scarring of intima and media in the renal vessels, and membrane multilayering in the peritubular capillaries. The interstitium also shows gradual fibrosis and generation of extracellular matrix. Tubules develop atrophic features. iNOS messenger RNA and protein were found in resident vascular smooth muscle and mesangial cells, as well as in invading macrophages and lymphocytes in patients with chronic allograft nephropathy. Frequency of acute rejection episodes, in particular vascular rejection, is a major risk linking acute and chronic graft failure. Inflammation as the driving process of atherosclerosis in general has been well accepted. Chronic inflammatory processes also may be the mechanism of function in the development of graft arteriosclerosis and late allograft dysfunction. Mediators of inflammation, including activated macrophages and lymphocytes, cytokines, chemokines, and growth factors, can be found at different stages of progressive chronic rejection.

Angiogenesis

Angiogenesis, the formation of new blood vessels from preexisting ones, is characteristic for an ongoing healing inflammatory process. This angiogenic response could be involved in chronic endothelial activation leading to arteriosclerosis-like chronic allograft nephropathy (Fig 2A and 2B). Shahbazi et al reported that genotypes encoding higher VEGF production were strongly associated with acute renal allograft rejection. Grone et al reported expression of VEGF in human recipients with renal vascular disease and chronic renal allograft rejection. In particular, macrophages are thought to play an important role in angiogenesis in the chronically rejecting allograft. Pronounced VEGF expression colocalized with monocyte/macrophage infiltration into the parenchyma of human renal allografts with evidence of interstitial fibro-
sis. This concurs with observations in rodent models of cardiac allotransplantation; sprouting of microvessels was increased markedly in the expanded intima of the donor vessels compared with the recipient’s own arteries. Also, in patients, neovascularization was colocalized with VEGF-producing inflammatory cells that had infiltrated the outer layers of the intima in cardiac allograft arteriosclerosis. Moreover, angiogenesis in the arteriosclerotic lesions provides a site of entry for leukocytes, thereby sustaining the ongoing inflammatory process.

**THERAPEUTIC INTERVENTIONS BASED ON THE NO SYSTEM**

Early inflammatory events may be an effective target for therapeutic intervention with long-term goals. One may postulate that blocking the initial inflammatory responses associated with ischemia/reperfusion injury and acute rejection may be of significant clinical importance to maintain graft morphology and function over time. The NO system plays a key role in kidney allograft rejection. Various factors that directly or indirectly are involved in the NO system could affect the pathophysiologic response to kidney transplantation/kidney transplantation outcome. We showed recovery from initial inflammatory responses and inhibition of acute graft rejection by supplementing the NOS cofactor BH₄ or the NOS substrate L-arginine, and by inhibiting the transcription factor NF-κB or iNOS, respectively. Hence, diminution of inflammatory prorejection conditions can be achieved by modulating the NO system in experimental renal transplantation. However, long-term effects of BH₄ supplementation or iNOS inhibition on renal allograft function have not yet been reported.

**L-Arginine-NO Pathway in Renal Transplantation**

In experimental acute renal failure, decreased renal plasma flow and glomerular filtration rate levels were associated with decreased tissue L-arginine levels, eNOS III expression, NO formation, and nitrite excretion. Therefore, L-arginine, the substrate of NO, was suggested to be beneficial in acute renal failure as well as hypertension, ureteral obstructive nephropathy, and cyclosporin A nephrotoxicity. The outcome of L-arginine supplementation in kidney transplant recipients was not uniform in experimental and human studies. Several studies focusing on reduced renal perfusion and filtration or on increased blood pressure levels reported beneficial hemodynamic effects of L-arginine supplementation immediately after kidney transplantation. L-arginine infusion also increased renal vasodilatation and natriuresis in renal transplant patients under long-term cyclosporine treatment, indicating that L-arginine counteracts the antinatriuretic effect of cyclosporin. However, Zhang et al reported that late L-arginine treatment either by infusion or long-term oral supplements failed to reverse cyclosporine-induced renal vasconstriction in patients with established chronic graft dysfunction. Similar negative findings were reported in heart transplant patients. Preliminary results in a kidney transplant model showed that long-term L-arginine supplementation, starting before the onset of chronic transplant failure, protected the graft from developing focal glomerulosclerosis and proteinuria.

**CONCLUSION**

Multiple strategies that can modulate the NO pathways need to be evaluated in terms of their efficacy in reducing the initial inflammatory response to injury and hence long-term graft survival. Balancing the cytoprotective and cyto-oxidative actions of NO will remain a major challenge in the coming years.

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