

Anti-inflammatory effects of tetrahydrobiopterin on early rejection in renal allografts: modulation of inducible nitric oxide synthase¹

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SPECIFIC AIM

To assess whether therapy with tetrahydrobiopterin (BH₄) diminishes oxygen radical stress and improves nitric oxide (NO) levels after allogenic kidney transplantation in a rat model. To relate such changes to modulation of iNOS function.

PRINCIPAL FINDINGS

1. Effect of sepiapterin on intra graft superoxide production in renal allografts

Oxidative stress is thought to play a major role in endothelial activation in transplanted organs. It has been suggested that NO synthase (NOS) enzyme can act as a source of superoxide (O₂⁻) production in vivo. To assess the contribution of NOS-mediated O₂⁻ production associated with early rejection of renal grafts, we performed syngenic (LEW to LEW) and allogenic (BN to LEW) kidney transplantations in rats and measured intragraft O₂⁻ production using lucigenin-enhanced chemiluminescence. As a result of ischemia-reperfusion injury, 24 h after syngenic kidney transplantation O₂⁻ production was modestly elevated in the isografts compared with O₂⁻ production in nontransplanted donor kidneys. As expected, elevation of O₂⁻ production in the allografts was much more pronounced, most likely due to the additional inflammatory reactions associated with allogenic transplantation. In both transplantation groups, we studied the effect of sepiapterin administration as a stable precursor, which is converted to BH₄ in vivo. Sepiapterin could not reduce the small increase in O₂⁻ production seen in the untreated isografts. After sepiapterin treatment, the threefold increase of O₂⁻ production in the allograft was decreased by ~ 50% (Fig. 1).

2. Effect of sepiapterin on intragraft NO release

Under conditions of insufficient availability of the NOS cofactor BH₄, the enzyme can directly reduce oxygen

and produce O₂⁻ instead of NO. If reduction of O₂⁻ levels in the allograft by sepiapterin were due to improved NOS function, supplementation of sepiapterin in vivo should lead to a concomitant increase in the production of NO. Therefore, we measured NO levels in the allografts using the NO spin trap Fe-(DETC)₂ and EPR spectroscopy. Treatment with sepiapterin improves NO bioavailability in kidney tissue twofold compared with that of untreated animals (Fig. 1).

3. Pattern of NOS protein expression

Since the effect of the NOS cofactor tetrahydrobiopterin was specific for the allografts, we evaluated the pattern of NOS expression in the allograft vs. that in isografts. Using immunohistochemical analysis of sections of 24 h renal transplants, we found that allograft transplantation was associated with iNOS expression. iNOS protein was focally expressed between the tubular structures in the inner and outer cortex in association with inflammatory cells. The constitutively expressed iNOS in renal tubuli had a similar intensity relative to the donor kidney.

4. Effect of BH₄ on iNOS function

To support the hypothesis that the elevation of iNOS expression in renal allografts could contribute to elevated O₂⁻ production, we performed in vitro experiments on recombinant iNOS using spin trap DEPMPO and EPR spectroscopy to demonstrate that iNOS can produce O₂⁻ in the absence of the cofactor BH₄.

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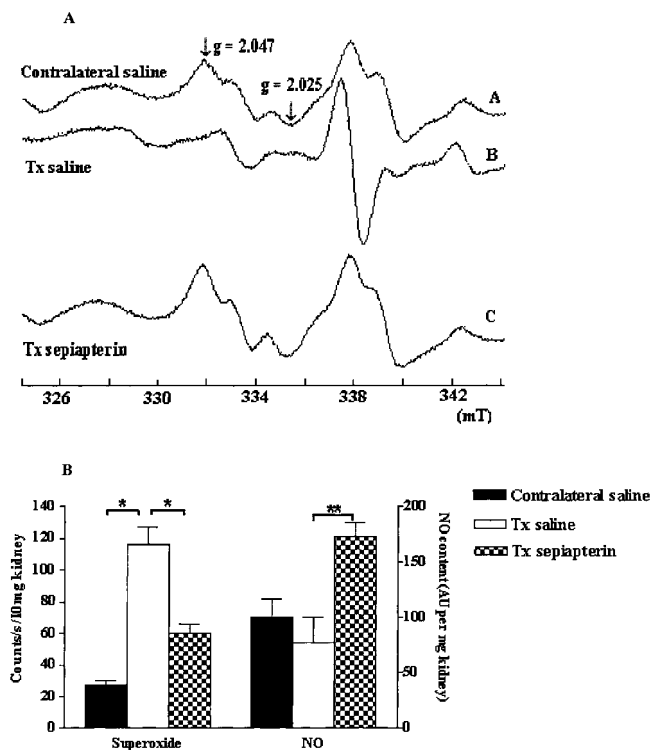


Figure 1. NO and superoxide measurement in the kidney. *A*) NO determination in a rat kidney: EPR signals obtained from rat kidney after allogeneic transplantation. *A*: Contralateral kidney of a rat injected with saline. *B*: Transplanted kidney of a rat injected with saline (*C*) transplanted kidney of a rat treated with sepiapterin. $g = g$ factor. *B*) Left: Renal cortical O_2^- levels in control kidney (donor) (filled bar); vs. saline (open bar) or sepiapterin (hatched bar) treated kidney grafts in the acute (24 h) phase after allogeneic transplantation (both groups $n=6$). O_2^- levels assessed by lucigenin-enhanced chemiluminescence measurements (100 μ M, which was shown not to generate auto-oxidation in these tissues) is expressed as counts/($s^{-1} \cdot 10$ mg dry weight $^{-1}$). Data are mean \pm SE; 1-way ANOVA; * $P < 0.05$ vs. saline treatment in transplanted kidney. Right: NO contents in transplanted and control rat kidney. Data shown are the means \pm SE, $n = 5$ for each group. NO content expressed as arbitrary units/g wet weight kidney tissue and as percentage of the mean value in the contralateral kidney of the saline-treated rats donor (filled bars), transplanted kidneys of rats treated with saline (open bars), and transplanted kidney of rats treated with sepiapterin (hatched bars). ** $P < 0.01$ vs. saline treatment in donor kidney.

Addition of NADPH (2.5 mM) to a solution containing BH_4 -free iNOS (1.5 μ M), calmodulin (10 μ g/ml), Ca^{2+} (5 mM), L-arginine (1 mM), and DEPMPO (50 mM) resulted in formation of the paramagnetic adduct DEPMPO-OOH. Addition of BH_4 reduced the superoxide signal by $\sim 70\%$ but did not abolish it. We conclude that iNOS was producing O_2^- even under the most optimal conditions. Within the NOS enzyme, the source of O_2^- can be localized by addition of site selective inhibitors. Addition of the reductase domain inhibitor diphenyliodoniumchloride (200 μ M) almost completely abolishes the O_2^- signal. In contrast, the heme domain inhibitor cyanide (10 mM), which inhibits electron flow downstream from the reductase do-

main, reduces the intensity of the O_2^- signal by $\sim 70\%$. These observations show that heme and reductase domain act as potential sources of O_2^- production. As BH_4 acts only on the heme domain, this explains why BH_4 cannot totally prevent superoxide generation by iNOS. The question then arises whether iNOS can still release NO when O_2^- is produced at the same time. Theoretically, this may lead to $ONOO^-$ production by the enzyme. In subsequent experiments using the formation of paramagnetic MNIC-MGD complexes to detect NO release from the iNOS enzyme, we found that only in the presence of adequate antioxidant capacity and of BH_4 would the iNOS enzyme release NO. If one of these two components is lacking, no NO is formed (Fig. 2). These data suggest that in an environment of sufficient antioxidant capacity, BH_4 supplementation may indeed reduce O_2^- and increase NO release by iNOS, as observed in our allografts.

5. Effect of sepiapterin on infiltration of inflammatory cells

We previously found that selective iNOS blockade in this transplantation model reduces inflammation. We therefore evaluated whether modulation of iNOS by the BH_4 precursor sepiapterin and its associated changes in NO/ O_2^- production result in less inflammation of the allograft. Infiltration pattern and amount of monocytes in the renal isograft and allograft were evaluated for the entire kidney cross section using an antibody directed against the monocyte/macrophage marker ED-1. In allografts (but not in isografts), sepiapterin treatment significantly reduced monocyte/macrophage influx compared with saline ($P < 0.05$). The mean number of monocytes/macrophages per vessel was decreased to levels seen in the saline-treated isografts.

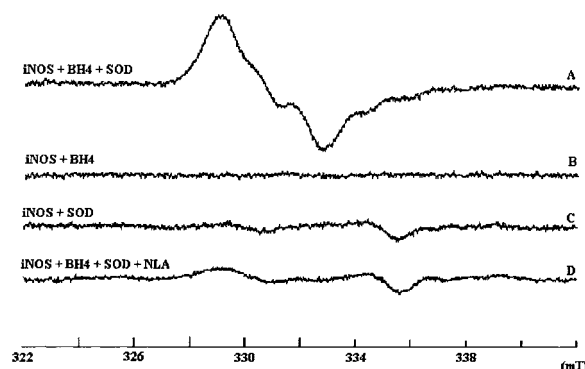


Figure 2. NO production by recombinant iNOS. Incubation of iNOS 0.75 μ M at 37°C in 50 mM Tris buffer pH 7.4 containing calmodulin 10 μ g/ml, $CaCl_2$ 5 mM, L-arginine 2 mM, BSA 25 μ g/ml, NADPH 2.5 mM, and with NO traps, iron-MGD complexes (0.6 mM). Spectra were recorded at 77K. All experiments were performed in triplicate. Typical spectra are shown in the *A*) presence of BH_4 1.5 μ M, and SOD 200 U/ml; *B*) absence of SOD; *C*) absence of BH_4 and the presence of SOD, *D*) with addition of NLA 3 mM in the presence of BH_4 and SOD.

CONCLUSIONS AND SIGNIFICANCE

The present study demonstrates that 24 h after ischemia-reperfusion, the renal production of O_2^- was increased in syngenic and allogenic renal transplants. However in the allogenic transplants O_2^- production was higher and associated with a higher number of infiltrating monocytes/macrophages in the vessel wall and expression of the inducible form of NOS. Treatment with sepiapterin, a substrate for BH_4 , reduced O_2^- production and increased NO production in allografts but not in the isografts. This improvement of redox state of the allograft was associated with a reduction of monocyte influx in the vessel wall. Since BH_4 is an essential cofactor of NOS and the transplant model is characterized by activation of iNOS, we investigated the possibility that BH_4 could have modified the production of NO and O_2^- by iNOS. Using the O_2^- spin trap DEPMPO and the NO spin trap Fe-MGD complex, we could demonstrate in recombinant BH_4 -free iNOS that cofactor-deficient iNOS is not an NO-producing enzyme but a O_2^- -producing enzyme. O_2^- production is derived from the heme as well as the reductase domain. Although BH_4 can reverse O_2^- production derived from the heme domain, it does not affect O_2^- production by the reductase domain. Only if SOD is added and O_2^- production by the reductase domain is inhibited, can the enzyme produce NO. Our renal transplant data suggest that under such conditions the iNOS enzyme may have anti-inflammatory properties.

The NOS enzymes requires optimal concentration of the cofactor BH_4 (and the substrate L-arginine) for their function. That the BH_4 precursor sepiapterin reduced O_2^- production as well as increased NO production in the renal allograft strongly suggests that there was a functional deficiency of BH_4 in the allograft.

The observation that sepiapterin had no effect on O_2^- formation or monocyte influx in the isografts is probably related to the fact that in this model of ischemia-reperfusion, O_2^- production is secondary to ATP depletion, hypoxanthine formation, and subsequent O_2^- production by xanthine oxidase. Any possible effect on eNOS function that may have been expected secondary to oxidative BH_4 degradation was probably overridden by this classical mechanism of ischemia-reperfusion-associated oxyradical formation. This observation underscores the specificity of our observations with sepiapterin in the allografts and points to a modulatory effect of BH_4 on iNOS function.

We also show that BH_4 induces a shift from O_2^-

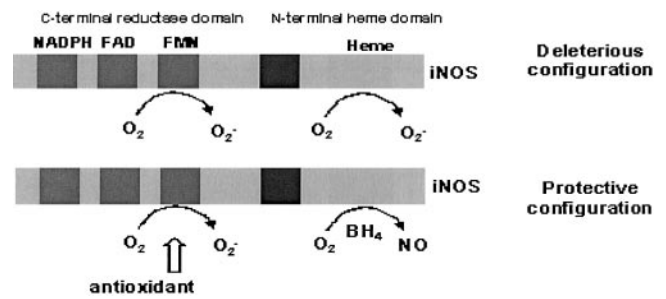


Figure 3. Schematic diagram. Working hypothesis: iNOS can be a superoxide as well as an NO-producing enzyme. iNOS mediated NO release depends on sufficient BH_4 availability and antioxidant capacity of the cellular microenvironment of the enzyme. If cofactor and antioxidant defense mechanisms are both optimal, iNOS enzyme may exert protective effects as demonstrated in the present study.

production by BH_4 -free iNOS to simultaneous NO and O_2^- production by BH_4 -repleted iNOS. When the antioxidant status of the microenvironment is insufficient to prevent the very rapid reaction between O_2^- and NO, rate constant 6.7×10^9 - 1.9×10^{10} (M.s) $^{-1}$, peroxynitrite formation will occur. A direct illustration is provided by NO trapping using Fe-MGD complexes. In this assay, paramagnetic MNIC-MGD is obtained only in presence of SOD, showing that the antioxidant status of the environment (i.e., SOD) determines whether enzymatic NO is sufficiently long-lived to diffuse away. These findings may help explain why inhibition of iNOS retards the development of vascular injury in models characterized by oxyradical stress (e.g., atherosclerosis and transplantation) whereas in models with 'intact' antioxidant capacity stimulation of iNOS mediates vasculoprotective effects.

The importance of the endothelial isoform of NO synthase has been well established. Endothelium-derived NO has been shown to be essential for vascular homeostasis and modulation of eNOS has thus become a target in prevention of cardiovascular disease. The role of iNOS in vascular biology, however, is less clear. Classically, iNOS has been regarded as an enzyme that produces nanomolar amounts of the NO radical, thereby causing cellular damage. The current study shows that iNOS can be a O_2^- , peroxynitrite as well as an NO-producing enzyme. The biological effects of iNOS not only depend on which radical species is released by the enzyme, but also on the antioxidant capacity of the cellular microenvironment of the enzyme (**Fig. 3**). [F]