Detection of Nitric Oxide Reduction during Ischaemia-Reperfusion by EPR Spectroscopy

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Abstract

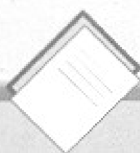
Introduction: Acute renal failure is a common consequence of sepsis due to concurrent renal ischaemia. The role of nitric oxide (NO) in endotoxaemia and in ischaemic injury in the kidney is not well defined.

Material and Methods: In this study we have used an animal model of sepsis induced by injection of bacterial lipopolysaccharide (LPS) in the rat and measured renal nitric oxide by X-band electron paramagnetic resonance (EPR) spectroscopy using the spin trap Fe^{2+}-N-methyl-D- glucamine dithiocarbamate [Fe(MGD)2] given by intravenous injection 6 minutes before sacrifice.

Results: The characteristic EPR spectrum of [Fe(NO)(MGD)2] was observed in kidneys of rats treated with LPS for 5h. Rat kidneys subjected to 20 min ischaemia and 5 min reperfusion had lower concentrations of [Fe(NO)(MGD)2] (1.0 ± 0.6 (M) compared to the contralateral nonischaemic kidneys (1.5 ± 0.9 (M, P<0.05).

Conclusion: This study shows reduced levels of NO after renal ischaemia in vivo.

Key Words: Nitric oxide, Electron paramagnetic resonance, Kidney, Ischaemia-reperfusion



 Arrived at 8/Dec/2001, Accepted at 12/Jan/2002

Introduction

Nitric oxide (NO) is an important regulator of cellular function. NO plays a role in a variety of communications between cells, including regulation of vascular tone, neuronal signal transmission, mediation of tissue injury and inflammation (1). In the kidney, NO exerts effects on renal haemodynamics (2) by a direct effect on renal tubular function or macula densa regulation of glomerular capillary pressure.

Renal ischaemia-reperfusion injury is the most common cause of acute renal failure (ARF). The role of NO in renal ischaemia-reperfusion injury has only recently been studied. Controversy exists regarding the role of NO in ischaemia-reperfusion injury in various organs including kidney where increasing NO production is reported to both improve and delay recovery from ischaemia-reperfusion (3, 4). NO synthesis may protect against or contribute to progression of renal failure. In the early experimental ischaemic or toxic ARF (5), L-arginine-derived NO improved renal function suggesting that endothelial constitutive nitric oxide synthase (cNOS) maintains renal blood flow after ischaemia reperfusion (6) On the other hand, plasma and urine nitrite + nitrate, the end products of NO metabolism, are reported to increase 24 hours after ischaemic ARF (7). NO may mediate rat proximal tubular hypoxia-reoxygenation injury and progression of renal failure (8).

Direct detection of NO in biological systems is difficult, because of the low concentrations generated by normal physiological processes such as vasodilation where the minimum effective concentration is of the order of 5 nM synthesised by cNOS (9). Concentrations of NO generated by inducible nitric oxide synthase (iNOS) are much higher (μM). Endotoxaemic shock after gram-negative bacterial sepsis causes ARF and can be mimicked experimentally by injection of bacterial lipopolysaccharide (LPS). In LPS-treated animals NO levels are increased systemically and the resultant hypotension decreases renal perfusion.

Electron paramagnetic resonance (EPR) spectroscopy in conjunction with spin trapping has been widely used to identify free radical formation. We have used spin-trapping techniques to identify increased hydroxyl and carbon-centred radical formation in renal ischaemia reperfusion injury (10). It has recently been reported that NO can be detected in animal tissues and blood using a spin trap comprised of a metal-chelator complex, [Fe(MGD)2]. Herein we apply EPR spectroscopy, spin concentration measurements and spin trapping by [Fe(MGD)2] to determine the effect of ischaemia-reperfusion on the synthesis of NO in kidneys.

Materials and Methods

Male Sprague Dawley rats weighing 270-400 g were used in all experiments and were allowed free access to standard food and water. Lipopolysaccharide (LPS, from Escherichia coli Serotype 026:B6) was obtained from Sigma Chemical Co. (St. Louis, USA), and Nembutal (sodium pentobarbitone) was from Boehringer Ingelheim Pty Ltd (Artarmon, Australia). All other chemicals used were of analytical grade. N-methyl-D-glucamine dithiocarbamate $(MGD)_2$ was synthesised after the method of Shinobu et al. (11) The $[Fe(MGD)_2]$ complex was prepared by adding MGD (326 mg/kg) and FeSO4 (34 mg/kg) to 0.5 ml H20 immediately prior to intravenous (i.v.) injection.

* *Experimental Protocol*

Rats in control and experimental groups were given an injection of LPS (8 mg/kg, i.p.). After 5 hours, control rats were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.). In the control group, kidneys were removed 5 minutes after i.v. injection of 0.5 ml $[Fe(MGD)_2]$ complex through a femoral vein. The time from LPS injection to kidney removal was exactly 5 hours. In the ischaemia-reperfusion group, the right kidney was made ischaemic for 20 min followed by reperfusion for 5 min. Ischaemia was produced by an atraumatic clamp placed on the right renal artery. At the end of ischaemia, 0.5 ml of $[Fe(MGD)_2]$ was injected intravenously and both kidneys were then collected after 5 min reperfusion as in the control group. After removal, the kidneys were immediately halved and one half freeze-clamped and stored at 70¯C. The other half was placed in 10%

phosphate buffered formalin for subsequent histological examination. For these kidneys, the fixative was changed to 70% ethanol at 24 hours.

* Histology

Sections were prepared from kidneys using routine histological methods. Paraffin sections (3-4 μm) were stained with haematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) for light microscopy (12) Neutrophils were identified morphologically in PAS-stained sections and counted in glomeruli in 20 random fields of renal cortex. Data are expressed as neutrophils per glomerulus.

* EPR Spectroscopy

Sample preparation: The NO generator, S-nitrosoglutathione (1 mM), was used to generate standard EPR spectra of nitric oxide. The sample was prepared by incubation of S nitrosoglutathione (0.6726 mg) in l ml H20 at 37 °C for 10 min in the presence of l ml of $[Fe(MGD)_2]$ complex solution containing 155 mg MGD and 28 mg FeSO4. The mixture was immediately frozen in liquid nitrogen. [Cu(MGD)2] was prepared by dissolving CuSO4.5H20 (3.192 mg) and MGD (70.32mg) separately in water before making up to a total volume 5 ml. 0.125 ml of this solution was added to 4.875 ml of glycerol to give a final concentration of 0.1 mM [Cu (MGD) 2] complex. 2, 2, 6, 6; tetramethylpiperidine- 1- oxyl (TEMPO) (1 mM) used as a standard for spin quantitation measurements was prepared by dissolving 1.56 mg of TEMPO in 10 ml of chloroform. Frozen tissue samples were placed in X-band EPR tubes without thawing.

Instrumentation: EPR spectra of kidneys (frozen tissue) and reference samples [Fe(NO)(MGD)2], [Cu(MGD)2] and TEMPO were measured on a Bruker ESP3OOE X and EPR spectrometer. Calibration of the microwave frequency and the agnetic field were performed with an EIP 845B microwave frequency counter and a ruker ER-035M gaussmeter. A dual rectangular TE104 cavity was used for spin oncentration measurements. The Bruker esp300e software (version 3.02) was employed or data collection.

Spin quantification: Quantification of the observed signals was performed by the comparison method in conjunction with a dual TE104 rectangular cavity and TEMPO as the reference sample. A frozen kidney tissue sample was placed in the back cavity (ca 120K) with TEMPO in the front cavity and the spectra of [Fe (NO) (MGD) 2] and TEMPO measured. The spectra of [Fe (NO) (MGD) 2] contained overlapping resonances from [Cu(MGD)2] and in order to allow quantitation of NO, the spectrum from [Cu (MGD) 2] was first subtracted from each kidney spectrum to remove the overlapping Cu signal. Quantification of the low molecular weight nitrogen based organic free radical was as described above for [Fe (NO) (MGD) 2].

* Statistical analysis

Values are reported as mean±SD Comparisons between groups were performed using the Wilcoxon matched-pairs test.

Results

Following LPS administration (5 hrs), an EPR spectrum of a control kidney (Fig. la), corresponding right kidney had received 20 min ischaemia and 5 min reperfusion), had resonances arising from [Fe (NO) (MGD) 2] (Fig. 1b) and [Cu (MGD) 2] (Fig. 1c).

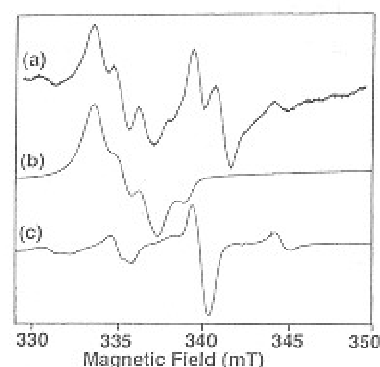


Fig. 1: EPR spectra of control LPS-treated rat kidney (a), [Fe (NO)(MGD)2](b) and [Cu(MGD)2] (c)

Multifrequency (Q-, X- and S- band) computer simulations of the EPR spectra from [Cu(MGD)2] and [Fe (NO) (MGD) 2] are shown in Figure 2 and reveal that the optimum frequencies to separate the spectra of [Cu (MGD) 2] and [Fe(NO)(MGD)2] is Q- band or

higher (Fig. 2c).

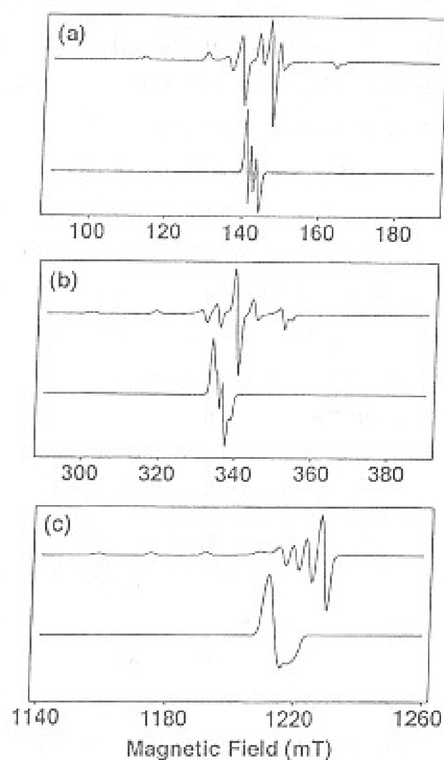


Fig. 2: Computer simulated EPR spectra of [Cu(MGD)2] and [Fe(NO)(MGD)2](a) S-band, (b) X-band and (c) Q-band

Unfortunately the concentrations of [Fe (NO) (MGD) 2] in the kidney samples was insufficient to give a Q-band EPR spectrum at 120K. Consequently, we resorted to the subtraction of the EPR spectrum of [Cu (MGD) 2] from the spectrum of frozen kidney to obtain the spectrum of [Fe (NO) (MGD) 2].

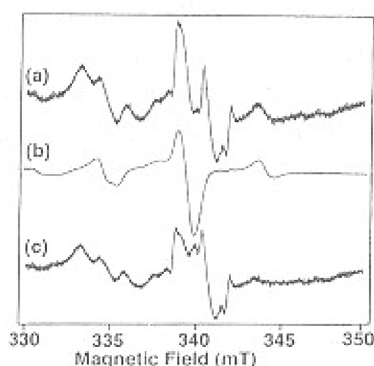


Fig. 3: EPR spectra of the rat kidney before (a) and after (c) subtraction of spectrum of [Cu(MGD)2] (b)

The concentration of [Fe (NO) (MGD) 2] and hence NO was obtained through spin concentration measurement. An example of this process is shown in Figure 3, before (Fig. 3a) and after (Fig. 3c) subtraction of the spectrum of [Cu(MGD)2] (Fig. 3b).

Typical EPR spectra from the left and right kidneys of LPS treated rats are shown in Figures 4a and 4b respectively. The right kidney received 20 min ischaemia and 5 min reperfusion, whilst the left kidney served as a control kidney. The EPR spectrum of the [Fe (NO) (MGD) 2] was clearly observed in Figures 4a and 4b.

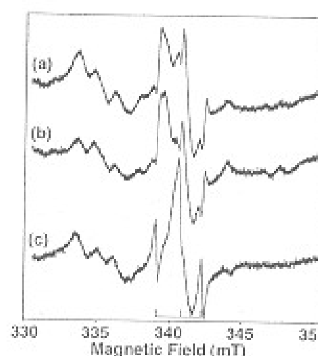


Fig. 4: Comparison of the EPR spectra of control (a), ischaemia-reperfused (b) kidneys and a control kidney after subtraction of [Cu(MGD)2](c)

The renal [Fe (NO) (MGD) 2] and nitrogen based radical concentrations in control kidneys and following 5 min reperfusion after 20 min ischaemia were determined by spin concentration measurements using TEMPO as a reference sample. The results are summarised in Table 1.

Table 1: NO concentration in kidneys of LPS treated rats before and after 20 min ischaemia and 5 min reperfusion. Significance of differences ($p<0.05$) between left and right kidneys in [Fe(NO)(MGD)2] concentration.

Spiecies	LPS treated Rats with unirateral renal (Roght Kidney) ischemia-reperfusion	
	Left Kidney	Right Kidney Ischemia-Reperfusion
[Fe(NO)(MGD)$_2$](μM)	1.5±0.9	1.0±0.6[a]
Unidentified free radical (μM)	0.3±0.07	0.26±0.16
[Cu(MGD)$_2$](μM)	0.8±0.3	1.0±0.7
Neutrophlis per glomerulus	1.0±0.4	1.2±0.2

Five hours after injection of LPS in animals not subjected to renal ischaemia, the concentration of [Fe

(NO) (MGD) 2] was 2.0 ± 1.4 (M (mean±SD) averaging the right and left kidneys. While this was not significantly different from the control, non-ischaemic kidneys in the experimental group (1.5 ± 0.9 (M) or the ischaemia reperfusion group (1.0 ± 0.6 (M), the scatter present in both groups increases the likelihood of a false negative. Comparing right (ischaemia-reperfusion) and left (control) kidneys in the same animal revealed that the [Fe (NO) (MGD) 2] levels were consistently lower following ischaemia reperfusion in all experiments (Fig. 5, $P<0.05$).

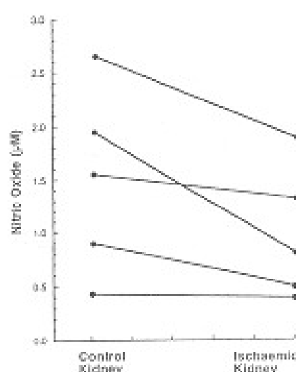


Fig. 5: Concentrations of NO in five control and five ischaemia-reperfused LPS treated rat kidneys. Significance of differences ($p<0.05$) between left and right kidneys

In addition to resonances from [Fe(NO)(MGD)2] and [Cu(MGD)2] spectra (Fig. 4a, b) from control kidneys and kidneys which had undergone ischaemia-reperfusion revealed a triplet (1:1:1) centred at g=2.0093, A (14N)= 15.3x10-4cm-1 (see stick spectra in Fig. 4). A low molecular weight nitrogen based organic free radical is the most likely species giving rise to this triplet. There were no changes in the number of glomerular neutrophils (Table 1).

Discussion

The EPR spectrum of [Fe(NO)(MGD)2] formed on trapping of NO by [Fe(MGD)2] was observed in kidneys of LPS-treated rats (Fig. la). Resonances from [Cu(MGD)2] were found to overlap those from [Fe(NO)(MGD)2]. This has been noted previously in spectra from urine of LPS-treated mice although it was not characterised (13). The origin of the copper is not known, however, rat kidney contains 7.4 mg/kg Cu (metallothionein, etc) (14) and sufficient copper in ceruloplasmin (12-24 pM) and superoxide dismutase (10-17 p.M) to account for the levels of [Cu(MGD)2] observed here (=1 (M). Subtraction of the [Cu(MGD)2] spectra from the spectra of kidneys and subsequent spin quantitation gave concentrations of NO in the pM range (Table 1). This is the concentration range expected for tissues in which iNOS is elevated. Increased renal concentrations of NO have been observed previously using EPR and spin trapping with [Fe(MGD)2] (15, 16) in the mouse and with iron (II) diethyldithiocarbamate in the rat.

Quantitation was performed in the studies of Fujii et al. (16) and Lai et al. (15) who found renal concentrations of NO of 11.5 and 10.9 nmol/g 2 h after subcutaneous injection of [Fe(MGD)2] in LPS-treated mice. The exposure to the spin trap was much longer, 2 h compared to 6 min in the present study, and hencemay reflect chronic cumulative NO production rather than acute concentrations as in our study. Ischaemia- reperfusion decreased renal concentrations of NO (Table 1). This is a novel finding that is in agreement with other studies which indirectly suggest reduced concentrations of NO in the immediate reflow after renal ischaemia (17). This fall in NO may result from decreased production or increased reaction of NO with compounds such as 02. Increased oxygen derivedfree radical production on reperfusion after renal ischaemia has been shown in litrature (10, 18, 19). The resulting production of peroxynitrite may accelerate reperfusion injury. The situation in the kidney may be different from other organs such as heart where increased concentrations of NO have been observed both during ischaemia and after reperfusion (20). There were no differences in neutrophil counts in control and reperfused kidneys.

In summary, EPR and spin trapping in vivo were used in this study to observe an acute fall in NO on reperfusion of the ischaemic rat kidney. This decrease in NO most likely reflects either reduced synthesis or increased degradation of NO in renal cells. The decrease in renal NO in early reperfusion may lead to an imbalance in vasoconstrictive and vasodilatory activity within the kidney resulting in reduced renal

blood flow and tissue perfusion which may lead to tissue injury and acute renal failure. In addition, there may be further tissue injury resulting from the degradation products of NO such as peroxynitrite derived from NO and superoxide, formed after ischaemia-reperfusion.

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