Nitric oxide generation by peripheral blood cells in chronic renal failure

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Accepted: 24 September 2001

ABSTRACT

Nitric oxide (NO), a labile free radical synthesised from L-arginine by the action of nitric oxide synthase (NOS), is said to be implicated in uraemic complications, such as infection and a tendency to bleed. In this study of NO production by peripheral blood cells, an increased level is seen in platelets from uraemic patients (both non-dialysed and haemodialysed) and a decreased level in leucocytes (neutrophils and monocytes). A negative correlation was noted between blood urea level and inducible NO in neutrophils and monocytes in uraemic patients not on dialysis. In contrast, haemodialysis appears to lead to an increase in inducible NO production in neutrophils and monocytes. Plasma NO levels were significantly increased in uraemic patients, compared with normal controls, and hemodialysis led to further increases. Superoxide dismutase (SOD) activity was significantly reduced in platelets, neutrophils and monocytes in the uraemic group. It is concluded that increased NO production by platelets may contribute to the bleeding tendency observed in uraemia, and high urea concentrations may contribute to the regulation of inducible NO production in leucocytes.

KEY WORDS: Kidney failure, chronic.
Nitric oxide.
Nitric-oxide synthase.

Introduction

Nitric Oxide (NO) is a labile free radical that acts as a paracrine and autocrine agonist, and is formed exclusively from L-arginine by the action of the enzyme nitric oxide synthase (NOS). There are at least two NOS subtypes: the calcium-dependent constitutive subtype, commonly expressed in endothelium and platelets (eNOS); and the calcium-independent inducible subtype (iNOS), seen in macrophages, neutrophils and vascular smooth muscle, which is induced by cytokines and endotoxins.[1,2] NO is produced by a variety of mammalian cells and mediates a range of biological actions that include vasodilation, neurotransmission, inhibition of platelet adherence and aggregation to macrophages, and neutrophil-mediated killing of pathogens.[1-3]

Superoxide anions (O_2^-) have been shown to contribute to the instability of NO because its effects are prolonged by the addition of superoxide dismutase (SOD) *in vitro*.[4] The reaction of NO with O_2^-, initially viewed as a route for NO inactivation, yields the potent oxidant peroxynitrite (ONOO) that decays to yield an hydroxyl radical (OH) – considered to be the strongest oxidant in biological systems.[5]

NO has been implicated in bleeding tendency[6] and infection,[7] both of which are well-known complications in uraemic patients, due to its role in platelet inhibition[3] and leucocyte cytotoxicity.[1,2] During haemodialysis, blood contact with a foreign surface initiates and promotes the activation of peripheral blood phagocytes and platelets, and this induces the release of many inflammatory mediators into the extracellular environment,[8] enhancing NO production due to activation of NOS.[9]

The effect of chronic renal failure on NO metabolism is incompletely understood. Both decreased and increased activities of L-arginine and the NO pathway have been suggested.[10-13] However, these studies were carried out either on experimental animals[11,13] or on human subjects,[10] and the patients were either undergoing haemodialysis[12] or on medication.[10] In addition, the results were generalised to all uraemic situations, which may be misleading or confusing.

The present study aims to clarify the situation by investigating different groups of patients with chronic renal failure (CRF), and by studying the haemodialysis group both before and after the session. In addition, it looks at the generation of NO by different blood cells containing the eNOS subtype (platelets) or the iNOS subtype (neutrophils, monocytes) in an attempt to shed some light on the effect of uraemia and/or haemodialysis on the production of NO in the clinical setting.

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Materials and methods

Subjects

Three groups of subjects participated in the present study: 10 patients with end-stage CRF on four-hour maintenance haemodialysis, three times a week (five men, five women; age range 45 to 65 years; serum creatinine 7.16 ± 1.19

Table 1. Superoxide dismutase activities in platelets, neutrophils and monocytes in all groups studied.

SOD activities (mU/mg protein)	Control	Non-dialysed	Pre-dialysis	Post-dialysis
Platelets	40.32±2.86	28.53±3.7*	32.04±3.47*	39.39±2.2†
Neutrophils	71.88±3.38	51.20±1.85*	51.58±2.37*	55±2.65*
Monocytes	59.72±2.01	44.83±2.19*	49.02±1.29*	52.27±1.47*

Data presented as the mean ± SE (n=10)

One unit of SOD activity is defined as the amount of enzyme which inhibits the rate of autoxidation of pyrogallol by 50 %

* Statistically significant difference from control (P<0.05)

† Statistically significant difference from pre-dialysis (P<0.05)

mg/dL); 10 CRF patients scheduled for haemodialysis for the first time were designated the non-dialysed group (six men, four women; age range 40 to 65 years; serum creatinine 11.19 ± 0.43 mg/dL); and 10 healthy volunteers who served as the control group (eight men, two women; age range 30 to 60 years; serum creatinine 0.88 ± 0.082 mg/dL). None of the patients had received nitrates, cyclooxygenase inhibitors, calcium antagonists, angiotensin converting enzyme inhibitors, antiplatelet drugs, or other drugs known to affect immune function. In the haemodialysis group, mean predialysis arterial blood pressure was 102 ± 5.7 mm Hg, and this decreased during the session.

Samples

Venous blood was collected directly into plastic tubes containing 3.5% sodium citrate. In the haemodialysis group, blood samples were drawn immediately prior to dialysis and at the end of the session. Citrated blood samples were centrifuged at 300 xg for 20 min. The platelet-rich plasma (PRP) was aspirated and centrifuged at 2500 xg for 15 min to obtain platelet-poor plasma (PPP). A discontinuous plasma-Percoll gradient was used to separate the blood cells.[14]

Assays

NO concentration in plasma and cell supernatant was determined by a modified procedure described by Ignarro *et al*.,[15] based on the diazotisation of sulphanilic acid by NO at acidic pH and subsequent coupling with *N*-(1-naphthyl)-ethylenediamine to yield an intensely coloured product. Absorbance was measured in a spectrophotometer at 458 nm.

Release of NO from platelets in PRP was studied under resting conditions (basal) and after activation with collagen at a final concentration of 10 µg/mL in buffer (25 mmol/L HEPES, 140 mmol/L sodium chloride, 5.4 mmol/L potassium chloride, 1 mmol/L calcium chloride and 1 mmol/L magnesium chloride [pH 7.4]). The mixture was incubated at 37°C for 15 min and the reaction was stopped by sonication (30 sec, x3). NO was detected by the method of Ignarro *et al*.[15]

In order to measure the NO released by leucocytes, neutrophils and monocytes were resuspended to give a total count of 10^6 cells/mL in RPMI-1640 medium (10% [v/v] fetal calf serum, 2 mmol/L glutamine, 100 µg streptomycin/mL, 100 units penicillin/mL, 0.87 mmol/L arginine and 15 mmol/L HEPES [pH 7.4]). The cells were studied under basal conditions and after activation. Neutrophils were activated with 1 µmol/L FMLP (formylmethionyl-leucyl-phenylalanine) and monocytes were activated with 10 ng/mL LPS (lipopolysaccharide). Cells were incubated for 12 h at 37°C in 5% CO_2. The reactions were stopped by sonication (30 sec, x3) and NO was detected in the supernatant by the method of Ignarro *et al*.[15]

To determine SOD activity, platelets, neutrophils and monocytes were washed in phosphate-buffered saline (x3), disrupted by sonication (30 sec, x3) and then centrifuged at 6000 xg for 30 min. SOD activity in the supernatant was measured using the method of Marklund and Marklund.[16] Supernatant was also used to determine protein concentration, using the modified method of Lowry *et al*.[17] Peripheral blood counts and differential counts were also obtained.[18]

Statistical analysis

Students *t*-test was used for comparisons between the groups, and a paired *t*-test for significance was used to compare the results obtained before and after dialysis.[19] Correlations between NO production, urea concentration and changes in blood pressure during haemodialysis also were perfomed.

Results

Figure 1 illustrates the rate of NO production by platelets, and shows a significant increase in all uraemic groups, compared with controls. Under basal conditions NO production by platelets was 108.7%, 65.5 % and 81.5 %, respectively, for the non-dialysed CRF patients and the pre- and post-dialysis levels in those on haemodialysis. (P<0.05; Figure 1). Following platelet activation these figures were 100%, 69.9% and 90.6%, respectively.

In the non-dialysed CRF group, the rate of NO production by neutrophils under basal and activated conditions, and by monocytes under activated conditions, was significantly decreased compared with controls (21%, 23.3% and 16.7%, respectively; P<0.05 [Figures 2 & 3]). However, in the haemodialysed group, the rate of NO production by neutrophils (basal and activated) in predialysis samples showed a significant increase compared with controls (80% and 24.9%, respectively; P<0.01 [Figure 2]), and a similar increase was seen in the monocytes (128 % and 62.9 %; P>0.01 [Figure 3]). Moreover, basal NO production by neutrophils and monocytes in post-dialysis samples was significantly increased (31 % and 82 %, respectively; P<0.01 [Figures 2 & 3]), compared with pre-dialysis samples.

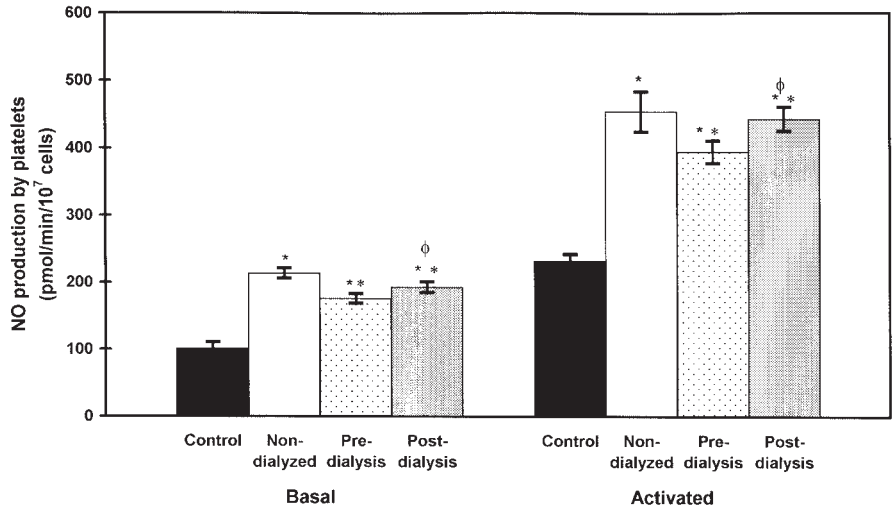

Fig. 1. NO generation by platelets in the groups studied.

Data represented as mean±SE (n = 10 in each group)

* Statistically significantly different from controls

★ Statistically significantly different from non-dialysed

ϕ Statistically significantly different from pre-dialysed

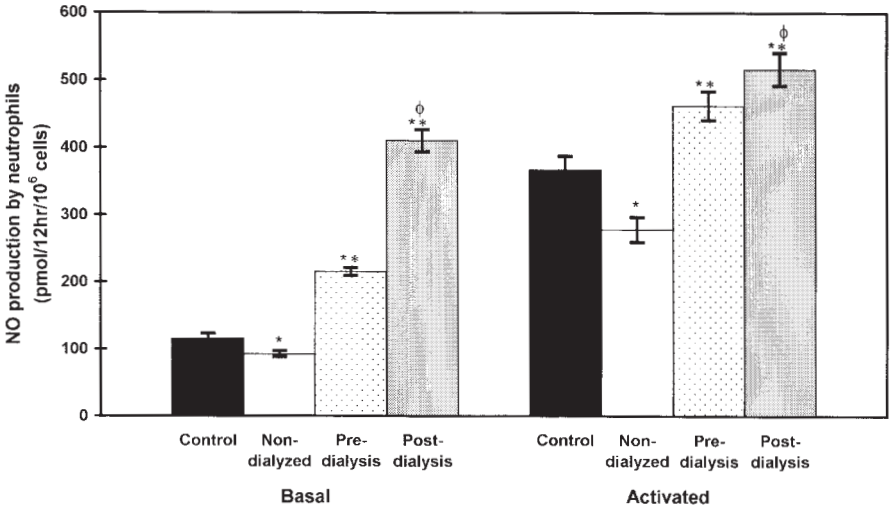

Fig. 2. NO generation by neutrophils in the groups studied.

Data represented as mean±SE (n = 10 in each group)

* Statistically significantly different from controls

★ Statistically significantly different from non-dialysed

ϕ Statistically significantly different from pre-dialysed

SOD activities were significantly decreased in the platelets from the non-dialysed and pre-dialysis groups, compared with the control group (P<0.05; Table 1), while the decreased activity in neutrophils and monocytes was seen in all CRF patient groups (P<0.05, Table 1).

Plasma NO levels in all patient groups were increased compared with controls (24.9%, 150.5% and 206.5%, respectively; P<0.05 [Figure 4]); however, the dialysed group showed higher levels than the non-dialysed group (P<0.01), and during the haemodialysis session the plasma NO level was elevated by 56% (P<0.01, Figure 4).

A negative correlation was found between serum urea concentration in the non-dialysed group and the inducible NO production by neutrophils (r = -0.74, P< 0.01) and monocytes (r = -0.62, P<0.025), and a significant positive correlation was found between the increase in plasma NO level during haemodialysis and the decrease in blood pressure that occured during the session (r= 0.80, P<0.01).

Discussion

In this study of CRF patients, we have shown that NO production by platelets is significantly higher than normal, under basal conditions and after activation with collagen, in both non-dialysed and haemodialysed groups of patients. This indicates that NO may play a role in the bleeding tendency observed in uraemics by counteracting vessel injury-induced vasoconstriction,[20] inhibiting platelet adhesion to damaged endothelium,[21] and interfering with the process of platelet-platelet interaction[3] by activating soluble guanylate cyclase that in turn increases platelet cyclic GMP.[3] The increased NO production indicates the up-regulation of eNOS.

These results are in agreement with Noris *et al.*,[12] who found that defective platelet aggregation was associated with exaggerated platelet NO synthesis in CRF patients, and that intraplatelet levels of cGMP were higher in uraemic patients.

The mechanisms responsible for this increased production by platelets are unclear, but it is possible that substances may accumulate in the plasma and platelets of uraemic patients that activate the L-arginine/NO pathway. To clarify this, we suggest that the increase in intraplatelet free-calcium concentration in CRF patients, as previously reported,[22,23] may provide a possible explanation because this increase is a key regulatory step in the control of calcium-dependent eNOS activity.

Table 2. Laboratory results for patients with chronic renal failure.

Parameter	Control (n=10)	Non-dialysed (n=10)	Pre-dialysis (n=10)	Post-dialysis (n=10)
Serum creatinine (mg/dL)	0.88 ± 0.08	11.19 ± 0.42*	7.16 ± 0.37*	3.67± 0.24*†
Blood urea (mg/dL)	31.77± 2.04	129.33± 15.78*	195.97± 10.17*	60.9 ± 2.96*†
White blood count (x10^9/L)	6.08± 0.43	4.85 ± 0.36*	5.91± 0.4 ND	
Neutrophils (%)	48.70± 3.59	63.40 ± 3.76*	59.7 ± 2.63*	ND
Absolute count (x10^9/L)	2.98 ± 0.33	3.04 ± 0.26	3.59 ± 0.39	ND
Monocytes (%)	4.00 ± 0.49	5.40 ± 0.49	6.3 ± 0.49*	ND
Absolute count (x10^9/L)	0.098 ± 0.03	0.25 ± 0.017	0.37 ± 0.04	ND
Lymphocytes (%)	35.60 ± 3.5	24.8 ± 3.4*	27.60± 2.90*	ND
Absolute count (x10^9/L)	2.11 ± 0.21	1.24 ± 0.233	1.55 ± 0.11	ND
Platelet count (x10^{11}/L)	2.15 ± 0.45	1.34 ± 0.15	1.39 ± 0.07	ND
Mean blood pressure (mmHg)	84.0 ± 1.40	104.00 ± 4.00*	102.0± 5.70*	90.0± 4.6*†

Data presented as the mean ± SE (n=10)

* Statistically significant difference from control (P<0.05)

† Statistically significant difference from pre-dialysis (P<0.05)

ND: Not determined

Our studies indicate that inducible NO production by leucocytes (monocytes and neutrophils) was significantly decreased in non-dialysed CRF patients, which reflects a decrease in iNOS activity, and leads us to suggest that a uraemic toxin[5] may inhibit iNOS expression or activation, as it was found that most end-stage uraemic plasma inhibited iNOS in a macrophage cell line.[24]

More indirect evidence for decreased inducible NO synthesis in non-dialysed patients comes from the work of Porter *et al.*,[25] who reported that impaired neutrophil functions in CRF were associated with increased superoxide anion production. As NO inhibits neutrophil production of reactive oxygen species by interacting with NADPH-oxidase[26] and xanthine oxidase,[27] the increased O_2^- production by neutrophils in CRF may be due to inhibition of NO and/or inhibition of SOD activity.

Urea has been regarded as an innocuous metabolite that accumulates during renal failure; however, it may contribute to the regulation of iNOS in CRF. Prabhakar *et al.*[28] showed that urea specifically and reversibly inhibited iNOS in a macrophage cell line in a dose-dependent manner by a post-transcriptional mechanism.[28] Our results showed a significant negative correlation between urea concentration in the non-dialysed group and inducible NO production by activated neutrophils and monocytes. This observation may have important implications for leucocyte dysfunction in uraemia.

In the haemodialysed group of patients (pre- and post-dialysis) studied, the rate of NO production by leucocytes was significantly higher than in the non-dialysed CRF and control groups, both under basal and activated conditions. We suggest that uraemic plasma from those on long-term dialysis may contain an enhancer molecule that is not present in other groups, and/or the inhibitory molecules that are present in non-dialysed plasma may be removed by haemodialysis as urea and L-arginine analogues. The significantly higher level of L-arginine found in uraemia[12,29] offers an explanation for increased NO synthesis in this condition.

The inducible NO-forming enzyme is dependent on the availability of extracellular L-arginine, and therefore it is conceivable that in patients with high L-arginine levels, excessive NO is formed due to activation of the inducible enzyme or availability of substrate. Moreover, the high plasma levels of interleukin-1 (IL-1) and tumour necrosis factor∝ (TNF∝)[30,31] in patients on haemodialysis appear to be additional reasons for enhanced formation of NO in uraemia, as IL-1 and TNF∝ are potent inducers of iNOS in various cellular systems.[32] TNF∝ is increased in haemodialysed patients due to reduced renal clearance,[33] and is consistent with enhanced monocyte activation on the dialysis membrane.[34]

During haemodialysis, NO production by platelets, monocytes and neutrophils was markedly elevated, indicating activation by the mechanical components of the dialyser. Rysz[9] attributed this increase of intradialytic NO production to the haemoincompatibility of the cuprophan membrane, which activates leucocytes, increases blood turbulance and enhances shear stress – which is known to augment NO release from neutrophils and platelets.[2,3] The use of heparin may be an additional factor in this enhanced NO production.[9]

Plasma NO levels were significantly elevated in all the uraemic groups. In the non-dialysed group, this may have been due to up-regulation of eNOS, perhaps representing a defence mechanism to limit the elevation of blood pressure in these patients, as suggested by an animal model of CRF.[13] In the haemodialysis group, increased plasma NO was due to up-regulation of iNOS by elevated levels of cytokines. During the session, plasma NO increased further due to the activation of leucocytes and platelets and the removal of urea and other inhibitors of iNOS such as asymmetrical dimethylarginine (ADMA).[24]

We found a significant positive correlation between the increase in plasma NO and the degree of hypotension during haemodialysis, in accordance with the work of Yokokawa[35] who found that NO production was increased in CRF patients who had a hypotensive episode during haemodialysis.

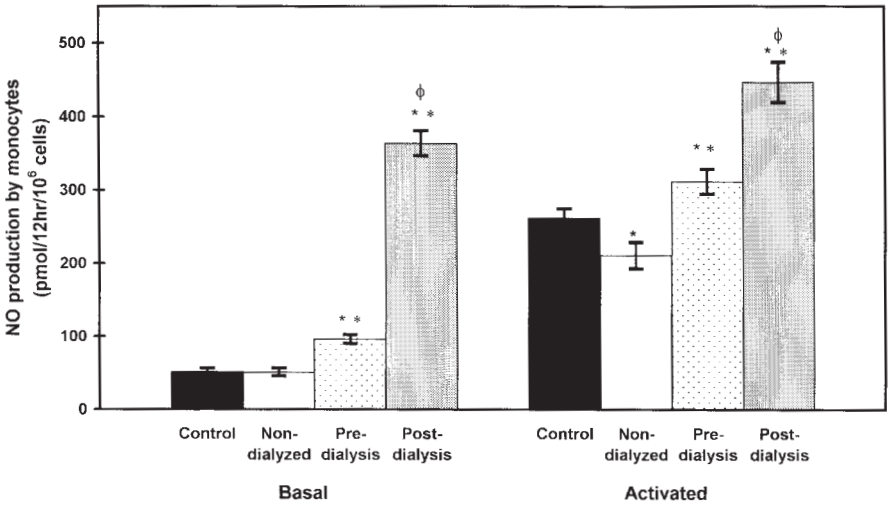

Fig. 3. NO generation by monocytes in the groups studied.

Data represented as mean±SE (n = 10 in each group)

* Statistically significantly different from controls

★ Statistically significantly different from non-dialysed

ϕ Statistically significantly different from pre-dialysed

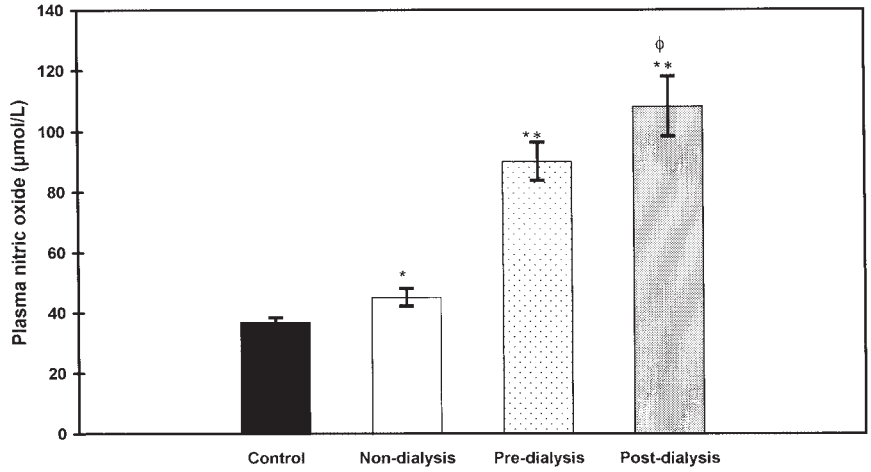

Fig. 4. Plasma NO level in the groups studied.

Data represented as mean±SE (n = 10 in each group)

* Statistically significantly different from controls

★ Statistically significantly different from non-dialysed

ϕ Statistically significantly different from pre-dialysed

SOD activity was significantly decreased in the cells studied in the uraemic patients, resulting in excessive formation of O_2^- that produces the damaging effect and inflammatory responses observed in CRF. As enhanced generation of reactive oxygen compounds takes place during haemodialysis,[36] the effects of NO release on intradialytic oxidative stress remain to be determined. However, NO has been shown to inhibit neutrophil production of reactive oxygen compounds by interacting with NADPH-oxidase and xanthine oxidase, while, on the other hand, NO can combine with O_2^- to form peroxynitrite – a potent oxidant that can cause tissue damage.[6] □

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