Early increase in blood nitric oxide, detected by electron paramagnetic resonance as nitrosylhaemoglobin, in haemodialysis


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Abstract The objective of this study was to determine intradialytic blood levels of nitric oxide (NO), in patients undergoing chronic haemodialysis. This was done by detection of nitrosylhaemoglobin by a sensitive technique of spin trap electron paramagnetic resonance at 0, 5, 15, 60, 180 and 240 min of a 4-h standard bicarbonate dialysis, using the same dose (6000 U) of heparin and different dialysis membranes. The study group included 12 patients treated with cellulose-derived dialysis membranes (nine with cuprophan and three with cellulose triacetate) and 10 patients treated with synthetic membranes (five with polysulfone and five with polyethylenimine). Control groups included 11 normal subjects and six patients with end-stage renal failure who were receiving intermittent peritoneal dialysis. Basal blood levels of nitrosylhaemoglobin in haemodialysis patients were significantly higher than normals, but similar to peritoneal dialysis patients. A significant increase (P<0.01) in nitrosylhaemoglobin level was detected at 15 min of haemodialysis irrespective of the membrane used. A decrease to basal levels at 180 min was observed in all but two cuprophan-treated patients who, in contrast to the others, had a symptomatic hypotenion at the end of the session and a further increase in blood nitric oxide. Patients undergoing peritoneal dialysis did not show any change in blood levels of nitrosylhaemoglobin during the first 180 min of the procedure. Thus, a constant increase in nitrosylhaemoglobin levels was observed early in haemodialysis patients, but not in peritoneal dialysis patients. Very preliminary evidence was obtained for a role of nitric oxide in the vascular instability at the end of haemodialysis in a few patients who had hypotensive episodes.

Key words: nitric oxide; biocompatibility; dialysis membranes; electron paramagnetic resonance

Introduction

Vascular endothelial cells [1] and several other cell lines, including phagocytes [2], synthesize and release nitric oxide (NO). NO counterbalances the vasoconstrictor action of endothelin (ET-1) and inhibits ET-1 production via a c-GMP-dependent pathway [3,4]. Evidence of a role for NO in diverse physiologic and pathologic processes is emerging [2]. The capability of this molecule to oxidize critical compounds, i.e. thiols, haeme groups and iron sulphurs, sustains its involvement in different biological processes. Besides vasodilation, its functions include inhibition of platelet aggregation and modulation of inflammatory and immune processes [2,5,6].

Plasma concentrations of arginine, the endogenous substrate of NO synthase, have been reported to be lower [7] or higher [8] than normal in uraemia. Moreover, it has been reported that uraemic plasma contains an endogenous compound, L-arginine, able to inhibit NO synthesis by interfering with the L-arginine/NO pathway [9]. However, exposure of cultured human endothelial cells to uraemic plasma results in potent induction of NO formation [8]. Besides an increased availability of substrate, which is still a matter of debate [7,8], it has been proposed that high plasma levels of cytokines, as a consequence of monocyte activation by dialysis membrane [10], enhance NO formation contributing to haemodialysis (HD) hypotension. The involvement of NO production in haemodialysis-induced hypotension was eventually confirmed by detecting NO2 and NO3 [11]. However, no data on intradialytic fluctuations of NO blood levels in HD patients have been reported to date. Therefore, 22 patients were carefully selected and intradialytic fluctuations of NO blood levels in HD patients were carefully selected and intradialytic fluctuations of NO blood levels in HD patients have been reported to date. Therefore, 22 patients were carefully selected and intradialytic fluctuations of NO blood levels in HD patients have been reported to date. Therefore, 22 patients were carefully selected and intradialytic fluctuations of NO blood levels in HD patients have been reported to date. Therefore, 22 patients, carefully selected for absence of intercurrent illnesses, entered a study aimed to detect basal values and intradialytic fluctuations of nitrosylhaemoglobin, as a surrogate marker of NO generation, by the sensitive technique of spin-trap electron paramagnetic resonance (EPR).

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Patients and methods

Patients

HD patients included 11 men and 11 women, with a mean age of 65.8 years (range 33–81 years), and a mean dialytic age of 83.8 months (range 17–215 months), undergoing 4 h of maintenance treatment three times a week. All were given 6000 U heparin continuously administered during the procedure. In these patients, clinically detectable infections within the last 4 weeks were excluded with certainty. The aetiology of renal failure was nephroangiosclerosis in 11 cases, chronic glomerulonephritis in five, reflux nephropathy in two, polycystic disease in three and unknown in one. Twelve patients were hypertensive. No patients were given steroids, anti-inflammatory drugs or ACE inhibitors. They were treated with bicarbonate haemodialysis. Dialysis fluid composition included sodium 140.00 mmol/l, chloride 107.25 mmol/l, potassium 2.00 mmol/l, calcium 1.75 mmol/l, magnesium 0.37 mmol/l, bicarbonate 35.00 mmol/l, and acetate 4.00 mmol/l. Nine patients were treated with cuprophan (Cu) membrane (Spiraflo NT 18–08, 1.72 s.m., thickness 8 μm), ultrafiltration rate (UFR) 8.6 ml/h/mmHg; Belco, Mirandola, Italy), three with cellulose triacetate (CTA) membrane (Nipro, Superflux 150 E, 1.5 s.m., 15 μm, UFR 10.4 ml/h/mmHg; Nissho Corp., Osaka, Japan), five with polymethylmethacrylate (PMMA) membrane (Filtryzer B2 1.5 H, 1.5 s.m., 20 μm, UFR 7.5 ml/h/mmHg; Toray Ind. Inc., Chuo-ku, Tokyo, Japan) and five with low flux polysulfone (PS) membrane (Fresenius F7, 1.6 s.m., 40 μm, UFR 6.4 ml/h/mmHg; Fresenius AG, Bad Homburg, Germany). Blood and dialysate flow rates were maintained at 300 and 500 ml/min, respectively.

Blood samples for nitrosylhaemoglobin detection were collected from the arterial line in heparinized vacuum tubes on ice at the start (t₀), at 15 (t₁₅) and at 180 min (t₁₈₀), and, in the six cases with haematocrit values allowing a greater sampling, also at 5, 60 and 240 min. Moreover, to exclude local increase of NO in the shunt environment, parallel measurements of venous and arterial nitrosylhaemoglobin were done at 15 min in three patients. Oxygen traces were replaced with argon and specimens were transferred into a liquid nitrogen container.

In order to better verify the effects of dialysis membranes on the parameters under study, three patients using Cu membrane for at least 4 months when initially studied were switched to TCA membrane for three dialysis sessions and PMMA membrane for three more sessions. Nitrosylhaemoglobin was detected at the third dialysis session. Dialyses were tested before and after dialysis using Limulus Amebocyte Lysate assay (Whittaker MA Bioproducts, Walkersville, MD). Lysate, prepared from the circulating amebocytes of horseshoe crab, Limulus polyphemus, was standardized to detect at least 0.125 EU/ml of FDA reference endotoxin.

Blood pressure (BP) was carefully checked at 30 min intervals from the start to the end of each dialysis session. BP fall was defined as significant if mean BP decreased to at least two-thirds of the initial values.

Control groups comprised 11 healthy subjects and six patients undergoing intermittent peritoneal dialysis (IPD). The last group included four women and two men with a mean age of 78 years (range 69–86), IPD mean dialytic time of 24.6 months (range 2–66), with end-stage renal failure due to nephroangiosclerosis (three cases), chronic glomerulonephritis (one), polycystic disease (one), and interstitial nephritis (one). Blood samples in IPD patients were collected at 0, 15 and 180 min during a standard IPD session (22.5 l dialysate containing 1.5% glucose, and 9 l dialysate with 4.25% glucose).

Detection of nitrosylhaemoglobin as a surrogate marker of NO generation

The principle of detection of NO by electron paramagnetic resonance (EPR) is based on the paramagnetic nature of the molecule which has the unpaired electron in the π orbital [2]. Under energy of microwave frequency and at adequate magnetic field strength, unpaired electrons of radicals are promoted to higher energy levels and relaxation to ground state produces characteristic spectra. In the case of NO, relaxation time is too rapid to be detected. In our detection system, haemoglobin (Hb) has been used as a ‘spin trap’ which interacts with unstable radicals. The more stable adduct, i.e. nitrosylhaemoglobin, can be measured by EPR. The identity of the radicals, defined by EPR, is characterized by the multiplicity of the hyperfine splitting, i.e. a triplet feature at 3.250–3.400 gauss with g values of 2.02, specific for the NO–Hb complexes [12]. The amount of radical present is proportional to the magnitude of the signal [12]. A computer system, on line with the EPR spectrometer (Bruker ESP 300), allowed calculation of spectra, measured as the derivative of signal integrals of interest normalized for the signal background and expressed as arbitrary units (AU) × 10⁻⁴.

Preliminary recovery assays using NO donors (such as sodium nitroprusside, SIN-1 and 5-nitroso-N-acetylpenicillamine) showed a linear dose–response relationship for the formation of EPR-detectable nitrosylhaemoglobin complexes, the signal centred at g = 2.02 being the major component of the spectra. Other components included a triplet pattern at g = 2.08, referred to NO bound to esacordinate haeme-iron, and a g = 6 peak related to methaemoglobin. Each signal component increased with NO donor concentration. However, the relative proportion of the g = 2.02 signal was found to be increasingly higher compared to the others. Specifically, at the levels measurable in vivo, i.e. 1 × 10⁻⁴ arbitrary units (AU), the g = 2.02 signal was 100-fold higher than g = 2.08, while g = 6 signal was virtually undetectable.

Total Hb in the blood samples did not correlate with the results of the nitrosylhaemoglobin assay, as preliminarily verified in five normals and 10 HD patients. After processing blood samples into liquid nitrogen the intra-assay variation coefficient was 0.9% within 4 weeks, and the inter-assay variation coefficient, as preliminarily assessed in 13 subjects with various degree of renal impairment, was 5.3% (three to six determinations per sample over a 1–3 month period).

Statistics

Results were analysed by a commercial software package (Abacus Concepts, Stat View, Abacus Concepts Inc., CA). Comparisons in normally distributed variables were addressed by analysis of variance (ANOVA) to compare multiple groups. If a statistically significant difference was found, differences between individual groups were tested by Student’s t-test. Linear regression analysis and Fisher’s exact test were also used for statistical analysis. Differences were considered significant at P < 0.05.
Results
Basal blood nitrosylhaemoglobin levels in HD patients were significantly higher than normal values ($P<0.01$ by ANOVA analysis) and similar to the control group of IPD patients (Figure 1). No differences were found in baseline nitrosylhaemoglobin between males and females nor between hypertensive and normotensive HD patients. None of patient age, dialytic age, or doses of antihypertensive drugs (calcium antagonists and beta-blockers) correlated with nitrosylhaemoglobin levels.

While IPD patients, examined as a control group, did not show any change in blood NO levels in the first 180 min of IPD procedure (Figure 2), a significant increase in nitrosylhaemoglobin level was detected at 15 min in HD patients ($4.70 \pm 2.13 \times 10^{-4} \text{ AU}, P<0.0001$) compared to baseline values ($2.88 \pm 1.67 \times 10^{-4} \text{ AU}$). Parallel measurements of venous and arterial nitrosylhaemoglobin in a few patients at 15 min were virtually identical: 4.98, 7.11, and 4.02 vs 5.01, 7.02 and 3.97. The increase at 15 min was followed by a decrease at 180 min ($2.94 \pm 1.79 \times 10^{-4} \text{ AU}, P<0.0001$ compared to $t_{15}$ values, non-significant compared to $t_0$ values), except for two Cu patients who showed a further increase. These two were the only patients of the study group who showed a significant decrease in blood pressure at the end of dialysis (with mean BP of 60 and 65 mmHg, respectively). Ultrafiltration rate (12.7 and 13.5 ml/min) fell in the range of the general population under study (12–16 ml/min). Underlying disease was nephroangiosclerosis in both cases. One patient, still hypertensive, was treated with clonidine. In both patients at least four episodes of hypotension a month could be detected.

The general profile of nitrosylhaemoglobin levels (Figure 2) showed that the values detected at 5 min and at the end of dialysis were similar (non-significantly different) to basal and 180 min values, respectively, and that the decrease in nitrosylhaemoglobin levels started approximately at 60 min.

The absolute values obtained with each dialysis membranes are summarized in Table 1. The increase in nitrosylhaemoglobin levels at 15 min ($\Delta t_{15-0}$) was not related to blood pressure medications, volume status/loss, haematocrit, cardiovascular problems, nephropathy, mean BP changes and dialytic age. Incidentally, nitrosylhaemoglobin $\Delta t_{15-0}$ in cellulose triacetate-treated patients ($4.01 \pm 2.77 \times 10^{-4} \text{ AU}$) was significantly greater than in the other membranes (Cu: $1.10 \pm 1.04 \times 10^{-4} \text{ AU, } P<0.003$; PS: $1.64 \pm 0.97 \times 10^{-4} \text{ AU, } P<0.02$; PMMA: $1.52 \pm 1.14 \times 10^{-4} \text{ AU, } P<0.02$).

The significance of this observation in the small sample of cellulose triacetate-treated patients was confirmed by switching three patients using the same Cu membrane for at least 4 months when initially studied, to CTA membrane for three dialyses and to PMMA for a further three sessions, and again examined on the third session. Figure 3 shows that the constant increase at 15 min was higher when patients were treated with CTA membrane.

<table>
<thead>
<tr>
<th>Pt no.</th>
<th>Membrane</th>
<th>$t_0$</th>
<th>$t_{15}$</th>
<th>$t_{180}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>CU</td>
<td>$3.74 \pm 0.62$</td>
<td>$4.95 \pm 0.73^{**}$</td>
<td>$3.42 \pm 0.75$</td>
</tr>
<tr>
<td>3</td>
<td>TCA</td>
<td>$2.13 \pm 0.87$</td>
<td>$6.26 \pm 1.13^{**}$</td>
<td>$3.20 \pm 0.46^*$</td>
</tr>
<tr>
<td>5</td>
<td>PS</td>
<td>$3.08 \pm 0.70$</td>
<td>$4.84 \pm 0.85^{**}$</td>
<td>$2.60 \pm 0.71^{**}$</td>
</tr>
<tr>
<td>5</td>
<td>PMMA</td>
<td>$2.46 \pm 0.39$</td>
<td>$4.36 \pm 1.06^{**}$</td>
<td>$2.76 \pm 0.78^{**}$</td>
</tr>
</tbody>
</table>
NO in haemodialysis

Fig. 3. Blood NO profiles in three patients treated with cuprophan membrane (+) for at least 4 months and then switched to cellulose triacetate (●) for three dialysis sessions and polymethylmethacrylate (*) for three more sessions. Values are expressed in arbitrary units $\times 10^{-4}$.

Discussion

Endothelial cells continuously release small amounts of NO, producing a constant level of active vascular smooth muscle relaxation. NO synthase inhibition induces vascular smooth muscle contraction and an increase in blood pressure in humans [13]. NO synthesis at vascular level is well established in the acetylcholine agonist model [14]. Other regulatory factors include ischaemia reperfusion mechanisms, shear stress and bradykinin [15,16]. In contrast to endothelial constitutive NO synthase, the inducible form in endothelial and macrophage cells produces large amounts of NO. NO synthase is normally absent in quiescent macrophages and is transcriptionally regulated [17] by cytokines released by immune cells in response to infection. Patients given interleukin-2 were found to excrete NO metabolites in urine [18]. Once synthesized, macrophage NO synthase continuously produces NO, but inhibitory cytokines [19] and mRNA-destabilizing sequences [20] prevent a widespread induction of NO synthase to avoid host damage.

The possible role of NO in several physiologic and pathologic conditions, including uraemia [8–10], is emerging, but no data on direct determination in blood of this powerful mediator are currently available. There are several assays that indirectly reflect the presence of NO. Measurement of cGMP, which assesses the effect of NO on guanylate cyclase, may be influenced by other hormones (such as ANP). Citrulline is a co-product of the action of NO synthase on L-arginine. Both indirect tests provide information on NO, but no indirect test, including detection of nitrite accumulation, which is affected by many variables including diet, alcohol intake, smoking and pollution, has been thought specific enough to be considered as an entirely satisfactory assay for NO [21]. One of the most reliable method of detection of NO in biological fluids is spin trap electron paramagnetic resonance [21,22]. This highly specific and sensitive method is based on the properties of NO to form a stable complex with reduced haeme iron (Fe(II)). The chemical and spectroscopic properties of NO–haemoglobin, formed by Hb trapping of NO, have been well established [22]. NO–Hb is unstable to aerobic oxidation. However, one valency hybrid $(a^+\bar{\text{N}}O\beta^+)$ has a unique aerobic stability. A diagnostic feature of the NO–Hb EPR spectrum is the characteristic three-line hyperfine pattern which is due to electron–nuclear hyperfine coupling to the NO nitrogen (hyperfine coupling constant $A = 16.5$ gauss) and originates primarily from the alpha-subunit in its low affinity penta-coordinate state. The triplet feature at 3.250–3.400 gauss with $g$ values of about 2.02 is specific for NO–Hb. These physicochemical characteristics support the theory that NO–Hb complexes might accumulate as a carrier and storage system for NO in vivo and serve as a surrogate marker for the generation of NO. Sampling represents a critical step in this assay. Thus, special precautions were used in collecting blood and samples were quickly processed into liquid nitrogen. The inter-assay variation coefficient did not exceed 6%.

It has been recently documented that an endogenous compound, i.e. $N^\text{G}-N^\text{G}$-dimethylarginine [9], which inhibits NO synthesis, accumulates in chronic renal failure. Due to its low concentration in uraemic plasma [8], the possibility for this compound to substantially interfere with either the constitutive or inducible NO-forming enzyme is a matter of debate [9]. Our data show that, even if effective, this inhibition must be counterbalanced by mechanisms sustaining an increased synthesis of NO. The causal substances are not identified yet, but some data support the role of cytokines, released as a consequence of the enhanced monocyte activation by dialysis membranes [8]. Other in vitro data suggest a role for dialysate composition [23].

The analysis of the changes in nitrosylhaemoglobin blood levels with the same conditions of standard haemodialysis showed a significant early increase irrespective of the membrane used (though with some prevalence for cellulose triacetate). The pathogenesis of this constant increase, occurring between the fifth and fifteenth minute of haemodialysis, is presently unclear. Due to the short time required, this event seems to be more consistent with the stimulation of a
constitutive form of NO synthase from platelets [6] or neutrophils [24] than with the induction of the inducible NO synthase of monocyte/macrophage, smooth muscle or endothelial cells. This hypothesis seems to be supported by in vitro experiments recently performed in our laboratory showing that neutrophils incubated for 15 min at 37°C on Petri dishes coated with dialysis membranes (i.e. Cu or AN69S) released about 10-fold more NO than cells incubated on uncoated disks. Values obtained for neutrophils incubated with dialysis membranes were comparable to NO levels obtained by suboptimal stimulation with 2 μM bradykinin or 10 ng/ml phorbol miristate acetate [25]. Nevertheless, further studies are needed to definitely ascertain the role of constitutive NO synthase.

Complement activation [26], stimulation of platelets and neutrophils [27] and release of oxygen free radicals from peripheral blood phagocytic cells [28–30] are known events occurring at 15 min of haemodialysis. More specifically, the interaction between superoxide and NO metabolism has been the subject of intensive studies [21,31,32]. Neutrophils and other diverse cell types generate both superoxide and nitric oxide [13]. These two compounds can react to form a new powerful oxidant, the peroxynitrite anion. This anion can increase luminol-enhanced chemiluminescence [24,31] and is thought to be an important mediator of the free radical-dependent toxicity because of its oxidizing properties towards different biomolecules [32]. For instance, the simultaneous generation of superoxide and nitric oxide initiates lipid peroxidation in human low density lipoproteins and has been thought to contribute to the pathogenesis of atherosclerosis [32].

Recently, Yokokawa et al. [11] showed increased NO generation in haemodialysis patients with hypotension. These authors had previously showed that heparin promoted vasodilator NO production by human vascular endothelial cells in culture [33]. Thus, they hypothesized that NO stimulated by heparin might play a role in vasodilatation leading to hypotensive episodes during haemodialysis [10]. While all their patients received an equal amount of heparin, only some of them showed increased plasma levels of NO2 and NO3, used as markers of NO production [10]. Our patients were also given the same doses of heparin, continuously administered throughout haemodialysis. Incidentally, a continuous increase in nitrosylhaemoglobin was detected only in the two patients from the study group who had a hypotensive episode. While the sample of symptomatic cases does not allow definite conclusions, our observation is consistent with previous suggestions of a role for NO in HD-related hypotension [8,10]. Heparin does not seem to be involved in these events, but individual sensitivity to heparin cannot be excluded.

In summary, a significant increase of nitrosylhaemoglobin, as a surrogate marker for the generation of NO, can be detected at 15 min of haemodialysis. This event, chronologically associated with the maximal increase in superoxide anion, might possibly contribute to lipid peroxidation in patients treated with dialysis membranes which promote the formation of oxygen free radicals.

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