Kidney International, Vol. 58 (2000), pp. 881–888

Bradykinin and nitric oxide generation by dialysis membranes can be blunted by alkaline rinsing solutions

Rosanna Coppo, Alessandro Amore, Paola Cirina, Bibiana Scelfo, Franca Giacchino, Luciano Comune, Mauro Atti, and Jean Louis Renaux

Nephrology and Dialysis Department, Regina Margherita Hospital, Torino, Nephrology and Dialysis Department, Ospedale Civile, Ivrea, and Hospal SpA, Bologna, Italy; and Hospal R & D International, Lyon, France

Bradykinin and nitric oxide generation by dialysis membranes can be blunted by alkaline rinsing solutions.

Background. Bradykinin (BK) generation following the first contact of blood with the dialysis materials is thought to enhance hypersensitivity reactions (HSRs). Some of the effects of BK are mediated by nitric oxide (NO). We have recently reported that the pH of diluted blood modulates the kinin system. The present study was aimed to investigate the role of the pH of culture media and filter-washing solutions and BK and NO generation, either in vitro and ex vivo.

Methods. BK was measured by a specific enzyme-linked immunosorbent assay (ELISA), and NO synthase (NOS) activity by ^{3}H-citrulline production after incubation with ^{3}H-arginine and nitrites by using the Griess reagent. In in vitro experiments, NOS activity was detected in endothelial cells (ECs) cultured with graded BK concentrations at various pH values. Blood from 30 patients in regular dialysis was ex vivo circulated in one single passage through minifilters prerinsed with pH 7 or pH 8 phosphate buffer (PB) solutions. The out-flowing blood was tested for BK and nitrite content and was incubated with cultured ECs to evaluate its capacity to modulate NOS activity.

Results. BK induced in vitro a dose-dependent increase in NOS activity of ECs, which was mediated by tyrosine kinase phosphorylation. NO generation was enhanced at pH 7.2, which remained unchanged at pH 7.6. In ex vivo experiments, blood out-flowing after one passage on filters washed with pH 7 PB solutions had increased BK levels ($P < 0.0001$), increased nitrites ($P < 0.05$), and enhanced EC NOS activity ($P < 0.05$) in comparison to data found when filters were washed with pH 8 PB. Only when the filters were rinsed with a solution at pH 7 did PAN DX and AN69 membranes show a distinct BK generation capability, and cuprophane a peculiar capability to enhance NOS. Such effects were prevented when dialyzers were prerinsed with pH 8 PB. Multiple regression analysis showed that the pH of the uremic blood was the driving factor for BK and NOS activation ($r = 0.54$, $P < 0.02$).

Conclusions. BK and NO generation are modulated by environmental pH. Rinsing the blood and dialysate compartments of filters with an alkaline solution prior to use may mitigate the activation of mediators likely to be involved in some HSRs.

Key words: hypersensitivity reactions, bio-incompatibility, nitric oxide, filter rinsing solutions, hemodialysis, AN69.

Received for publication July 28, 1999
and in revised form January 12, 2000
Accepted for publication March 14, 2000

The first contact of blood with the dialysis circuit and filter surface during hemodialysis may enhance various hypersensitivity reactions (HSRs) [1]. The severity of this syndrome (also called first-use syndrome, immediate HSRs, or anaphylactoid reactions) ranges from life threatening to milder symptoms, including hypotension, nausea, faintness, itching, flushing, swelling of tongue or throat, wheezing, or bronchospasm. The frequency of severe episodes was estimated to be 3.3 out of 1000 patients per year with hollow-fiber filters and 10 times less with flat-sheet dialyzers [2]. However, milder HSRs have been reported in up to 24% of the dialysis centers in the United States in 1992 [3], and a recent survey on 20,000 patients found an annual prevalence rate of 7 HSRs per 1000 patients [4]. Several enhancing factors have been considered in the development of this syndrome, including ethylene oxide residues, bacterial lipopolysaccharide fragments, capillary geometry, and cuprophane (CU) membranes [1, 5–8]. The observation of an increased frequency of HSRs after using the high-flux (AN69) membrane, particularly in patients undergoing treatment with angiotensin-converting enzyme inhibitors (ACE-Is), has led to the hypothesis that some HSRs result from activation of the contact-phase system by the negatively charged AN69 surface with enhanced bradykinin (BK) generation [9–13]. By impairing kinin degradation, ACE-I drugs could further increase BK to pathological levels, giving rise to the clinical features. This hypothesis is supported by an overlapping of the HSR syndrome with the symptoms described following contact-phase activation [5], and by the observation that the metabolism of exogenous BK added to sera of patients dialyzed on AN69 membranes was delayed by the in vitro addition of an ACE-I [14]. A recent study in sheep confirmed that polyacrylonitrile membranes and ACE-I affect BK levels [15].

The contact-phase system activation and the consequent increase in BK could lead to increased production and release of nitric oxide (NO), which is thought to mediate the vasodilation induced by BK [16, 17]. The endothelium plays a key functional role in generating factors modulating vascular smooth muscle tone, among which NO is of primary importance. NO is a short-living gas produced by means of a specific enzyme, NO synthase (NOS), capable of increasing the level of cytosolic cGMP in smooth muscle vascular cells leading to vasodilation [18]. Endothelial cells (ECs) are provided with two isoforms of NOS. The constitutive one (cNOS) is preformed and produces small physiological puffs of NO, while the inducible isoform (iNOS) is synthesized de novo and releases high and sustained amounts of NO, which can lead to severe vasodilation or to vascular sclerosis as we reported [19].

A recent in vitro work by our group has shown that contact-phase activation by dialysis membranes is a pH-dependent phenomenon and that the generation of kallikrein in diluted plasma can be inhibited by keeping the pH above 7.4 [20].

The present study aimed to obtain new insight into the modulation of BK and NO by pH in both in vitro and ex vivo settings, focusing on the enhancing effect of the first contact of the blood with the dialysis membranes.

We report that the use of alkaline rinsing solutions before circuit connection could blunt BK and NO generation, possibly contrasting the development of HSRs.

METHODS

Experimental design of in vitro studies

The investigation aimed at evaluating in cultured ECs the effects of culture medium pH (in the 6.9 to 8 range) and the addition of purified BK (from 100 to 10,000 fmol/mL) on NO generation. To get further insight into the intracellular signaling involved, the activation of tyrosine kinases by BK and its involvement in NOS activation were investigated.

Endothelial cells. The murine EC line t End. 1 (kindly provided by Prof. F. Bussolino, Institute of Chemistry, University of Turin, Turin, Italy) was maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma Co., St. Louis, MO, USA) containing 10% fetal calf serum (FCS; Sigma), penicillin, streptomycin, and amphotericin (Sigma) as previously described [21]. These cells, used at the 98th to 166th passages for the experiments described herein, were derived from a thymic hemangioma expressing the polyoma middle T antigen. t End. 1 cells retain a wide array of the functional properties of normal ECs. They proliferate at confluence without aspects of overgrowth; take up acetylated low-density lipoproteins; express CD31, vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin; respond to interleukin-1β (IL-1β) tumor necrosis factor-α (TNF-α) and specific EC growth factors; and produce interleukin-6 and chemokines [22].

Measurement of nitric oxide synthase activity. Endothelial cells were frozen in 1 mL of reaction buffer [20 mmol/L HEPES, 0.5 mol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L dithiothreitol, pH 7.2] and were homogenized on ice with three 20-second bursts in a Polytron homogenizer. Each reaction used 100 μL of homogenate in a mixture containing 2 mmol/L NAPDH, 1.5 mmol/L $CaCl_2$, 1 to 100 μmol/L L-Arg, and 2.5 μCi (= 0.4 μmol/L) L-[2, 3, 4, 5-^{3}H] arginine monohydrochloride (62 Ci/mmol; Amersham International, Bucks, UK). After a 30-minute incubation at 37°C, the reaction was stopped by adding 2 mL of 20 mmol/L HEPES, 2 mmol/L disodium EDTA, pH 6. The whole reaction mixture was applied to 2 mL columns of Dowex AG50WX-8 (Na^+ form; Aldrich, Milan, Italy) and eluted with 4 mL of water. At pH 6, arginine is negatively charged, while citrulline is neutral. The Dowex resin is a cationic exchanger which binds arginine, but not citrulline, under these conditions. The radioactivity corresponding to [^{3}H]citrulline content in 6 mL eluate was measured by liquid scintillation counting. The protein content of cells was assessed with the modified micro-Lowry method (Sigma). NOS activity was expressed as pmol of citrulline generated per minute of incubation per milligram of cell protein. NOS activity was expressed as the fold increases in comparison with values obtained with the same cell line under basal conditions.

In some experiments, the culture medium for ECs (pH 7.4) was modified by the addition of 0.01 mol/L HCl or 0.01 mol/L NaOH. In others, ECs were conditioned with BK (Peninsula Lab Inc., Merseyside, UK) at graded concentrations from 100 to 10,000 fmol/mL for 30 minutes, 60 minutes, and 6 hours. More than 97% of cells were viable by Trypan blue staining at the end of the experiments. To ascertain whether the effect of BK on NOS activity was mediated by the phosphorylation of tyrosine kinase, NOS activity was measured in ECs incubated with BK 5000 fmol/mL with or without genistein 75 μmol/L (Sigma), an inhibitor of tyrosine kinase phosphorylation.

Tyrosine kinase phosphorylation. t End.1 endothelial cells treated with BK (5000 fmol/mL) were incubated for either 45 minutes or 6 hours. At the end of incubation, cells were scraped, rapidly pelletted, and resuspended in lysis buffer (1 mol/L Tris HCl, pH 7, 1 mol/L NaCl, 0.1 mol/L EDTA, 1% NP40, 200 mmol/L Na_3VO_4, 200 mmol/L phenylmethylsulfonyl fluoride, and 100 mmol/L leupeptin). Lysates were incubated for 30 minutes on ice and centrifuged at 5000 × *g* at 4°C for two minutes. Two micrograms of cell lysate (boiled in SDS-PAGE buffer) were run on 7.5% SDS-PAGE gels together with low molecular weight markers (Rainbow, Amersham

PLC, Bucks, UK) at 250 V/10.0 mA for 30 minutes, using the Phast System apparatus (Pharmacia, Uppsala, Sweden), and then transferred to nitrocellulose membranes. The blots were blocked with 2% bovine serum albumin in phosphate-buffered saline 0.15 mol/L, pH 7.4, 0.1% Tween (TPBS) overnight at 4°C. The blots were then incubated with 1:1000 antiphosphorylated tyrosine kinase (Transduction Laboratories, Lexington, KY, USA) diluted in 2% bovine serum albumin/TPBS for 60 minutes while being shaken. After washing with TPBS and incubation with horseradish peroxidase-labeled secondary antibody (Pierce, Rockford, IL, USA) diluted to 1:2000 in TPBS, the blots were incubated for 45 minutes under shaking. After additional washes, the blots were incubated with Super Signal Ultra Chemiluminescent (Pierce) and exposed on x-ray film.

Experimental design of ex vivo studies

The blood out-flowing from patients was circulated, as previously described [20], in one single passage (discussed later in this article) through minidialyzers made of various membranes and rinsed with 0.1 mol/L, pH 7 or pH 8 phosphate buffers (PBs). During the blood passage through the filters, the dialysate compartments were filled with PBs. Three minutes after the patients were connected, the out-flowing blood was tested for pH, BK, nitrites (the method description is discussed later in this article) and incubated for six hours with EC cultures, as performed in previous protocols [26]. NOS activity was then measured (the method description is described previously in this article). Hence, the study was designed to allow the analysis of BK generation in plasma, NO release by circulating blood cells (likely monocytes), and modulation of NOS activity in EC cultures by uremic blood out-flowing from minidialyzers washed with solutions at different pH values.

Patients. Thirty patients, aged 16 to 56 years, on regular dialysis treatment for more than six months, gave their informed consent for the ex vivo study. The hemoglobin level was higher than 11 g/dL, and none of the patients had been treated with ACE-I or steroids over the preceding four-week period.

The research was conducted according to the guidelines and principles of Helsinki declaration.

Ex vivo circuit. Five commercially available dialysis membranes were tested: Polyacrylonitrile (PAN DX; Asahi, Tokyo, Japan; and AN69; Hospal Cobe, Meyzieu, France), CU (Azko, Hamburg, Germany), cellulose triacetate (TA; Baxter, Columbia, MD, USA) and polysulfone (PS; Fresenius, Wuppertal, Germany). AN69 ST (Hospal R & D Int., Lyon, France), an experimental membrane that is still in the development stage, was also tested. AN69ST is provided with the same technical data as AN69, but with a less electronegative zeta potential (-3 ± 1 mV vs. -70 ± 5 mV) obtained by coating the AN69 membrane with 5 mg polyethylenimine/m^2. The hollow-fiber minidialyzers (Hospal R & D Int.) used in these experiments were developed as a small-scale mode (1/50) of a standard hollow-fiber dialyzer. The characteristics of the minidialyzers used in the experiments are summarized in Table 1. Note that AN69 and AN69ST minidialyzers have identical technical characteristics.

Before a test run, the minidialyzers were rinsed by circulating the same solution (PB pH 7 or 8) successively through the blood and dialysate compartments at a flow rate of 5 mL/min. Then the dialysate compartment was clamped. The arterial line drained patient's blood to the minidialyzer at the flow rate of 2 mL/min without any anticoagulant. Samples were collected at time 0 and blood perfused through the tubing set alone before entering the minifilters and at outlet three minutes after patients' connection. Samples were tested for measures of pH, BK, nitrites, and activity of inducing NOS in cultured ECs. All of the patients were tested with membranes washed first with pH 7 and then with pH 8 PB. Each membrane was tested on five different patients.

Gas analysis. The gas analyzer ABL 5 pH meter (Radiometer Laboratory, Neuilly Plaisance, France) was used to measure blood pH.

Determination of plasma BK. The test is described in detail elsewhere [23]. Briefly, undiluted blood (1 mL) was collected at the outlet of the minidialyzer in cold absolute ethanol (4 mL). After homogenization and incubation at 4°C, the ethanolic extract was centrifuged ($1075 \times g$, 15 min). The supernatant was evaporated to dryness using a Speed Vac system (Savant, Farmingdale, NJ, USA) before the extraction step. Ethanolic residues were suspended in 2 mL of cold trifluoroacetic acid (TFA) 0.1% in water and centrifuged ($1075 \times g$, 15 min). The clear supernatant (1 mL) was loaded on a column containing C8-silica (Waters, Mildford, MA, USA) conditioned by successive washing with acetonitrile (3 mL) and TFA 0.1% (2 mL). After washing with 3 mL of TFA 0.1%, bound kinins were eluted with 2 mL of 30% acetonitrile/0.1% TFA solution. This eluate was evaporated to dryness in a Speed Vac system. The residue was dissolved in 1 mL of 50 mmol/L Tris-HCl buffer, pH 7.4, containing 100 mmol/L NaCl and 0.05% Tween-20. A competitive enzyme immunoassay (EIA) was used to assay BK. This assay used highly specific polyclonal rabbit IgG raised against the carboxy-terminal of BK, digoxigenin-labeled BK as tracer, and alkaline phosphatase labeled Fab fragments antidigoxigenin (Boehringer Mannheim, Mannheim, Germany) to detect and quantitate immune complexes. Each sample was measured in triplicate. On a molar basis, the polyclonal anti–BK-purified IgG exhibited no cross-reactivity with des-Arg9-BK. Typical calibration curves were characterized by a half-maximal saturation value of 0.78 pmol/mL.

The sensitivity of the test was 25 fmol/mL.

Table 1. Characteristics of polyacrylonitrile (PAN DX, AN69, and AN69ST), cuprophan (CU), cellulose triacetate (TA), and polysulfone (PS) minidialyzers

	PAN DX	AN69	AN69ST	CU	TA	PS
Effective length *cm*	12	18	18	18	12	18
Number of fibers	170	170	170	170	170	170
Effective surface area cm^2	163	231	231	207	128	195
Contact time *seconds*	25	34	34	27	15	24
Internal diameter of fibers μm	240	240	240	215	200	203

Contact time of blood with the dialysis membrane tested depends on the length and diameter of fibers.

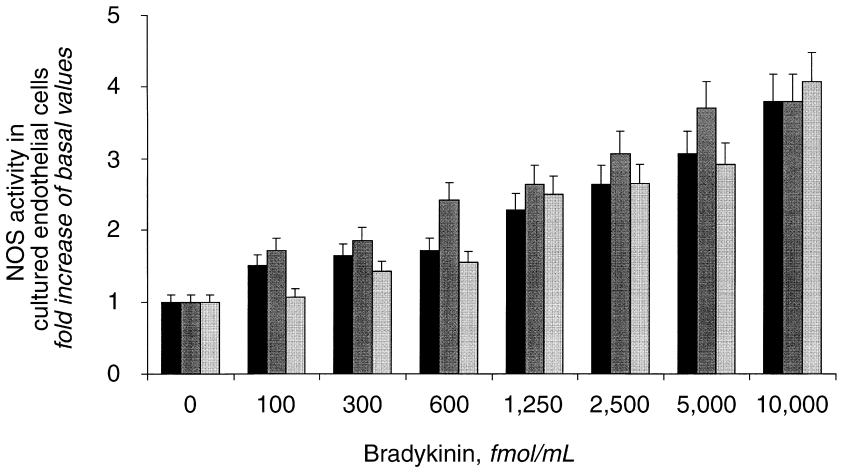

Fig. 1. Dose-dependent release of nitric oxide (NO) by endothelial cell (EC) cultures in response to increasing concentrations of bradykinin (BK). Nitric oxide synthase (NOS) activity in EC cultures was evaluated after 30 (■), 60 (▩), and 360 (□) minutes of incubation with graded amounts of BK, ranging from 0 to 10,000 fmol/mL. Data are expressed as fold increases of values found in unconditioned cells. Columns report the mean ± SD of four experiments, each in triplicate.

Blood nitrite measurement. Sera were mixed with the same volume of Griess reagent (mixture of 1:1 vol/vol of 0.1% naphthyl ethylenediamine dihydrochloride in water and 0.1% sulfanilamide in 5% phosphoric acid) by repeated pipetting (8 times), and then adsorbance at 543 nm was immediately measured by a spectrophotometer. OD values referred to a standard curve with known nitrite concentrations.

Statistical analysis

All results were expressed as means ± SD. For calculation of BK means, all values >10,000 fmol/mL were considered as 10,000 fmol/mL, and values <25 fmol/mL were assumed as 25 fmol/mL. Statistical analysis was performed by means of analysis of variance (ANOVA) using an appropriate post hoc test for paired and unpaired observations according to needs. The simple or stepwise multiple regression analysis was used whenever appropriate. $P < 0.05$ was considered as significant.

RESULTS

In vitro studies

Relationship between BK and NOS activity in cultured ECs. Graded concentrations of purified BK, ranging from 0 to 10,000 fmol/mL, incubated with EC cultures for 30, 60, and 360 minutes, induced a dose-dependent increase in NOS activity (Fig. 1).

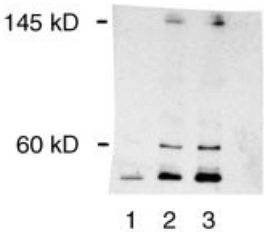

Fig. 2. Western blot of phosphorylated tyrosine kinases. ECs were incubated with 5000 fmol/mL BK for 45 minutes and 6 hours. Lane 1, unconditioned cells. Lane 2, BK (5000 fmol/mL) for 45 minutes. Lane 3, BK (5000 fmol/mL) for six hours. Two bands of 60 and 145 kD were found in lanes 2 and 3.

The binding of BK to its receptor on ECs induced phosphorylation of two tyrosine kinases of 60 and 145 kD, respectively. The activation of this intracellular pathway was evident after 45 minutes and even more obvious after 6 hours (Fig. 2). The phosphorylation of tyrosine kinases induced by BK was crucial for the enhancement of NOS activity, since it was blunted by the addition of genistein, a tyrosine kinase inhibitor. BK 5000 fmol/mL induced a 3.5-fold increase in basal NOS activity of ECs. When cells were incubated with genistein together with BK, no significant modification in the basal NOS activity was found.

Relationship between NOS activity and environmental pH. NOS activity was greatly modulated by the EC cul-

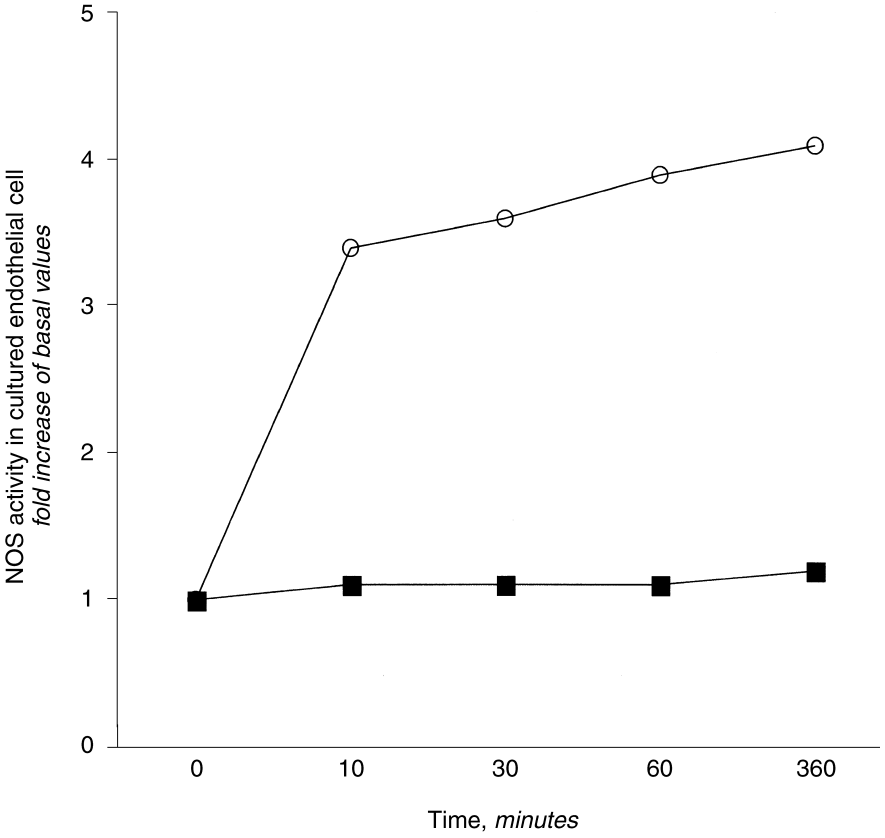

Fig. 3. Effect of the culture medium pH on NOS activity in EC cultures. Time courses of NOS activity were performed in ECs cultured in pH 7.2 (○) or pH 7.6 (■) media. Data are expressed as fold increases of values found in unconditioned cells. Each point reports the mean ± SD of four experiments, each in triplicate.

Table 2. Gas analysis, bradykinin and nitrite concentrations in blood ex-uremic patients circulated in a single passage through minifilters

	Basal values	pH 7 PB washing solution	pH 8 PB washing solution	Statistical significance, *P*
Bradykinin *fmol/mL*	36 ± 19	3912 ± 4835	190 ± 267	< 0.0001
NOS activity *pmol/min/mg protein*	0.06 ± 0.05	0.11 ± 0.09	0.07 ± 0.05	< 0.01
Nitrites *μmol/mL*	4.7 ± 3.1	7.7 ± 2.4	6.3 ± 2.0	< 0.05
Blood pH	7.35 ± 0.05	7.12 ± 0.1	7.42 ± 0.04	< 0.0005

NO synthase activity in endothelial cells was conditioned with blood circulated through minifilters. Data before ex vivo circuit connection (basal values) and at the third minute after connection are reported. Each value represents the mean ± SD of 30 determinations (5 for each of the 6 membranes tested). Each patient was tested on the same membrane washed first with pH 7 and then with pH 8 phosphate buffers (PB). *P* is the statistical significance of the difference between data obtained after using pH 7 and pH 8 rinses.

ture medium pH. The time-course from 10 to 360 minutes showed that the NOS activity of cells cultured at pH 7.2 increased after 10 minutes, lasting 6 hours, while the basal NOS activity of EC cultured at pH 7.6 remained unchanged (Fig. 3).

Ex vivo studies

Gas analysis. Gas analysis of patients' blood before the ex vivo tests showed a metabolic acidosis compatible with dialysis controlled uremia (pH 7.35 ± 0.05; bicarbonates 24.8 ± 3.9 mEq/L).

After one single passage through the minidialyzers, the blood pH values were significantly higher when the devices had been washed with pH 8 than pH 7 PB ($P < 0.0005$; Table 2). No correlation was found between gas analysis baseline values, including bicarbonate levels and pH values of out-flowing blood. No patient had a blood pH value lower than 7.4 after ex vivo circulation on filters washed with pH 8 PB solution. No difference was found comparing data obtained with various membranes (data not given).

Bradykinin generation. The levels of BK generated in the blood ex uremic patients passed through minidialyzers were significantly higher when filters were prerinsed with pH 7 than pH 8 washing solutions ($P < 0.0001$; Table 2). BK generation (Table 3) was distinctly high in the blood circulated through classic polyacrylonitrile membranes—PAN DX and AN69—when filters were washed with pH 7 PB (BK > 10,000 fmol/mL in each tested patient for both membranes), while it was almost

Table 3. Bradykinin concentration (fmol/mL) in blood ex-uremic patients circulated in a single passage through minifilters

	pH 7 PB washing solution	pH 8 PB washing solution
AN69	>10,000 ± 0	474 ± 692
	$P1 < 0.0001$	$P2 < 0.001$
AN69 ST	128 ± 105	48 ± 25
PAN DX	>10.000 ± 0	263 ± 63
	$P1 < 0.0001$	$P2 < 0.0005$
Polysulfone (PS)	47 ± 27	57 ± 38
Triacetate (TA)	43 ± 31	26 ± 2
Cuprophan	90 ± 37	47 ± 41

Each value represents the mean ± SD of 5 determinations. Each patient was tested on the same membrane washed first with pH 7 and then with pH 8 phosphate buffers (PB). *P*1 denotes statistical significance of the difference with basal values, and *P*2 is the statistical significance of the difference between data obtained after using pH 7 and pH 8 rinses.

Table 4. Nitric oxide synthase (NOS) activity (pmol/min/mg protein) in endothelial cells conditioned with blood circulated through minifilters

	pH 7 PB washing solution	pH 8 PB washing solution
AN69	0.15 ± 0.02	0.09 ± 0.02
	$P1 < 0.05$	$P2 < 0.05$
AN69 ST	0.03 ± 0.01	0.02 ± 0.01
		$P2 < 0.05$
PAN DX	0.04 ± 0.01	0.06 ± 0.02
Polysulfone (PS)	0.08 ± 0.05	0.02 ± 0.01
Triacetate (TA)	0.06 ± 0.05	0.03 ± 0.03
Cuprophan	0.23 ± 0.02	0.16 ± 0.02
	$P1 < 0.01$	$P2 < 0.05$

Each value represents the mean ± SD of 5 determinations. Each patient was tested on the same membrane washed first with pH 7 and then with pH 8 phosphate buffers (PB). *P*1 denotes statistical significance of the difference with basal values, and *P*2 is the statistical significance of the difference between data obtained after using pH 7 and pH 8 rinses.

completely blunted when filters were washed with pH 8 PB. It should be noted that no significant BK generation was observed with the AN69ST membrane provided with reduced negative charges. Triacetate, polysulfone, and CU membranes induced only a slight and nonsignificant release in BK when washed with either pH 7 or pH 8 PB solutions.

Nitric oxide synthase activity in cultured ECs. Nitric oxide synthase activity in cultured ECs incubated with blood out-flowing after one single passage through minifilters was statistically different when filters had been previously washed with pH 7 and pH 8 solutions ($P < 0.01$; Table 2). Considering the various membranes (Table 4), NOS activity was distinctly enhanced by CU ($P < 0.01$ vs. precirculation values). This effect was significantly blunted by washing the filters with pH 8 solution ($P < 0.05$). NOS activity of blood circulated on pH 7 PB washed AN69 membrane was also increased ($P < 0.05$ vs. precirculation values), while it remained unchanged when filters were washed with pH 8 PB. A similar, although nonsignificant, effect was observed for the other membranes.

Nitrite generation. The circulation of blood ex uremic patients through minifilters washed with pH 7 PB generated significantly higher amounts of nitrites than with pH 8 washing solutions ($P < 0.05$; Table 2). When single membranes were analyzed, no group of data attained levels of statistically significant difference.

Multiple regression analysis of ex vivo results. The data analysis reported in Tables 3 and 4 indicates that the membrane type was a major discriminating factor for BK and NOS generation when filters were washed with pH 7 PB, while no correlation was found when filters were washed with pH 8 PB.

To investigate the interrelationships between BK generation in plasma, nitrites released by circulating blood cells and NOS activity enhancement in cultured EC, the data were processed by multiple regression analysis. Assuming blood pH as an independent variable, a significant correlation was found among data obtained when filters were washed with pH 7 solutions (total $r = 0.54$, $P < 0.02$). This relationship was no more significant when considering data obtained using filters washed with pH 8 solutions.

DISCUSSION

Focusing on the modulation of BK and NO by environmental pH, we investigated in vitro and ex vivo settings searching for the chance to down-regulate these mediators potentially involved in the development of HSRs during dialysis.

We previously demonstrated that the generation of kallikrein in diluted plasma was enhanced by pH values below 7.4 [20]. In the present study, the NO release in EC cultures was modulated by the culture medium pH, that is, strongly enhanced at pH 7.2 while remaining unchanged at pH 7.6. BK induced a dose-dependent prompt release of NO by ECs. The effects at 30 and 60 minutes are likely to be mediated by cNOS, while 6 hours are needed for a de novo synthesis of iNOS, responsible for long-lasting release of high amounts of NO [18, 19, 24, 25]. The enhancement of NOS by BK was mediated by the phosphorylation of tyrosine kinases since the addition of genistein, a specific inhibitor of tyrosine kinase phosphorylation, completely abolished the effect of BK on NOS activity.

Having demonstrated in vitro the modulating effect of environmental pH on BK and NO generation, we tried to get a deeper in vivo insight by means of an experimental ex vivo approach mimicking initial dialysis conditions. For this purpose, minidialyzers, designed as 1/50 scale replicas of clinical devices used in a previous study of our group [20], were used to investigate the effects of pH on the activation of BK and NO pathways in circulating uremic blood. The ex vivo experimental

devices and conditions were not designed to reproduce a routine hemodialysis, but to provide a valuable model for the selective investigation of the initial contact between the diluted blood and the dialytic material [26], which seems to be crucial for HSR enhancement.

We first investigated the role of pH of washing solutions. In the routine practice of dialysis, the blood compartment is primed with saline in preparation for a treatment. The dialysate compartment is generally rinsed with bicarbonate dialysate following priming. During rinsing and immediately after the beginning of dialysis, equilibration between blood and dialysate compartment takes place. One should expect a sudden alkalinization in the blood compartment, conversely, transfer of CO_2 from bicarbonate solution dialysate to blood first occurs, leading to a greater magnitude of change in pCO_2 than in bicarbonates and to a further acidification of the diluted patient's blood [27]. The pH of diluted blood during the first minutes of dialysis can be below 7.2 when using saline solution for dialyzer priming, while pH 8 bicarbonate washing solutions allowed maintenance of the diluted blood pH above 7.4 at the first contact with the dialysis materials [28]. Hence, immediately after the start of dialysis, the pH of the diluted patient's blood can meet the conditions for BK generation and NOS activation. To provide the clearest information on the role of the pH of the rinsing solutions, extreme conditions of pH 7 and pH 8 buffers were used in our experiments, which led to a significantly different gas-analysis pattern in the outflowing blood. Since in our previous work we found a direct relationship between pH and BK generation in a large pH scale ranging from 7.1 to 7.8 [20], these two pH values were chosen in ex vivo experiments with dialyzed patient to limit the drawing of blood and show the maximal effect. Interestingly, when using pH 8 washing buffer, no patient had a blood pH lower than 7.4, which seems to be a critical point for both BK release [20] and, from our in vitro experiments, for NOS activation. Since the electronegative charge of membranes could be a major factor in BK activation, heparin (highly negatively charged molecule) [29] was not used.

We found a striking difference between values in uremic blood passed through the minidialyzers previously washed with pH 7 and pH 8 solutions. While alkaline buffer rinses inhibited a significant release of BK and NO—in most cases irrespective of the type of dialysis membrane used—significant generation of these mediators was observed with pH 7 solution washes. BK generation was particularly enhanced by membranes with negatively charged surfaces, including PAN DX and AN69, in agreement with previous reports [14]. The relevance of the membrane's electrical charge was further stressed by the finding that the AN69 ST dialyzer (made by reducing surface electronegative charges of the original AN69) failed to induce a significant release of BK. The possibility of binding of precursors of vasoactive substances to various membranes was considered. However, in preliminary experiments, we failed to detect any kinin or BK adsorption on the membranes used in the ex vivo tests (J.L.R., personal observations).

The investigation of the NO generation in ex vivo settings was limited by the experimental design, which to avoid the development of HSRs in patients, did not allow for the contact between BK generated during the passage through the minifilters and the patient's endothelium. Hence, in the experimental conditions chosen, the release of nitrites—NO end products—detected in blood circulated ex vivo through the filters cannot be of endothelial origin, but must derive from blood cells only, most likely monocytes [18]. In the ex vivo experimental conditions chosen, the nitrites levels were slightly increased when pH 7 washing solutions were used, but no clear relationship between BK and NO generation was found. These findings do not rule out the hypothesis of a greater NO generation from vascular endothelium in clinical settings [30] following BK release. Indeed, the incubation of EC with patients plasma (containing BK generated by the contact of blood with AN69) induced a significant increase in NOS activity, similar to what observed when ECs were incubated with purified BK. Of interest, the phenomenon is pH dependent, as was observed only when filters were washed with pH 7 PB. CU membrane increased NOS activity, possibly because of a complement-dependent activation, irrespective of BK pathway. Indeed, in a previous work of our group in a sham dialysis model with bicarbonate buffer using healthy donors' blood, we found that CU strongly enhanced NOS activity as early as after five minutes of circulation. The enhanced NOS activity was due to tumor necrosis factor released by leukocytes following complement activation, as hypothesized by Hakim et al [8]. In this model of donors' blood sham dialysis, where the circulating blood pH was carefully kept at >7.4, we demonstrated a lack of NOS activation by polymethylmethacrylate [19] and AN69 (A.A. and R.C., unpublished observation).

The comparison of these two series of data suggests that blood pH is critical for enhancing a BK-mediated NOS activation when AN69 membranes are used. Even though CU does not activate the BK system, it enhances NOS activity by a complement-mediated pathway that can be modulated by the pH of the washing solutions as well.

The different activation of BK and NOS by AN69 and CU membranes might account for their different clinical associations with HSRs.

Hence, the membrane type was a discriminating factor for BK and NOS activation. The multiple regression analysis definitely clarified the relationships among the variables examined, demonstrating that pH of blood out-

flowing from the minifilters was the factor governing the activation of significantly related variables, including BK release, nitrite generation, and enhancement of ECs to synthesize NOS. Since these relationships were evident only when dialyzers were washed with pH 7 solutions, the pH of the rinsing solutions used to prepare the filters for dialysis is likely to play a pivotal role in the enhancement of mediators, which are strictly related to HSRs and bioincompatible reactions. When using pH 8 washing solutions, the events were so blunted that no correlation could be observed.

In conclusion, the observations made in vitro and ex vivo suggest that attention should be focused on the pH and pCO_2 of the solutions used to wash dialysis filters before clinical use. Rinsing the blood and dialysate compartments of filters with an alkaline solution prior to use may mitigate the activation of mediators likely to be involved in some adverse reactions during dialysis.

ACKNOWLEDGMENTS

Parts of this work were presented as oral communications at the 1997 American Society of Nephrology meeting and at the 1998 European Dialysis and Transplant Association meeting.

Reprint requests Rosanna Coppo, M.D., Divisione Nefrologia e Dialisi, Ospedale Regina Margherita, Piazza Polonia, 94, 10126 Torino, Italy. E-mail: nefrologia@oirmsantanna.piemonte.it

REFERENCES

1. Poothullil J, Shimuzu A, Day RP, Dolovich J: Anaphylaxis from the products of ethylene oxide gas. *Ann Int Med* 82:58–60, 1975
2. Villaroel F: Incidence of hypersensitivity reactions in hemodialysis. *Artif Organs* 8:278–280, 1984
3. Tokars JI, Alter MJ, Favero MS, Moyer LA, Miller E, Bland LA: National surveillance of dialysis associated diseases in the United States, 1992. *ASAIO J* 40:1020–1031, 1994
4. Bright RA, Torrence ME, Daley WR, McClellan WM: Preliminary survey of the occurrence of anaphylactoid reactions during haemodialysis. *Nephrol Dial Transplant* 14:799–800, 1999
5. Cochrane CG, Revak SD, Wueppek KD: Activation of Hageman factor in solid and fluid phases: A critical role for kallikrein. *J Exp Med* 138:1564–1573, 1973
6. Pegues DA, Beck-Sague CM, Woollen SW, Greespan B: Anaphylactoid reactions associated with reuse of hollow fiber hemodialyzers and ACE inhibitors. *Kidney Int* 42:1232–1237, 1992
7. Bigazzi R, Atti M, Baldari G: High-permeable membranes and hypersensitivity-like reactions: Role of dialysis fluid contamination. *Blood Purif* 8:190–198, 1990
8. Hakim RM, Breillat J, Lazarus M, Port FK, Eknoyan G: Complement activation and hypersensitivity reactions to dialysis membrane. *N Engl J Med* 311:878–882, 1984
9. Verresen L, Waer M, Vanrenterghem Y, Michielsen P: Angiotensin converting enzyme inhibitors and anaphylactoid reactions to high flux membrane dialysis. *Lancet* 336:1360–1362, 1990
10. Tielemans C, Madhoun P, Lenaers M, Schandene L, Goldman M, Vanherweghem JL: Anaphylactoid reactions during hemodialysis on AN69 membrane in patients receiving ACE inhibitors. *Kidney Int* 38:982–984, 1990
11. Schulman G, Hakim R, Arias R, Silverberg M, Kaplan AP, Arbeit L: Bradykinin generation by dialysis membrane: Possible role in anaphylactoid reactions. *J Am Soc Nephrol* 3:1563–1569, 1993
12. Verresen L, Fink E, Lemke HD, Vanrenterghem Y: Bradykinin is a mediator of anaphylactoid reactions during hemodialysis with AN69 membranes. *Kidney Int* 45:1497–1503, 1994
13. Fink E, Lemke HD, Verresen L, Shimamoto K: Kinin generation by hemodialysis membrane as a possible cause of anaphylactoid reactions. *Braz J Med Biol Res* 27:1975–1983, 1994
14. Blais C Jr, Marc-Aurele J, Simmons WH, Loute G, Thibault P, Skidgel RA, Adam A: Des-Arg9-bradikinin metabolism in patients who presented hypersensitivity reactions during hemodialysis: Role of serum ACE and aminopeptidase P. *Peptides* 20:421–430, 1999
15. Kreiter DH, Grude M, Lemke HD, Fink E, Bonner G, Shcolkens BA, Schultz E, Muller GA: Anaphylactoid reactions during hemodialysis in sheep are ACE inhibitor dose-dependent and mediated by bradykinin. *Kidney Int* 53:1026–1035, 1998
16. Volpe AR, Giardina B, Preziosi P, Carmignani M: Biosynthesis of endothelium-derived nitric oxide by bradykinin as endogenous precursor. *Immunopharmacology* 33:287–290, 1996
17. Busse R, Fleming I: Molecular response of endothelial tissue to kinins. *Diabetes* 45(Suppl 1):S8–S13, 1993
18. Kroenke KD, Fehesel K, Kolb-Bachofen V: Nitric oxide: Cytotoxicity versus cytoprotection: How, why, when and where? *Nitric Oxide* 1:107–120, 1997
19. Amore A, Coppo R: Nitric oxide production with polymethyl methacrylate, in *Polymethyl methacrylate: A flexible membrane for a tailored dialysis: Contrib Nephrol* (vol 125), edited by Ronco C, Basel, Karger, 1998, pp 182–196
20. Renaux JL, Thomas M, Crost T, Loughraieb N, Vantard G: Activation of the kallikrein-kinin system in hemodialysis: Role of membrane electronegativity, blood dilution and pH. *Kidney Int* 55:1097–1103, 1999
21. Bussolino F, De Rossi M, Sisca A, Colotta F, Wang JM, Boccietto E, Padura IM, Bosia A, Dejeand E, Mantovani A: Murine endothelioma cell lines transformed by polyoma middle T oncogene as target for and producers of cytokines. *J Immunol* 147:2122–2129, 1993
22. Garlanda C, Parravicini C, Sirono M, De Rossi M, Wainstok DE, Calamanovici R, Carozzi F, Bussolino F, Colotta F, Mantovani A, Vecchi A: Progressive growth in immunodeficient mice and host cell recruitment by mouse endothelial cells transformed by polyoma middle T: Implications for the pathogenesis of opportunistic tumors. *Proc Natl Acad Sci USA* 91:7291–7295, 1994
23. Decarie A, Drapeau G, Closset G, Couture R, Adam A: Development of digoxigenin-labeled peptide: Application to chemiluminoenzyme immunoassay of bradykinin in inflamed tissues. *Peptides* 15:511–518, 1994
24. Amore A, Bonaudo R, Ghigo D, Arese M, Costamagna C, Cirina P, Gianoglio B, Perugini L, Coppo R: Enhanced production of nitric oxide by blood-dialysis membrane interactions. *J Am Soc Nephrol* 6:1278–1283, 1995
25. Amore A, Cirina P, Mitola S, Peruzzi L, Bonaudo R, Gianoglio B, Coppo R: Acetate intolerance is mediated by enhanced synthesis of nitric oxide by endothelial cells. *J Am Soc Nephrol* 9:1431–1436, 1997
26. Mulvihill J, Crost T, Renaux JL, Cazenave JP: Evaluation of haemodialysis membrane biocompatibility by parallel assessment in an ex vivo model in healthy volunteers. *Nephrol Dial Transplant* 12:1968–1973, 1997
27. Symreng T, Flanigan MJ, Lim VS: Ventilatory and metabolic changes during high efficiency hemodialysis. *Kidney Int* 41:1064–1069, 1992
28. Linder G, Bergonzi G, Renaux JL, Guarneri F, Bacchi M, Borgatti PP, Cristinelli L: Confronto tra bicarbonato e salina come soluzione priming dei dializzatori: Effetto sul pH e sulla pCO_2 del sangue diluito. *Giorn It Nefrol* 6:1–13, 1997
29. Moskowitz R, Schwartz RJ, Michell B, Ratnoff OD, Astrup T: Generation of kinin-like agents by chondroitin sulfate, heparin, chitin sulfate, and human articular cartilage: Possible pathophysiologic implications. *J Lab Clin Med* 76:790–798, 1970
30. Roccatello D, Mengozzi G, Alfieri V, Pignone E, Menegatti E, Cavalli G, Cesano G, Rossi D, Formica M, Inconis T, Martina G, Paradisii L, Sena LM, Piccoli G: Early increase in blood nitric oxide, detected by electron paramagnetic resonance as nitrosylhemoglobin in hemodialysis. *Nephrol Dial Transplant* 12:292–297, 1997