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Adherence of uremic erythrocytes to vascular endothelium decreases endothelial nitric oxide synthase expression

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Adherence of uremic erythrocytes to vascular endothelium decreases endothelial nitric oxide synthase expression.

Background. High prevalence of atherosclerotic cardiovascular events accounts for much of the mortality among patients suffering from end-stage renal disease (ESRD). Endothelial dysfunction as a pathogenic mechanism might contribute to increasing the cardiovascular risk of ESRD. Reduced endothelium-dependent vasodilation has consistently been observed in chronic renal failure patients. Since nitric oxide (NO) is the principal endothelium-derived vasodilator, a reduction in the NO bioavailability may be envisaged in ESRD patients.

Methods. To clarify whether exposure to erythrocytes from ESRD patients might modulate NO release by the endothelium, we evaluated endothelial NO synthase (eNOS) protein levels (Western blot), eNOS mRNA quantity (real-time PCR), and NOS activity (conversion of L-[3H] arginine in L-[3H] citruline) in endothelial cultures stimulated by erythrocytes from healthy subjects and ESRD patients.

Results. A time-dependent decrease in eNOS protein levels was evident in cultures treated with erythrocytes from ESRD patients. This observation was consistent with the decreased eNOS mRNA quantities induced by erythrocytes from such patients. Moreover, compared to controls, NOS activity exhibited a significant reduction after incubation with erythrocytes from ESRD patients. The observed eNOS reduction induced by erytrocytes from ESRD patients was totally abolished by annexin V, able to mask red blood cell (RBC) surface-exposed phosphatidylserine.

Conclusion. These findings suggest that adhesion of erythrocytes from ESRD patients to vascular endothelium may cause a decrease in the levels of eNOS mRNA and protein, and inhibition of NOS activity. This might contribute to endothelial dysfunction, and may play a role in the pathogenesis of cardiovascular disease in ESRD patients.

Key words: endothelial nitric oxide synthase, erythrocyte, uremia.

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The vascular endothelium has long been thought to be a single lining of the blood vessels but it is now recognized to be a complex organ with paracrine, autocrine, and endocrine functions [1–3]. Healthy endothelium plays a pivotal role in the maintenance of vascular homeostasis and in the prevention of atherosclerosis [4]. Many of the favorable and antiatherogenic effects of intact endothelium are exerted by nitric oxide. NO is continuously synthesized from L-arginine in the endothelial cells by constitutive eNOS activity [5]. NO is a potent vasorelaxing substance that regulates vascular tone and vasomotor function [6, 7]. In addition, NO inhibits platelet aggregation [8], leukocyte adhesion to the endothelium [9], and vascular muscle cell proliferation [10], all key events involved throughout the course of atherosclerosis [3].

Patients suffering from end-stage renal disease (ESRD) experience accelerated atherosclerosis [11], and the high prevalence of atherosclerotic cardiovascular events accounts for much of the morbidity and mortality in this patient population [12]. Perturbations of the endothelial physiology (endothelial dysfunction) might be a pathogenic mechanism contributing to the markedly increased cardiovascular risk of ESRD [13]. Reduced endothelium-dependent vasodilation and raised plasma levels of endothelium-derived mediators have consistently been observed in chronic renal failure patients [14–18]. Since NO is the principal endothelium-derived vasodilator, the finding of attenuated endothelium-dependent vasodilation suggests a reduction in the NO bioavailability in chronic uremic patients [14–16]. The mechanisms of reduced NO bioactivity in renal failure remain unclear, however.

Defective eNOS is thought to be a crucial parameter characterizing endothelial cell dysfunction [19]. Decreased eNOS mRNA and protein levels and inhibition of NOS activity have been shown to occur following the adhesion of sickle erythrocytes to the vascular endothelium [abstract; Phelan et al, *Blood* 86(Suppl 1):418a, 1995] [20, 21]. Erythrocyte-endothelial interaction is especially

Table 1. Demographic and clinical characteristics of the study population

	Control ($N = 12$)	Hemodialysis ($N = 12$)	P value
Male	6	7	NS
Smoker	1	1	NS
Age *years*	57 ± 7.5	61.6 ± 10.4	0.225
Systolic blood pressure *mmHg*	125.4 ± 10.7	123.2 ± 16	0.712
Diastolic blood pressure *mmHg*	77 ± 7.2	76.7 ± 10	0.927
Total cholesterol *mg/dL*	165.7 ± 33.5	167 ± 39.6	0.930
Triglycerides *mg/dL*	131.3 ± 34	161 ± 99.8	0.341
Haemoglobin *g/dL*	14.3 ± 1.3	11.1 ± 1.6	<0.001
Creatinine *mg/dL*	1 ± 0.1	9.7 ± 2	<0.001
Body mass index	24.4 ± 1.2	24 ± 1.9	0.547

pronounced in sickle cell disease, and may be relevant to reported vascular damage [22]. In both renal failure patients and experimental models in vivo, there is some evidence of a reduced expression of endothelial NOS and a resultant deficiency of NO [23]. Because red blood cells from patients on hemodialysis have an increased propensity to adhere to human endothelial cells through surface-exposed phosphatidylserine [24], it is possible that increased adhesiveness to vascular endothelium of uremic erythrocytes might affect the endothelial NO synthetic pathway. In the present study, we examined the effect of uremic erythrocytes on the expression and the functional activity of eNOS in human umbilical vein endothelial cells.

METHODS

Study population

Red blood cells (RBC) were obtained from ESRD patients on chronic maintenance hemodialysis and from healthy control subjects. Table 1 shows the main clinical and biochemical characteristics of the participants. ESRD patients had been on regular dialysis treatment for more than 6 months (mean time on dialysis 47 ± 38 months), and were recruited from among those presenting at the dialysis center of the University of Chieti. The cause of ESRD was chronic glomerulonephritis in 6 patients, chronic interstitial nephritis in 4, and nephroangiosclerosis in 2. Exclusion criteria included age younger than 18 years or older than 75 years, diabetes mellitus, uncontrolled hypertension, active infection, malignant or inflammatory disease, blood transfusion over the past 3 months, and iron and folic acid deficiency. All patients were on a stabilized erythropoietin dosage, and were undergoing dialysis 3 times weekly with a 4-hour dialysis session using bicarbonate dialysate and first-use synthetic membranes (polysulphone, $N = 7$; polyacrylonitrile, $N = 5$). Healthy control subjects enrolled in the study were not receiving any medication. Subjects with any abnormalities on routine physical examination, standard laboratory tests, ECG, or chest x-ray were not included in the study. Written informed consent was obtained from all taking part in the study.

Materials

Phosphate-buffered saline (PBS), glutamine, fetal calf serum (FCS), and trypsin were from Mascia Brunelli (Milan, Italy); heparin, collagenase, gelatin, endothelial cell grown factor (ECGF), HEPES, EDTA, NADPH, Nonident P-40, sodium deoxycholate, SDS, L-NAME, $CaCl_2$, and BH_4 were from Sigma Chemical Co. (St. Louis, MO, USA); ionomycin from Boeringher Mannheim (Mannheim, Germany), M199 medium from Biospa (Milan, Italy); and DMEM/F12 from Gibco BRL (Grand Island, NY, USA). Fluorescein isothiocyanate-labeled annexin V (FITC-AnV) was from Bender MedSystems (San Bruno, CA, USA). Heavy- and light-chain specific (goat) peroxidase was from Calbiochem (San Diego, CA, USA), 4–12% NuPAGE Novex Pre-Cast gel system was from Invitrogen (Carlsbad, CA, USA). Hybond ECL nitrocellulose membranes and Enhanced Chemiluminescent (ECL) was from Amersham Pharmacia Biotec (Bucks, England). Bio-Rad Image Processing and Analysis System was from Bio-Rad (Hercules, CA, USA). [^{3}H] arginine monohydrocloride 30 to 60 Ci/mmol was from Perkin Elmer (Boston, MA, USA). Resin DOWEX AGWX8-200 was provided by Aldrich (Steinheim, Germany). All chemicals for real-time polymerase chain reaction (PCR) were from Applied Biosystems (Foster City, CA, USA).

Antibodies: mouse monoclonal anti-β-actin (1:10000) was from Sigma; mouse monoclonal anti-eNOS (1:2500) was from BD Transduction Lab (Lexington, KY, USA); and antimouse IgG (1:10,000) was provided by Calbiochem.

Preparation of cells

Erythrocytes. Blood was drawn by venipuncture into evacuated tubes containing EDTA. After centrifugation at 700 *g* for 5 minutes at 4°C, the plasma and buffy coat were removed, the RBC pellet was washed 3 times with PBS to remove traces of serum, and then resuspended to 0.5% hematocrit in DMEM/F12 with 0.1% of FCS.

Endothelial cells. Umbilical cords were obtained from randomly selected healthy mothers giving birth at Chieti University Hospital. Primary cultures of human umbilical vein endothelial cells (HUVEC) were obtained as previously described [25]. After perfusion of umbilical cords with 0.1% collagenase at 37°C, HUVEC were grown on 0.2% gelatin-coated tissue culture plates in M199 endothelial growth medium supplemented with 20% FCS, 10 μg/mL heparin, and 50 μg/mL ECGF (complete medium). In all experiments cells were used between the 3rd and 6th passage in vitro.

Experimental protocol

Endothelial cells (EC) were plated (3.5×10^5 cells mL^{-1}) in Petri dishes (100 mm^2) or 6-well plates in complete M199 endothelial growth medium, and grown to confluence. After 72 hours, the EC monolayer was washed twice with PBS to remove traces of serum, and incubated for 3, 6, 18, and 24 hours with DMEM/F12 with 0.1% of FCS (basal), in the presence or absence of red blood cells (RBC 0.5%) from healthy controls (control) or from patients with end-stage renal disease (ESRD).

In another set of experiments, RBC suspensions from uremic patients (obtained after centrifugation as described above) were incubated for 5 minutes with FITC-AnV (100 nmol/L) to mask RBC surface-exposed phosphatidylserine (PS) [24] before they were added to HUVEC monolayers.

eNOS Western blot analysis

As described in the experimental protocol, HUVECs were first stimulated and then lysed in lysis buffer containing PBS, 1% Nonident P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 4% protease inhibitor cocktails. Protein concentrations in the cell lysates were measured by spectrophotometric assay (Bradford protein assay; Bio-Rad). Equal amounts of protein (30 μg) were analyzed by Western blot (WB) with 4–12% NuPAGE Novex Pre-Cast gel system (Invitrogen). The nitrocellulose membranes were then blotted in TBS-milk and incubated overnight with mouse monoclonal anti-β-actin or mouse monoclonal anti-eNOS. The nitrocellulose membranes were washed in TBS-Tween 20 (0.1%, v/v), and incubated with anti-mouse IgG for 2 hours. After further washing with TBS, blots were developed using ECL, and quantified using a computerized densitometric system (Bio-Rad Image Processing and Analysis System; Bio-Rad).

eNOS mRNA quantification by real-time PCR

RNA preparation. Total RNA was extracted from the HUVEC using NucleoSpin RNAII (Macherey-Nagel GmbH & Co., Duren, Germany).

RT reaction. Two micrograms total RNA was employed to synthesize of first strand cDNA using the High-Capacity cDNA Archive Kit (PE Applied Biosystems, Foster City, CA, USA).

Real-Time PCR. A single-tube real-time PCR assay was optimized for the quantization of e-NOS gene expression versus GAPDH or 18s rRNA ("housekeeping" genes) with specific primers and probes using TaqMan™ technology on an ABI Prism 9700HT Sequence Detection System Instrument (Applied Biosystems) connected to Sequence Detector Software (SDS version 2.0; Applied Biosystems) for collection and analysis of data.

According to the recommendations of the manufacturer, 25 μL reactions were performed in a MicroAmp Optical 96-well reaction plate using 12.5 mL TaqMan Universal PCR Master Mix 2X, 1.25 mL Assay-on-Demand™ Gene Expression Product 20X for e-NOS target genes (TaqMan MGB probe, FAM™ dye-labeled; Applied Biosystems), and 1.25 mL Pre-Developed TaqMan Assay Reagent 20X for GAPDH reference genes (TaqMan MGB probe, VIC™ dye-labeled). For each sample, 5 μL of the cDNA reaction mix, corresponding to 200 ng of RNA transcript, was added.

PCR was performed at 50°C for 2 minutes and at 95°C for 10 minutes, and then run for 45 cycles at 95°C for 15 seconds and at 60°C for 1 minute. Each sample was tested in triplicate and repeated twice. The means of Ct values calculated by SDS Software for e-NOS and GAPDH in each reaction well were used for further calculations.

For each sample, the fold decrease in e-NOS gene expression after RBC incubation was evaluated with 2^{-DDCt} according to the comparative Ct method, where DDCt was the normalized mean Ct value of untreated cells − the normalized mean Ct value of treated cells. For validation of the comparative Ct method, a relative efficiency plot e-NOS/GAPDH was evaluated.

Nitric oxide synthase (NOS) activity

HUVEC grown to confluence in 100-mm diameter Petri dishes were stimulated by RBC from healthy subjects or RBC from ESRD patients and, as a positive control, by ionomycin (2 μmol/L) for 18 and 24 hours. After incubation, cells were detached by trypsin/EDTA (0.05/0.02% v/v), washed with PBS, then resuspended in 1 mL of reaction buffer (HEPES 20 mmol/L, EDTA 0.5 mmol/L, dithiothreitol 1 mmol/L, pH 7.2) and sonicated on ice with 3 10-second bursts. In each test tube, the following reagents were added to 100 μL lysate at the final concentrations: 2 mmol/L NADPH, 1.5 mmol/L $CaCl_2$, 0.1 mmol/L BH_4 (tetrahydrobiopterin), 2.5 μCi L-[^{3}H]arginine = (0.4 μmol/L) [26, 27]. After a 15-minute incubation at 37°C, the reaction was stopped by adding 2 mL HEPES-Na pH 6 containing 2 mmol/L EDTA; the entire reaction mixture was applied to 2-mL columns of Dowex AG50WX-8 (Na^+ form) and eluted with 4 mL of water. The radioactivity corresponding to the [^{3}H]citrulline content in the eluate was measured by a liquid scintillation analyzer (Packard Bio Science Company, Meriden, CT, USA). NOS activity was expressed as pmol citrulline/min/mg cell protein. In some experiments, L-NAME (1 mmol/L) was added 40 minutes before adding L-[^{3}H]arginine.

Statistical analysis

Results are presented as mean ± SD. For comparison of data, analysis of variance (ANOVA) test followed by Bonferroni's analysis was used. Significance was defined as a P value less than.05.

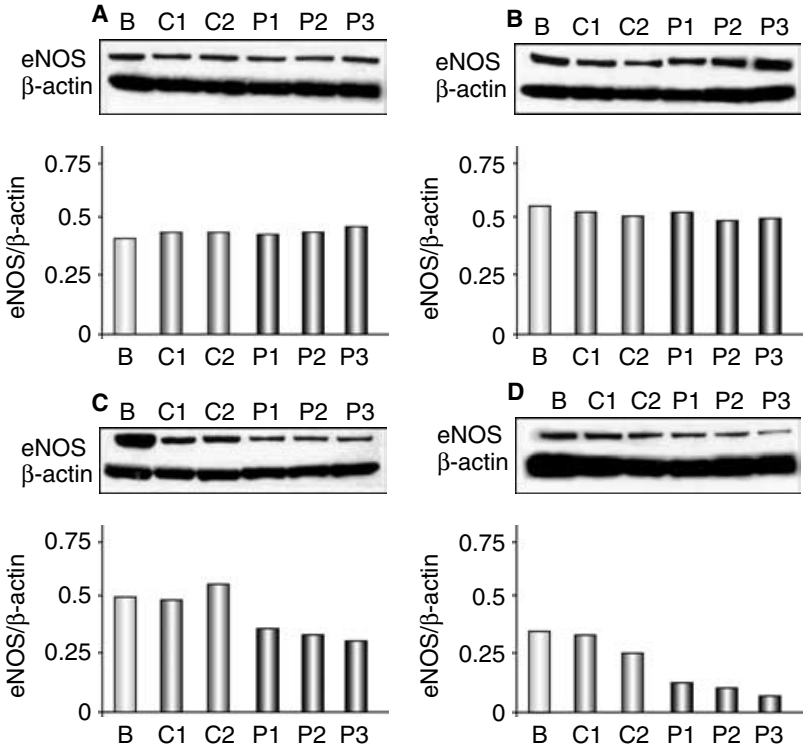

Fig. 1. Representative protein blotting of eNOS and β-actin after 3 hours (*A*), 6 hours (*B*), 18 hours (*C*), and 24 hours (*D*) of HUVEC stimulation with basal medium (▮, B), RBC from healthy controls (▮, C1, C2), or RBC from ESRD patients (▮, P1, P2, P3). Signals of eNOS were quantified and normalized to those of β-actin using a bioimaging analyzer.

RESULTS

In a recent study [24] we demonstrated an enhanced adherence of human uremic erytrocytes to human endothelial cells in culture, possibly via a direct interaction between matrix thrombospondin and increased RBC PS exposure that may serve as a recognition site for the HUVEC monolayers. To clarify whether the increased adherence of washed erythrocytes from ESRD patients might potentially contribute to endothelial dysfunction in uremia, we first evaluated eNOS protein levels (by Western blotting analysis) in HUVEC stimulated with RBC from control and ESRD subjects.

Subsequently, to better understand the possibility of a transcriptional regulatory role being played by RBC from control and ESRD subjects in eNOS synthesis, we also quantified eNOS mRNA by real-time PCR. Because increased PS exposure might play its part in the regulation of endothelial NO release mechanisms, we evaluated both eNOS protein and mRNA levels in HUVEC stimulated with erythrocytes from patients in presence or absence of FITC-AnV, used to mask RBC surface-exposed PS.

Next, in the same experimental conditions, the impact of incubation with RBC (control and ESRD) on NOS activity was also examined. To this end we monitored the conversion of L-[3H] arginine into L-[3H] citrulline.

When the effect of washed RBC (control and ESRD) on eNOS protein levels was followed-up for 24 hours, a distinct time-dependent effect was observed. As shown in representative examples in Figure 1, RBC from ESRD patients (as compared to both basal and control conditions) caused a significant reduction in the eNOS level. Automated image analysis enabled the band intensity of all the samples to be quantified: after 18 and 24 hours, the mean densitometric intensity in samples stimulated by RBC from ESRD patients was, respectively, almost 1.7 and 3 lower than the band intensity in samples stimulated by control RBC (Fig. 2). Furthermore, our data demonstrate that, as compared to endothelial cells treated with RBC from ESRD patients, the addition of AnV (100 nmol/L) to RBC suspensions induced, after both 18 and 24 hours of incubation, a significant increase in eNOS protein levels, which reached those observed at basal conditions (Fig. 3).

We next evaluated the ability of RBC (control and ESRD) to modulate the eNOS mRNA quantity in HUVEC cultures (Fig. 4). After 24 hours of incubation, as compared with cells exposed to RBC from control subjects, RBC from ESRD patients slightly but significantly decreased eNOS mRNA quantity. This inhibitory effect on eNOS mRNA levels by uremic erythrocytes was totally abolished by RBC preincubation with AnV (Fig. 4). We also examined the effect of RBC (control and ESRD) on eNOS mRNA levels after longer time incubation than 24 hours. After 48 hours of stimulation with RBC from ESRD patients, eNOS mRNA quantity significantly

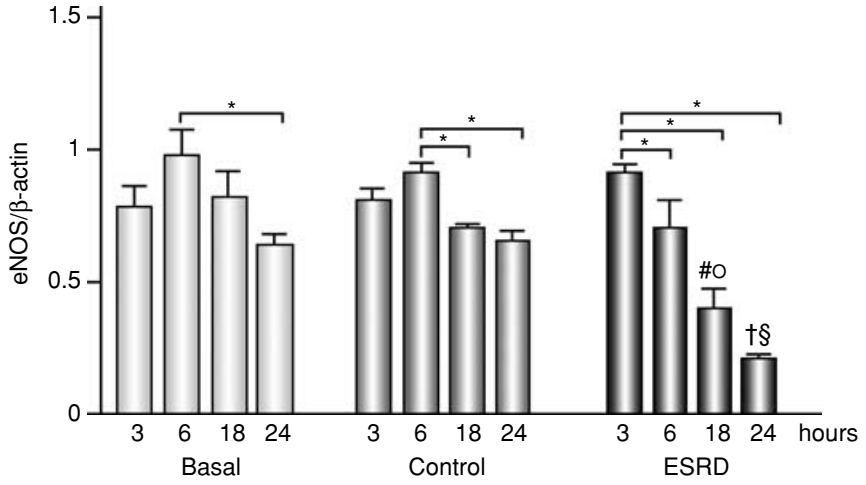

Fig. 2. Time effect of basal medium (▯), RBC from controls (▮, $N = 12$) or RBC from ESRD patients (▮, $N = 12$) on eNOS levels in HUVEC cultures. Signals of eNOS were quantified and normalized to those of β-actin using a bioimaging analyzer. Results are mean ± SD values of three experiments. $^{*}P < 0.05$, $^{\#}P < 0.05$ vs. Basal 18 hours; $^{\circ}P < 0.05$ vs. Control 18 hours; $^{\S}P < 0.05$ vs. Basal 24 hours; $^{\dagger}P < 0.05$ vs. Control 24 hours.

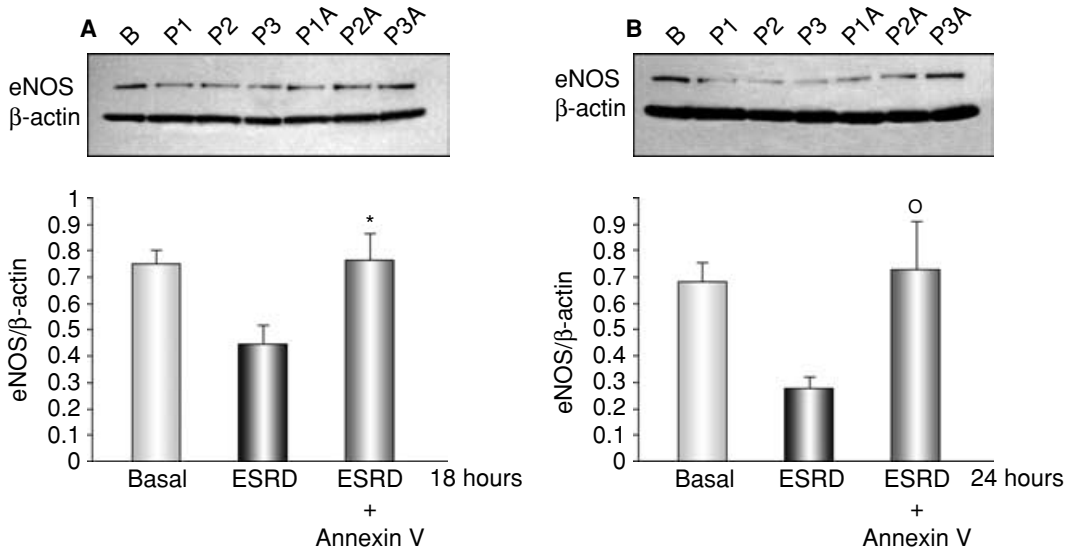

Fig. 3. Effect of RBC preincubation with annexin V (AnV) on eNOS protein levels. RBC from ESRD patients (▮, $N = 12$), RBC from ESRD patients (AnV (▮, $N = 12$) after 18 hours (*A*) or 24 hours (*B*) of HUVEC stimulation. $^{*}P < 0.05$ vs. ESRD 18 hours, $^{\circ}P < 0.05$ vs. ESRD 24 hours.

decreased compared to control (0.47 ± 0.05 and 0.31 ± 0.01 mRNA eNOS quantity, control-RBC and ESRD-RBC, respectively; $P < 0.05$).

The impact of RBC (control and ESRD) on NOS activity was also examined (Fig. 5). Compared with endothelial cells exposed to basal medium (basal), after 24 hours of incubation, RBC from healthy controls significantly increased eNOS activity (0.37 ± 0.03 vs. 0.19 ± 0.03 pmol/min/mg total protein, $P < 0.05$). On the contrary, after 18 and 24 hours of incubation, no difference between endothelial cells exposed to RBC from ESRD patients or basal medium could be detected in terms of NOS activity. Again, compared to controls, NOS activity showed a reduction after 18 hours that became significant at 24 hours of HUVEC incubation with RBC from ESRD patients (0.23 ± 0.02 and 0.16 ± 0.01 vs. 0.29 ± 0.03 and 0.37 ± 0.03 pmol/min/mg total protein, 18 and 24 hours, respectively; $P < 0.05$ at 24 hours). When HUVEC cultures were exposed to ionomycin (a molecule that induces NO production via mobilization of intracellular Ca^{2+}) in order to assess the integrity of the eNOS activation system, cells exhibited a significant increase in NO production ($P < 0.05$). Preincubation with L-NAME (which causes a selective inhibition of NOS activity) caused a significant inhibition in stimulated NOS activity ($P < 0.05$).

DISCUSSION

Several studies indicate that in patients suffering from ESRD NO production is reduced [28]. Given the important role of NO in maintaining vascular function and integrity, deficient NO production may be one of the key steps leading to the functional and structural vascular changes in renal failure. The present study focuses on the

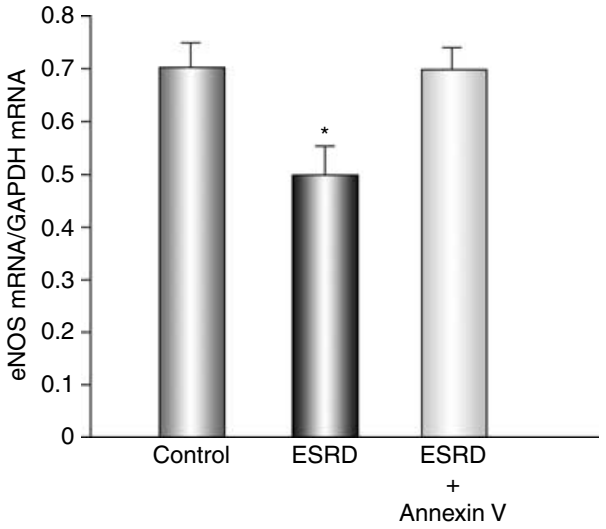

Fig. 4. Effect of RBC from controls (▯, $N = 12$), RBC from ESRD patients (▮, $N = 12$)**, or RBC from ESRD patients preincubated with AnV** (▯, $N = 12$) **on eNOS mRNA quantity in HUVEC cultures by real-time PCR analysis**. Data of eNOS gene expression were normalized versus GAPDH gene expression. Results are mean ± SD values of three experiments. *$P < 0.05$ vs. Control and vs. ESRD + AnV.

ESRD erythrocyte adherence effects on eNOS expression and activity in cultured HUVEC, since decreased eNOS expression and a resultant deficiency of NO might be one of the conjectural mechanisms leading to endothelial dysfunction in ESRD [14].

Our study demonstrated that, in cultured HUVEC, erythrocytes from patients with ESRD inhibited the eNOS expression, activity and, consequently, NO production, whereas erythrocytes from healthy subjects had no such role. This inhibitory effect can be attributed to increased exposure of membrane PS in RBC from ESRD patients, as demonstrated by AnV preincubation effect on eNOS level in our cellular model. Since our observations, along with others [28], support the hypothesis of reduced NO production in ESRD patients. Regarding the possible role of comorbidities/medications in the interactions between erythrocytes and the endothelium, our patients were not affected by morbidities or treated with drugs known to influence adherence of RBC to endothelium, potentially impairing NO production. This supports the hypothesis of an essential role of uremia in increased erythrocyte adherence to endothelium and consequent potential down-regulation of NO synthesis in ESRD patients.

On the other hand, several reports indicate that the NO bioavailability is reduced in ESRD [16]. Recently, much attention has in fact been paid to the evidence that reduction in NO bioavailability, which can be explained not only by a mechanism of NO inactivation but also by a reduced NO release, may have a crucial role in the loss of endothelial function [29]. Nitric oxide can be inactivated by the superoxide radicals (O_2^-) [30], and a variety of pathologic conditions, including chronic renal failure [31], can be associated with the presence of increased O_2^- plasmatic levels. NO and O_2^- can easily react to produce peroxynitrite, a potent long-living oxidant [30], as demonstrated by the presence of nitrotyrosine in biological fluids such as plasma and urine of patients with chronic renal failure.

This hypothesis does not eliminate the possibility that in these patients might coexist both reduced NO release and reduced bioavailability.

However, some studies have also demonstrated an increased NO release in ESRD patients [32]. This suggests that in this pathologic condition, in spite of possible increased NO synthesis, NO availability was not increased and, indeed, very likely reduced. For, as mentioned above [29], in pro-oxidant conditions, a great amount of NO can be rapidly converted into peroxynitrite [32].

Thus, the finding of reduced NO bioavailability, as demonstrated by functional studies, does not provide insight into the mechanisms causing endothelial dysfunction, because reduced bioavailability can be a result of decreased NO production, increased NO degradation, or both.

Our results and several others indicate that in uremia NO production might be reduced [33, 34]. In such patients this may occur by a variety of mechanisms, among which may be included reduced activity of NOS secondary to deficiency of substrate (L-arginine) or critical cofactors for NO synthesis (tetrahydrobiopterin), or inhibition of NOS by endogenous inhibitors accumulating in renal failure, such as asymmetrical dimethylarginine (ADMA) [34]. However, it appears that there is no evidence of any substrate deficiency in NOS in uremia [16]. Furthermore, the physiologic significance of ADMA accumulation in ESRD patients still needs to be definitively established [13].

Since ESRD is associated with accelerated atherosclerosis, it seems reasonable to assume that a circulating "uremic factor" could be responsible for these changes. The vascular endothelium is a likely target for the actions of circulating elements, such us modified RBC and/or plasmatic factors. However, previous cell culture studies did not unequivocally show that uremic plasma factors only affect the eNOS activity in endothelial cell lines [35–37]. As matter of fact, Jankowski et al have recently demonstrated that the eNOS activity was unaffected by accumulation of plasmatic phenylacetic acid in ESRD patients [38].

In our recent paper, supporting the hypothesis of RBC involvement in vascular complication of uremia, we have shown [24] that PS externalization may promote increased uremic erythrocyte adhesion to cultured endothelial cells, and we surmised that this might contribute to endothelial dysfunction in ESRD through an inhibitory effect on eNOS expression and activity. The

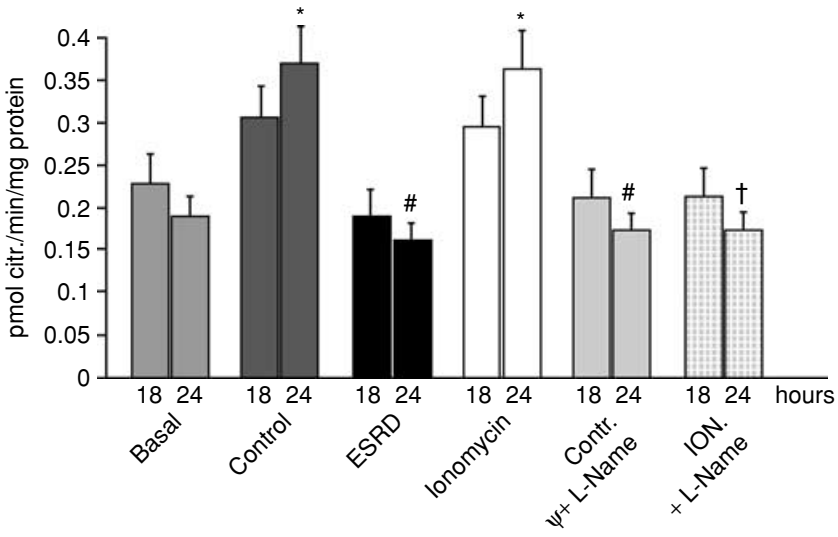

Fig. 5. Effect of RBC from controls (N = 12) **or RBC from ESRD patients** (N = 12) **on NOS activity at 18 hours and 24 hours**. Results are mean ± SD values of three experiments. $^{*}P < 0.05$ vs. basal 24 hours, $^{\#}P < 0.05$ vs. control 24 hours, $^{\dagger}P < 0.05$ vs. ionomycin 24 hours.

present study may offer one explanation of the decreased NO production in patients with ESRD because erythrocytes from patients inhibited eNOS expression and activity in cultured HUVEC.

The mechanism whereby ERSD erythrocytes can regulate gene expression in the endothelium is under investigation. The membrane lipids of uremic red blood cells have been shown to change quantitatively and qualitatively [24, 39], and lipids are widely used in nature as second messengers in pathways leading to gene induction. Recently, it has been shown that PS-liposomes inhibit NO production upstream of the transcription of inducible NO synthase (iNOS) mRNA, and that the inhibition of p38MAP kinase is crucial for this effect [40]. To investigate the interactions of the eNOS enzyme with phospholipids present in membrane and the functional consequences of these interactions on enzyme catalytic activity, Venema et al [41] have shown that binding of eNOS to PS vesicles prevents the subsequent binding of the enzyme to calmodulin, leading to inhibition of eNOS catalytic activity. In this regard, we evaluated both eNOS protein and mRNA levels in HUVEC stimulated with RBC from patients in presence or absence of annexin V, which has a propensity for binding to the PS domain, rendering it unavailable for other biological processes [24]. Preincubation with FITC-AnV totally abolished the ESRD-RBC inhibitory effect on eNOS levels (both protein and mRNA), supporting the idea that the observed decreased NO production might be mediated by increased exposure of PS on the membrane of uremic red cells.

The hypothetical defective activity of eNOS in ESRD, as also suggested by our study, might have several consequences. Reduced NO production, given the key role of NO in maintaining vascular function and integrity, may represent one crucial step leading to the functional and structural vascular changes encountered in uremia, and may, thus, be implicated in the pathogenesis of uremic vasculopathy. In addition, chronic NOS inhibition might have secondary effects, such as increased expression of adhesive molecules and oxidative stress, resulting in endothelial damage and accelerated atherosclerosis [42]. Inhibition of eNOS may also play a causal role in the increased peripheral resistance and elevated blood pressure in ESRD [37]. Finally, the endogenous inhibitory action of uremic RBC on eNOS may be implicated in the altered biology of endothelial progenitor cells (EPCs) observed in uremia [43]. EPCs are mobilized from bone marrow under various stimuli, and are critical for neovascularization and angiogenesis [44]. Because eNOS has an essential role in the mobilization of EPC [45], deficient NO production might lead to decreased mobilization of EPCs from bone marrow [43].

CONCLUSION

Our data demonstrate that adhesion of uremic RBC to endothelial cells in culture may cause a decrease in the levels of eNOS mRNA and protein, potentially due to an increased exposure of membrane PS, and inhibition of NOS activity. This mechanism may contribute to increased atherosclerosis and cardiovascular morbidity in ESRD patients.

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