Kidney International, Vol. 68 (2005), pp. 2173–2179

Effects of nutritional status on the L-arginine–nitric oxide pathway in platelets from hemodialysis patients

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Background. Malnutrition is a common feature in chronic renal failure and adversely affects patient morbidity and mortality. We here investigate the effects of nutritional status on the L-arginine–nitric oxide signaling pathway and platelet function in chronic renal failure patients on regular hemodialysis.

Methods. Platelet aggregation was correlated with plasma amino acid profiles, L-arginine transport, and nitric oxide synthase (NOS) activity determined by conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline and accumulation of intracellular cyclic guanosine monophospate (cGMP) in platelets from malnourished and well-nourished chronic renal failure patients on regular hemodialysis ($N = 78$).

Results. Transport of L-arginine (pmol/10^9cells/min) via y^+ L system was increased in well-nourished (104 ± 15) compared to controls (57 ± 11) or malnourished chronic renal failure patients (55 ± 13). Basal NOS activity (pmol/10^8cells) was enhanced in well-nourished chronic renal failure patients (0.51 ± 0.01) compared to controls (0.18 ± 0.01) or malnourished chronic renal failure patients (0.08 ± 0.03). In addition, basal cGMP levels are elevated in platelets from well-nourished chronic renal failure compared to malnourished uremic patients. Platelet aggregation induced by collagen is impaired in well-nourished chronic renal failure patients compared to malnourished patients and controls. Plasma L-arginine levels are reduced in chronic renal failure patients and even lower in malnourished patients.

Conclusion. Our findings provide the first evidence that L-arginine transport via the high affinity system y^+ L and nitric oxide synthesis are only stimulated in platelets from well-nourished chronic renal failure patients, leading to impaired platelet aggregation. The absence of this adaptive response in the L-arginine–nitric oxide pathway in platelets from malnourished chronic renal failure patients may account for the enhanced occurrence of thrombotic events in these patients.

Key words: L-arginine transport, nitric oxide, platelets, uremia, malnutrition.

Received for publication April 23, 2005
and in revised form June 2, 2005
Accepted for publication June 20, 2005

Chronic renal failure is a complex syndrome characterized by significant abnormalities such as endothelial dysfunction, hypertension, elevation of circulating cytokines and alteration of platelet function, and associated with a disturbance in the L-arginine–nitric oxide pathway [1–9]. Nitric oxide is an endogenous modulator with diverse biologic functions and is produced from the cationic amino acid L-arginine by a family of nitric oxide synthases (NOS) [10]. The majority of studies in animal models and humans suggest that systemic production of nitric oxide is increased in uremia, while inhibition of glomerular nitric oxide is involved in the genesis of chronic renal failure [1–8]. The prolonged bleeding time in uremic patients may be the consequence of increased nitric oxide synthesis, since in animal models this haemostatic defect can be reversed by infusions of the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA) [1, 2, 6].

Malnutrition is a frequent comorbid factor in chronic renal failure patients and exacerbates cardiovascular mortality in these patients [11–13]. Among the earliest indications of nutritional deficiency are low concentrations of plasma amino acids [14], including L-arginine. Several studies have demonstrated that both reduced serum albumin concentration and low body mass index (BMI) are strong predictors of cardiovascular mortality in uremic patients [15–18]. Malnutrition in uremic patients results in elevated levels of circulating cytokines, further exacerbating the oxidative and inflammatory milieu in uremia [13, 17, 19–21].

Platelets possess both inducible NOS (iNOS) and endothelial NOS (eNOS) and interact with endothelial cells [22, 23]. L-arginine, nitric oxide, and nitric oxide donors

inhibit, while L-NMMA potentiates platelet aggregation [22, 24]. Platelet NOS is activated during platelet adhesion to collagen and aggregation induced by adenosine diphophosphate (ADP), collagen, and arachidonic acid [22, 24, 25].

Our group has demonstrated that, unlike red blood cells and leukocytes [26], L-arginine is transported only by system y^+ L in human platelets [27], although there are reports of a weaker activity of system y^+ in these cells [28]. System y^+ L mediates high-affinity, Na^+-independent cationic and Na^+-dependent neutral amino acid transport [26]. Molecular studies indicated that y^+ LAT and 4F2hc combine to induce y^+ L system activity [29, 30]. Similar to endothelial cells, L-arginine transport via system y^+ L in platelets is rate-limiting for the generation of nitric oxide [31, 32].

We reported previously that L-arginine transport into blood cells is increased, while circulating plasma concentrations of L-arginine are reduced in chronic renal failure patients [5, 25, 31, 33, 34]. However, these previous studies did not examine the nutritional status of uremic patients. In the present study, we have examined the transport of L-arginine in platelets in both well-nourished and malnourished chronic renal failure patients on hemodialysis. Additionally, platelet function, basal NOS activity and cyclic guanosine monophosphate (cGMP) in platelets, inflammatory status, and plasma concentrations of L-arginine and related amino acids were investigated in these patients.

METHODS

Subjects

Seventy-eight chronic renal failure patients on hemodialysis and 42 age-matched healthy volunteers participated in the study (Table 1). No patient was on antiplatelet treatment. Most hypertensive patients were using converting enzyme inhibitors and β blockers with only a small percentage on calcium channel blockers. Patients were treated for at least 6 months with hemodialysis three times per week. Blood samples were drawn by venipuncture before a 4-hour dialysis session. The exclusion criteria were heart failure, infection, dyslipidemia, and recent blood transfusion. The Pedro Ernesto Hospital Ethical Committee approved this work, and informed consent was obtained from each of the patients.

Anthropometric measurement

Anthropometric measurements were performed using BMI [i.e., the ratio of postdialysis body weight (kg) divided by height square (m^2)]. BMI values less than 18.5 kg/m^2 were considered malnutrition [35]. BMI was 16.7 ± 1 kg/m^2 in malnourished uremic patients, 22 ± 2 kg/m^2 in well-nourished uremic patients, and 23 ± 3 kg/m^2 in controls.

Table 1. Characteristics of healthy controls and chronic renal failure patients

Data	Controls	Malnourished patients	Well-nourished patients
Number of patients	42	36	42
Age *years*	54 ± 7	47 ± 13	54 ± 14
Gender *male/female*	26/16	24/12	27/15
Months on dialysis	—	24 ± 16	22 ± 12
Dialysis session *minutes*	—	240 ± 0	236 ± 6
Kt/V urea	—	1.3 ± 0.13	1.3 ± 0.08
Hypertension	—	28	37
Vasoactive drugs	—	1.3 ± 0.13	1 ± 0.7
Body mass index	23 ± 3	16.7 ± 1[a,b]	22 ± 2
Albumin *g/dL*	4 ± 0.7	3.4 ± 0.6[a,b]	3.7 ± 0.2
Erythropoietin dose *units/week*	—	4421 ± 797	4285 ± 530
Hemoglobin *g/dL*	13 ± 7	10 ± 1.2[a]	10 ± 1.3[a]
Total cholesterol *mg/dL*	192 ± 5	163 ± 23	176 ± 28
Fibrinogen *g/L*	2.49 ± 0.8	3.47 ± 0.32[a,b]	4.46 ± 0.27[a]

[a] $P < 0.05$ vs. controls; [b] $P < 0.05$ vs. well-nourished patients.

L-(^{3}H)-arginine influx in platelets

Venous blood sample was anticoagulated with a citric acid–dextrose anticoagulant (ACD) (mmol/L) (73.7 citric acid, 85.9 trisodium citrate, and 111 dextrose). As described previously [27], plasma-rich plasma (PRP), obtained by centrifugation (180*g*, 15 minutes) of whole blood, was centrifuged at 800g for 15 minutes. Pellet washed once with ACD was resuspended in Krebs' buffer (mmol/L) (119 NaCl, 4.6 KCl, 1.5 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 15 $NaHCO_3$, and 11 glucose, pH 7.4). Washed platelets (1×10^9 platelets/mL) were incubated at 37°C and L-(^{3}H)-arginine influx (1 to 50 μmol/L) measured over 5 minutes. L-leucine (10 mmol/L), a substrate for system y^+ L, was used to resolve total L-arginine transport in platelets into system y^+ L and transport with diffusion kinetics. Transport was terminated by rapid centrifugation, followed by two washes with Krebs' buffer, recentrifugation and lysis with Triton for β scintillation counting. Platelets were counted using a Coulter counter.

Measurement of platelet NOS activity

Basal NOS activity was determined from the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline [31]. Platelet suspensions (1×10^8 platelets/mL) were incubated at 37°C in the presence of L-[^{3}H]-arginine (2 μCi/mL) plus unlabeled L-arginine (1 μmol/L) for 45 minutes. All reactions were stopped by rapid centrifugation (2000*g*, 15 seconds), followed by two washes with Krebs' buffer. The platelet pellet was lysed with 0.1% Triton and applied to a Dowex cation exchange resin column. L-[^{3}H]-citrulline was eluted with 2 mL water and radioactivity measured by liquid scintillation counting.

Assay of platelet cGMP levels

cGMP content was determined in washed platelets at baseline using a commercial enzyme-linked immunosorbent assay (ELISA) method (Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, washed platelets (1×10^8/mL) were preincubated with 200 μmol/L isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, for 30 minutes. Ice-cold perchloric acid (0.3 mol/L) was added to the platelet suspension, and the platelets were lysed by sonication followed by rapid freezing in liquid nitrogen. Cell debris was then pelleted by centrifugation (2000*g*, 20 minutes). The supernatants containing cGMP were collected and stored at −80°C until ready for assay using the ELISA method.

Platelet aggregation protocol

Platelet aggregation was evaluated on PRP by optical densitometry. Briefly, blood samples were anticoagulated with 3.8% trisodium sodium and centrifuged at 180*g* for 15 minutes at room temperature. Platelet-poor plasma (PPP) was obtained by centrifuging the leftover blood at 800*g* for 10 minutes. The platelet concentration in PRP was adjusted with PPP to a constant count of 2.5×10^8/mL. Aggregation was induced by collagen (2 mg/L) and responses monitored for 5 minutes in a four-channel aggregometer (Chrono-Log, Havertown, PA, USA). Tests were performed at 37°C with a stirring speed of 900 rpm. Maximal aggregation was expressed in percentage.

Determination of plasma levels of fibrinogen and tumor necrosis factor-α (TNF-α)

Briefly, plasma samples were isolated. The concentration of fibrinogen was measured by Clauss Method and TNF-α levels were determined by ELISA method (Amersham, Pittsburgh, PA, USA).

Measurement of plasma amino acid concentrations by high-performance liquid chromatography (HPLC)

As described previously [33, 36], individual amino acids were measured by reverse-phase HPLC, using the fluorescence of the orthophthalaldehyde derivatives with an automated sample processing device (Asted) (Anachem, Luton, UK). The separation resolved all the known plasma amino acids over a 45-minute analysis period. Fluorescence was measured at 340 nm excitation and 440 emission wavelengths using a Jasco 4100 fluorimeter (Jasco, Thermo Separation Products, Stone, UK).

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were of the highest analytic grade.

Statistics

Data are expressed as the means ± SEM of measurements in number of control subjects or chronic renal failure patients. Statistical significance was determined at $P < 0.05$ using one-way analysis of variance (ANOVA) and post hoc Tukey tests (GraphPad Prism Program, San Diego, CA, USA). Curves were fitted with Enzfitter (Elsevier), with a nonlinear least squares fit to the Michaelis-Menten equation.

RESULTS

Patient profile and biochemical and nutritional status

Malnourished chronic renal failure patients presented with lower BMI index and albumin compared to well-nourished patients and controls. Hemoglobin was reduced in the two groups of chronic renal failure patients. Malnourished and well-nourished uremic patients did not differ in relation to the use of vasoactive drugs. Fibrinogen plasma concentration was increased in eutrophic compared with malnourished uremic patients. Control patients had lower plasma fibrinogen levels when compared to both groups of chronic renal failure patients (see Table 1).

L-arginine influx in platelets

Analysis of the nutritional status of chronic renal failure patients revealed that L-arginine transport via system y^+ L was increased primarily in well-nourished chronic renal failure patients, with similar transport rates measured in controls and malnourished chronic renal failure patients (Fig. 1). The transport of L-arginine with kinetics of diffusion was not different in chronic renal failure patients (malnourished 0.25 ± 0.1 hour^{-1} and well-nourished: 0.4 ± 0.1 hour^{-1}) and controls (0.4 ± 0.1 hour^{-1}).

Basal NOS activity and cGMP content in platelets

Basal NOS activity, assaying production of L-[^{3}H]-citrulline from L-[^{3}H]-arginine was increased in platelets from well-nourished chronic renal failure patients compared with controls and malnourished chronic renal failure patients (Fig. 2A). Basal cGMP levels in platelets were also enhanced in well-nourished chronic renal failure patients compared with controls and malnourished chronic renal failure patients (Fig. 2B), confirming the increase in NOS activity measured in platelets from well-nourished patients.

Platelet aggregation

Platelet aggregation in response to collagen was significantly impaired in eutrophic chronic renal failure patients compared to malnourished patients and controls (Fig. 3).

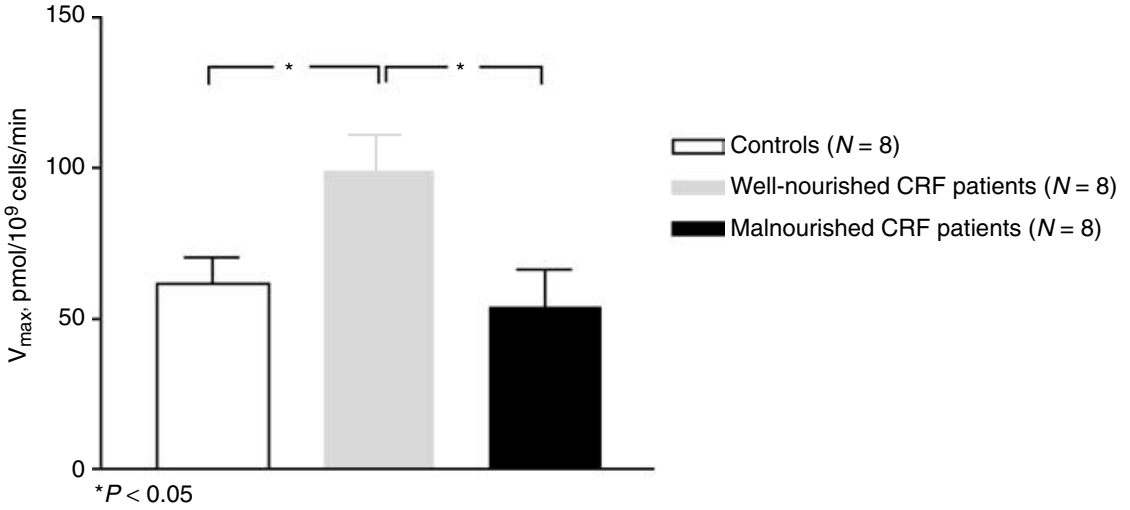

Fig. 1. Effects of malnutrition on L-arginine transport in platelets from chronic renal failure (CRF) patients on hemodialysis. Kinetics of L-arginine transport in platelets isolated from age-matched, healthy controls ($N = 8$) and chronic renal failure patients on hemodialysis ($N = 16$). V_{max} values for system y^+ L mediated L-arginine transport are shown. Data denote the mean ± SEM.

Plasma amino acid profile in well-nourished versus malnourished uremic patients

Blood samples were separated into plasma for analysis of amino acid levels. The results for L-arginine and other amino acids are shown in Figure 4. Plasma L-arginine concentrations were significantly lower in all chronic renal failure patients (94 ± 8 μmol/L) compared with controls (146 ± 14 μmol/L). The reduction in plasma L-arginine levels was more pronounced in malnourished (76 ± 12 μmol/L) compared to well-nourished chronic renal failure patients (107 ± 8 μmol/L) ($P < 0.05$). Malnourished patients also revealed a reduction of plasma L-lysine concentration compared to well-nourished chronic renal failure patients. Plasma concentrations of L-ornithine were elevated in all chronic renal failure patients, while L-citrulline concentrations were only increased in well-nourished chronic renal failure patients compared with controls and malnourished chronic renal failure patients (Fig. 4).

TNF-α levels in well-nourished and malnourished uremic patients

As shown in Figure 5, circulating TNF-α levels were not significantly different in control and well-nourished uremic patients. However, in uremic patients with malnutrition TNF-α levels were significantly increased compared to controls.

DISCUSSION

Cardiovascular disease is the major cause of death in hemodialysis patients. Uremic malnutrition is closely associated with cardiovascular disease risk in chronic renal failure patients. The present results provide the first

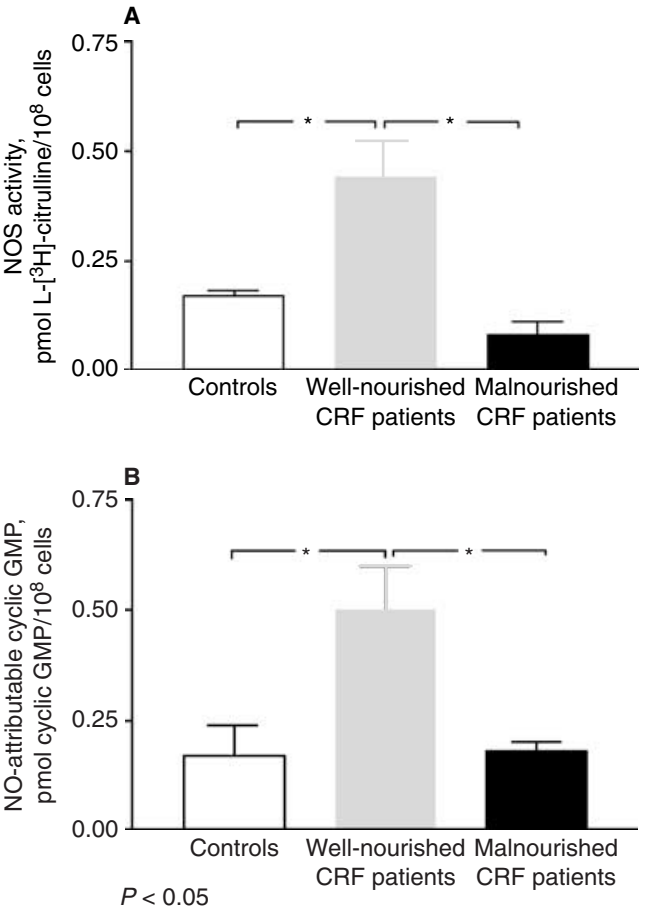

Fig. 2. Effects of malnutrition on basal L-[^{3}H]-citrulline production (*A*) and cyclic guanosine monophosphate (cGMP) levels (*B*) in platelets from age-matched, healthy controls ($N = 6$ to 8) and well-nourished ($N = 6$ to 10) and malnourished ($N = 6$ to 8) chronic renal failure (CRF) patients on hemodialysis. Data denote the mean ± SEM. NOS is nitric oxide synthase.

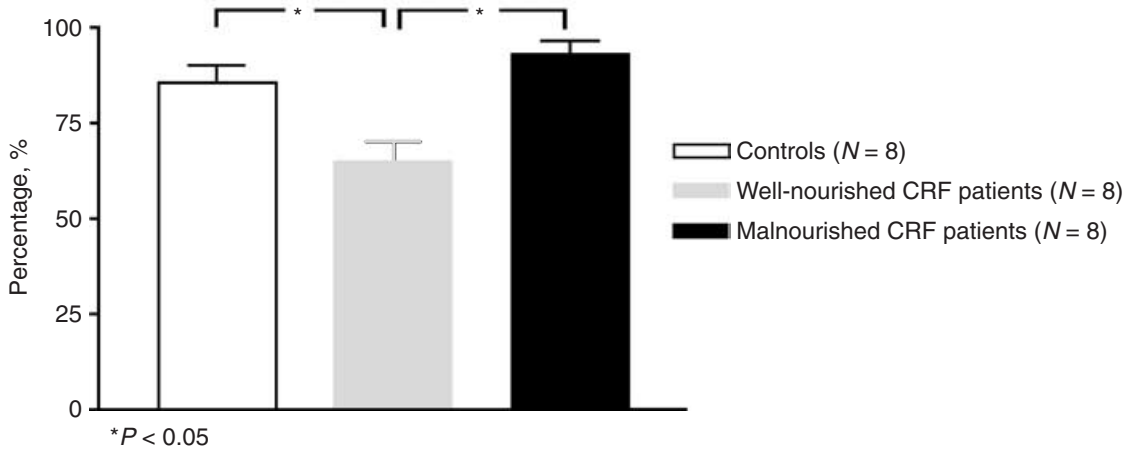

Fig. 3. Platelet aggregation in platelet-rich plasma induced by collagen. Platelets were obtained from age-matched, healthy controls ($N = 8$), well-nourished ($N = 8$), and malnourished ($N = 6$) chronic renal failure patients (CRF) on hemodialysis. Data denote the means ± SEM.

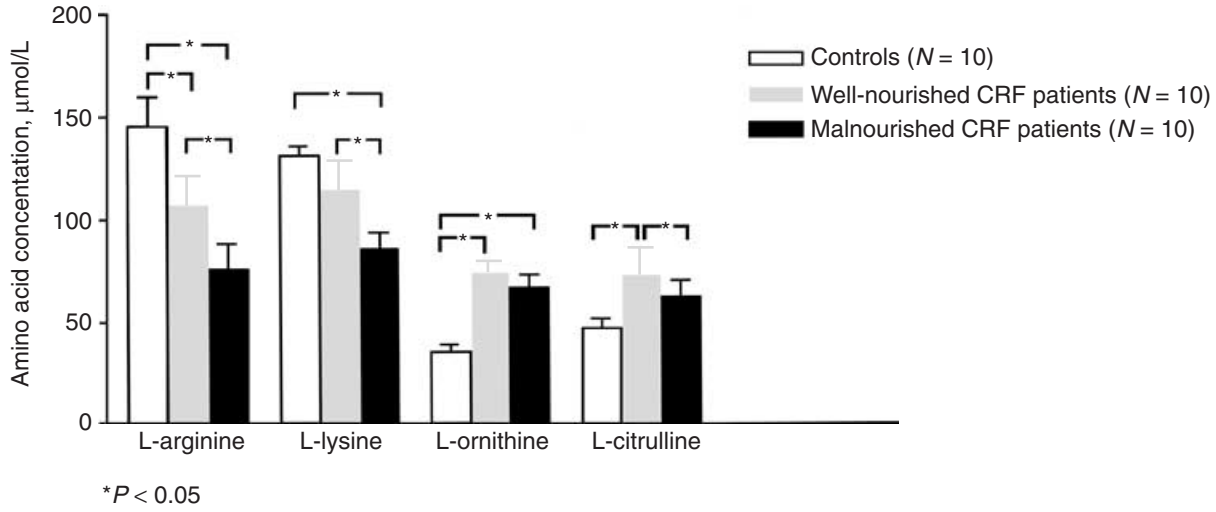

Fig. 4. Plasma amino acid concentrations in chronic renal failure (CRF) patients. High-performance liquid chromatography (HPLC) measurements of amino acid concentrations in plasma from age-matched, healthy controls ($N = 10$) and well-nourished ($N = 10$) or malnourished ($N = 10$) chronic renal failure patients on hemodialysis ($N = 20$). Data denote the means ± SEM.

evidence in human platelets that stimulation of both L-arginine transport via system y^+ L and nitric oxide synthesis is associated with reduced platelet aggregability only in well-nourished chronic renal failure patients. The absence of an adaptive increase in the L-arginine–nitric oxide pathway in platelets from malnourished chronic renal failure patients may account for the thrombotic events in these patients. It is possible that the elevation in L-arginine transport in chronic renal failure provides the necessary supply of substrate for maintaining increased systemic synthesis of nitric oxide in uremia [3, 5]. Increased nitric oxide production in uremic platelets may be responsible for the inhibition of platelet aggregation observed in well-nourished chronic renal failure patients. It has been suggested that reduced platelet aggregation serves as a protective mechanism against thrombosis in a prothrombotic, uremic milieu. Indeed, uremic patients receiving erythropoetin present with a significant improvement in platelet aggregation paralleled by an accelerated atherosclerosis [5].

We previously reported systemic arterial hypertension induces a disturbance in system y^+ L transport activity in human blood cells [7]. The present findings clearly demonstrate that L-arginine transport via system y^+ L is activated in well-nourished chronic renal failure patients with corresponding changes in NOS activity and cGMP accumulation compared to malnourished chronic renal failure patients or age-matched, healthy controls. As L-arginine is the substrate for NOS, activation of L-arginine transport via system y^+ L in platelets from well-nourished patients could provide the necessary substrate for sustaining elevated nitric oxide production [22]. Up-regulation

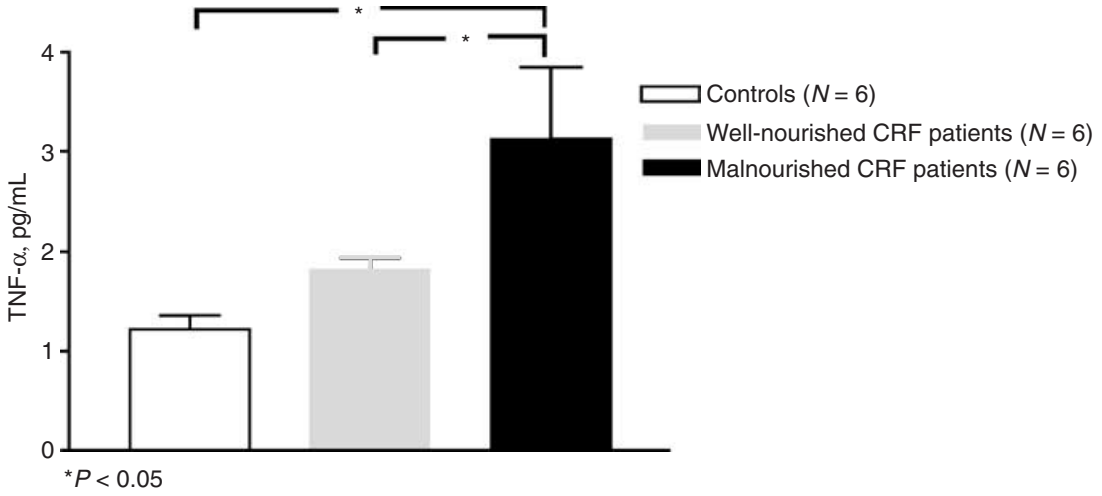

Fig. 5. Tumor necrosis factor-α (TNF-α) levels in plasma from control and chronic renal failure (CRF) patients. Data denote mean ± SEM of measurements in controls ($N = 6$), well-nourished ($N = 6$), and malnourished ($N = 6$) uremic patients.

of systemic nitric oxide synthesis may serve as a protective mechanism against hemodynamic and hemostatic disorders of uremia [3]. Alternatively, increased nitric oxide produced by circulating platelets could sustain the bleeding tendency, a well-known complication of uremia [3, 27].

Although molecular studies have shown that association of y^+ LAT with 4F2hc induces system y^+ L transport activity [29, 30, 37], the mechanisms involved in the regulation of this transport system have not been fully investigated. The elevated plasma levels of TNF-α in malnourished uremic patients (Fig. 5) are associated with diminished system y^+ L activity. Whether there is a direct link between platelet y^+ L activity and TNF-α plasma levels remains to be established. Moreover, we cannot exclude the possibility that cytokines reduce y^+ L activity in human platelets.

The intriguing observation that malnourished hemodialysis patients do not exhibit an activation of system y^+ L or nitric oxide synthesis, highlights the importance L-arginine availability [38] and may explain, in part, the increased risk of cardiovascular morbidity and mortality in this cohort of uremic patients. It is possible that the profound alterations in amino acid metabolism detected in malnourished uremic patients affect the obligatory amino acid exchange mechanism for system y^+ L in platelets. It is well known that the uptake of the amino acids by this transport system depends largely on the intracellular substrate composition. Clinical experience indicates that bleeding and thrombotic tendencies coexist in the general population of uremic patients [39]. Thus, lack of activation of L-arginine uptake and nitric oxide production in the subgroup of malnourished patients may have clinical implications with respect to the reported tendency to thrombosis in uremia.

Increased plasma L-citrulline levels are a common finding in chronic renal failure patients [33], and this finding has traditionally been associated with diminished L-citrulline uptake and low production of L-arginine by the failing kidney. However, as L-citrulline is the coproduct of L-arginine metabolism via NOS, elevated L-citrulline levels are consistent with increased nitric oxide production in well-nourished chronic renal failure patients [3].

CONCLUSION

The present study showed that L-arginine transport via system y^+ L in platelets was only up-regulated in well-nourished uremic patients and associated with increased nitric oxide production and reduced platelet aggregability. The mechanisms involved in the modulation of system y^+ L by the nutritional status of uremic patients remains to be elucidated and may provide insights into the pathophysiology and potential interventions in uremia.

ACKNOWLEDGMENTS

This work was funded by a Wellcome Trust Collaborative Research Initiative Grant (A.C.M.R., J.C.E., and G.E.M.) and PQ-CNPq (Brazil). We thank Clive Lane (Royal Liverpool Hospital) for the plasma amino acid analyses and Professor Richard Bruckdorfer for his advice in monitoring platelet aggregation.

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