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Kidney Int. Author manuscript; available in PMC 2009 September 16.

Published in final edited form as:
Kidney Int. 2001 April ; 59(4): 1466–1472. doi:10.1046/j.1523-1755.2001.0590041466.x.

Circulating endothelial nitric oxide synthase inhibitory factor in some patients with chronic renal disease

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Abstract

Circulating endothelial nitric oxide synthase inhibitory factor in some patients with chronic renal disease.

Background—Chronic renal disease (CRD) is associated with hypertension and reduced synthesis of nitric oxide (NO). Here, we investigated whether there is a circulating endothelial NO synthase (eNOS) inhibitory factor(s) in some patients with CRD that might directly influence endothelial NOS.

Methods—Human dermal microvascular endothelial cells (HDMECs) were incubated for six hours with 20% plasma from subjects with normal renal function ($P_{Cr} = 0.8 \pm 0.2$ mg%), and patients with moderate renal insufficiency of various causes ($P_{Cr} = 4.0 \pm 1.5$ mg%) and impact on NOS activity, transport of L-arginine, and abundance of eNOS protein were measured. Plasma concentrations of asymmetric and symmetric dimethyl L-arginine (ADMA and SDMA) were also measured.

Results—There was no effect of any human plasma on L-arginine transport. The NOS activity was variable in CRD patients and fell into two subgroups: CRD I, individual values similar to control, and CRD II, individual values lower than control mean. The effect of CRD plasma on NOS activity in cultured cells was not related to the primary disease, but was predicted by plasma ADMA levels since plasma ADMA was elevated in CRD II versus both control and CRD I. Blood urea nitrogen and creatinine levels were uniformly elevated in CRD plasma. The abundance of eNOS protein was unaffected by plasma.

Conclusion—High plasma levels of ADMA in CRD patients are independent of reduced renal clearance, suggesting an alteration in ADMA synthesis and/or degradation. High ADMA is a marker and is partly responsible for the inhibition of eNOS activity in cultured cells and may also result in reduced eNOS activity in vivo, with consequent hypertension.

Keywords

cell culture; human dermal microvascular endothelial cells; L-arginine transport; asymetric dimethylarginine; end-stage renal disease

Hypertension is common in chronic renal disease (CRD) patients and is an important risk factor in the accelerated deterioration of renal function to end-stage renal disease (ESRD) [1]. Nitric oxide (NO) deficiency has been suggested to play a role in hypertension secondary to renal failure [2]. In support of this, we have reported a reduction in the total amount of NO produced

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in CRD patients [3] as well as in ESRD patients on both peritoneal dialysis and hemodialysis [4,5].

Nitric oxide is produced in many locations in the body, and it is the continual synthesis of vasodilatory NO by vascular endothelial cells that plays a major role in control of vascular tone, blood pressure (BP), and blood flow [6]. Hypertension occurs in mice with knockout of the endothelial NO synthase (eNOS) gene [7]. Hypertension and other cardiovascular complications have been reported in humans with some (but not all) eNOS gene polymorphisms [8–10]. An accumulation in plasma of methylated arginine analogues, which function as NOS inhibitors, could cause impaired eNOS activity, as originally suggested by Vallance et al in ESRD patients [2]. We recently reported that plasma from ESRD patients has eNOS inhibitory activity when incubated with cultured vascular endothelial cells and that plasma asymmetric dimethylarginine (ADMA) levels are uniformly elevated [11].

In the present study, we investigated whether plasma from patients with CRD exhibited a similar eNOS-inhibitory potential that might contribute to hypertension. In vitro studies were conducted using cultured human endothelial cells from the skin microvasculature to test the hypothesis that in CRD, circulating factors impair endothelial NO production.

METHODS

Plasma was collected from 5 normal subjects and 11 CRD patients [4 patients with diabetes mellitus (DM), 2 with obstructive nephropathy (ON), 1 with IgA + solitary kidney, 1 with a solitary kidney, 1 with focal and segmental glomerulonephritis (FSGS), 1 with chronic interstitial nephritis (CIN), and 1 with Wegener's granulomatosis; Table 1] and was stored individually. Blood samples were collected into heparin-coated tubes, spun cold, aliquoted, and frozen at −80°C within 20 minutes of collection and were thawed immediately prior to use. The renal function in all CRD patients was similar at approximately 30% of normal glomerular filtration rate, as reflected by plasma creatinine and blood urea nitrogen (BUN; Table 2). The demographic and clinical characteristics of the study populations are shown in Tables 1 and 2.

Human dermal microvascular endothelial cells (HDMECs) and endothelium growth medium (EGM-MV) were obtained from Clonetics Corporation (San Diego, CA, USA). HDMECs (passages 4 through 7) were maintained in EGM-V media as described previously [11]. Cells were subcultured onto 12-well plates and, when confluent, were incubated for six hours in modified Eagle's medium (MEM) containing 20% human plasma (either individual CRD patient plasma or pooled normal control). Cells were then studied for arginine transport or NOS activity.

Transport of L-arginine into endothelial cells was measured by the method of Gazzola et al in the presence of 50 μmol/L L-arginine with 1 μL L-[^{3}H]arginine (1 μCi) in 0.5 mL Krebs-HEPES buffer per well [12]. The time course of L-arginine transport in the HDMECs is shown in Figure 1. Since L-^{3}H-arginine uptake is linear up to five minutes, in all subsequent experiments, L-arginine transport was measured at the three-minute incubation time.

Nitric oxide synthase activity was determined in living cells by measuring L-[^{3}H]arginine conversion to L-[^{3}H]citrulline according to the method of Davda et al [13], with minor modifications that we have described previously [11]. NOS activity and L-arginine transport were expressed in terms of the total cell protein, determined by the Bio-Rad detergent method, with bovine serum albumin (BSA) as a standard.

In separate studies, HDMECs were grown in T75 flasks for measurement of total intracellular arginine concentration and eNOS protein abundance by Western blot. Confluent HDMECs

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were incubated for six hours with pooled control plasma (from 4 normal subjects) and plasma from CRD patients with normal plasma NOS activity and CRD patients with low plasma NOS activity (all $N = 3$). Arginine was measured on cell lysates by reverse-phase high-performance liquid chromatography (HPLC) using the AccQ Tag method, as described by us previously, except that the column temperature was maintained at 41°C [4]. Abundance of eNOS protein was measured on cell lysates (75 μg protein in 50 μL) by Western blot. Proteins were electrophoretically separated on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE; 200 V, 65 min), transferred to nitrocellulose (Bio-Rad trans-blot SD semidry transfer cell, 105 min, mA). After blocking the membrane for one hour in 3% nonfat milk (NFM) TBS-T, the eNOS was detected with mouse monoclonal antibody (N30020; Transduction Laboratories, Lexington, KY, USA) at 1:1000 dilution for one hour at room temperature, and secondary antibody, goat anti-mouse IgG-horseradish peroxidase (Transduction Laboratories M15345), 1:2000 dilution for one hour at room temperature. Membranes were then stripped and reprobed for β-actin (Sigma mouse mAb, A5441, 1:60,000 dilution for 1 hour at room temperature; secondary antibody: anti-mouse IgG-horseradish peroxidase, 1:60,000 dilution for one hour at room temperature). A separate membrane was probed with rabbit polyclonal antibody to iNOS (Santa Cruz #651), 1:200 dilution at room temperature for one hour and secondary antibody (goat, anti-rabbit; Santa Cruz #2004) 1:2000 dilution for one hour at room temperature. Equal loading was also verified by Ponceau red staining of the membrane, prior to probing with specific antibodies. Protein abundance was visualized by ECL on Kodak X-OMAT AR film and quantitated by image analysis (Optimas 6.2, Bothell, WA, USA) of the integrated optical density.

L-arginine analogues, asymmetric dimethyl L-arg (ADMA), and symmetric dimethyl L-arg (SDMA) were measured in plasma using reverse-phase HPLC with the AccQ Tag method, as we described previously [4]. The limit of detection of ADMA is 0.15 μmol/L, and SDMA is 0.1 μmol/L in human plasma.

Arginine transport was expressed as pmol arginine transported/min/mg of protein and NOS activity as pmol arginine converted to citrulline/min/mg of protein [11]. Each assay was run in triplicate, and experiments were repeated at least three times. Individual numbers per group are given in the table or figure legends. Results are expressed as mean ± SEM. Statistical analysis was performed with the use of Student unpaired *t* test and one-way analysis of variance (ANOVA). Values of $P < 0.05$ are considered to be significantly different.

RESULTS

As shown in Table 1, the CRD and control populations were matched for age and body surface area (BSA). CRD patients had significant systolic hypertension (Table 2), even though most CRD patients were on one or more antihypertensive medications (Table 1). The primary cause of the renal disease was variable (Table 1), and there was a similar loss of renal function in all CRD patients, with glomerular filtration rate (24-hour creatinine clearance) being approximately 30% of the normal value (Table 2).

The effect of six-hour incubation with 20% control or CRD patient plasma on NOS activity in HDMECs is shown in Table 3. Data for controls are averaged, and the 11 CRD patient plasmas are shown both as group average and individually. There was no difference in the average values of the NOS modulatory effect of plasma between controls and the entire CRD group, although the variation was greater in the latter. However, when the NOS modulatory effect of each individual CRD plasma was compared statistically to the control mean value, we found that six CRD patient plasmas had no impact on eNOS activity (CRD I), while plasma from five CRD subjects exerted a significant reduction in eNOS activity (CRD II) versus control (Table 3, $P < 0.05$). As shown in Figure 2, as with the NOS modulatory effect of plasma, there

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was no difference between the average plasma level of ADMA in all CRD patients and in controls. However, when the CRD plasma was separated into two subgroups depending on the NOS modulatory effect, plasma ADMA was high in the low NOS activity group (CRD II) versus both control and the group of CRD plasma with normal NOS activity (CRD I). The NOS activity of plasma was predictive of the plasma ADMA level on a blinded basis and vice versa. In contrast to the variability in the plasma ADMA level, the plasma concentration of the SDMA was uniformly elevated in all CRD patients. As shown in the lower panels of Figure 2, plasma levels of creatinine and BUN are similarly elevated versus controls in all CRD patients. Therefore, the difference in plasma levels of ADMA between CRD plasma subgroups I and II is not primarily related to the level of loss of renal function.

To establish that increasing plasma ADMA could inhibit eNOS activity in vitro, HDMECs were incubated for six hours in synthetic solutions containing different concentrations of ADMA. We found that NOS activity was significantly inhibited by 2.5 μmol/L ($P < 0.05$) ADMA, while 1 μmol/L ADMA had no effect (16.6 ± 0.4 vs. 18.8 ± 0.8 vs. 19.8 ± 0.7 pmol/min/mg · protein in control). It is important to point out that after a one in five dilution, the level of ADMA in patient plasma is < 1 μmol/L, even in CRD II, suggesting that other eNOS-inhibitory material is also present.

To determine whether differences in NOS activity of CRD patient plasma are related to stimulation of iNOS in some cases, HDMECs were pretreated with dexamethasone (10 μg/mL for 1 hour), which inhibits iNOS transcription [6]. We have found that dexamethasone pretreatment prevents lipopolysaccharide (LPS; 10 μg/mL) + interleukin-1β (IL-1b; 100 U/mL) + interferon-γ (IFN-γ; 100 U/mL)-stimulated iNOS activity in these cells (data not shown). Data given in Table 3 show that prior dexamethasone pretreatment had no effect on the NOS modulatory effect of any of the human plasmas.

There was no difference in L-arginine transport into HDMECs after six hours of incubation between the mean value for CRD patient plasma (190 ± 19 pmol L-arginine/min/mg · protein) or any individual CRD plasma value compared with control (198 ± 24 pmol L-arginine/min/mg · protein). The intracellular concentrations of L-arginine were calculated from L-arginine content expressed as μmol/g total cell protein and factored for volume, assuming 1 mg intracellular protein = 6 μL intracellular water [14]. The intracellular L-arginine concentration was 445 ± 28 μmol/L in cells treated with control plasma, similar in cells treated with low NOS activity plasma (510 ± 24 μmol/L), and slightly higher in cells treated with normal NOS activity CRD plasma (578 ± 33 μmol/L, $P < 0.02$ vs. control). As shown in Figure 3, Western blot analysis of eNOS protein level from HDMECs showed that there was no impact of CRD II plasma (from 3 of the subjects) on eNOS abundance versus either CRD I or controls (both $N = 3$). Equal loading was verified by both β-actin and Ponceau red staining. Furthermore, iNOS was undetectable in any of the cell lysates after six hours of incubation with any human plasma.

DISCUSSION

The main finding from this study is that the eNOS-inhibitory potential in plasma from patients with CRD is variable and is predicted by the plasma ADMA concentration. Plasma from approximately 50% of CRD patients inhibited eNOS activity in cultured cells, but there was no obvious relationship between the effect on NOS activity of the plasma and the primary disease. For example, plasma from two patients with diabetic nephropathy showed eNOS-inhibitory activity, while two others were normal (Table 3). Several of the patients had immunemediated diseases (for example, CIN, IgA nephropathy), and increased intrarenal NO generation via iNOS has been reported during the acute phase of immunologically mediated glomerular diseases [15], although we have recently reported that total NO production is low in rats with chronic, post-glomerulonephritis CRD (abstract; Wagner et al, *J Am Soc*

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Nephrol 11:632A, 2000). Nevertheless, we were concerned that iNOS-activating factors in some CRD plasma samples might obscure falls in eNOS activity. However, pretreatment of cultured cells with dexamethasone, which inhibits iNOS transcription [6], had no effect on NOS activity after incubation with CRD patient plasma for six hours. Furthermore, there was no evidence of iNOS protein, by Western blot, after six hours of incubation of HDMECs; thus, we are confident that we are measuring eNOS activity.

All CRD patients in this study were on one or more antihypertensive drugs at the time plasma was drawn, and we considered the possibility that the medications might influence the NOS modulatory effect of plasma and perhaps account for the variable effect of plasma from CRD patients. However, there was no obvious association between any given drugs and the eNOS activity of plasma. The drugs used included diuretics (furosemide and hydrochlorothiazide), calcium-channel blockers (amlodipine and diltiazem), β-receptor blocker (metoprolol), and angiotensin II-converting enzyme inhibitors (enalapril, captopril, and lisinopril). Studies by others have shown that these drugs either have no effect on NO production, in the case of β-receptor blockers [16] and the channel blocker verapamil [17] or may increase NO production (diuretics, calcium channel blockers, and angiotensin II-converting enzyme inhibitors) in vascular endothelial cells [17–19]. Therefore, the inhibition of eNOS activity by some CRD patients' plasma is not likely to be related to their antihypertensive therapy.

To determine why the plasma from some CRD patients had NOS inhibitory effects, we measured the concentrations of the L-arginine analogues ADMA and SDMA. It has been reported that ADMA can competitively bind NOS and inhibit NOS activity [2]. Although numerically higher, the average plasma level of ADMA in all 11 CRD patients was not statistically different from the normal control. However, we did find differences when we separated the CRD patients into subgroups according to the level of NOS modulatory effect of their plasma (based on a statistical analysis). All patients whose plasma had NOS inhibitory effects had high plasma concentrations of ADMA (CRD II). In contrast, those whose plasma had no impact on NOS activity had normal levels of ADMA (CRD I). It is important to point out, however, that plasma from the CRD II subgroup, which lowers eNOS activity, is diluted one in five for the six-hour incubation so that the ADMA concentration in contact with the cells is only approximately 0.5 μmol/L. Since this is a concentration that we have shown will not influence eNOS activity in vitro, it is likely that in vivo, elevated plasma ADMA is a partial cause of eNOS inhibition and is also a marker for other eNOS inhibitors in the plasma of some patients with CRD.

Why should high plasma levels of ADMA occur in some but not all CRD patients? The assumption has been that this occurs secondary to reduced renal clearance, as reported for ESRD patients [2,4,5]. However, in the present study, there was no correlation between the level of residual renal function and the plasma levels of ADMA. Both BUN and creatinine levels were uniformly high in all CRD patients, despite marked differences in plasma ADMA. Recently, an increase in plasma ADMA levels has been reported in hypercholesterolemic states in the absence of reduced renal clearance [20]. This suggests that alterations in synthesis and/or catabolism of methylated arginines may also contribute to functional NOS inhibition in some circumstances, including, perhaps, CRD. There is evidence that degradation of methylarginines is regulated by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). DDAH is widely distributed in many tissues and is present in high concentrations in kidney and aorta of the rat [21]. Colocalization of DDAH and NOS has been reported in the kidney [22], and inhibition of DDAH (which allows accumulation of ADMA) inhibits endothelium dependent relaxation [23]. Of note, while ADMA is a substrate for DDAH, the inactive SDMA is not [24]; therefore, an altered DDAH activity may contribute to the elevated ADMA in CRD II patients as in an animal model of CRD [25]. Endothelial cells also contain S-adenosylmethionine– dependent methyltransferases, which are responsible for methylating the

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proteins that subsequently break down to form ADMA and SDMA. These methyltransferases are up-regulated in endothelial cells in response to low-density lipoprotein cholesterol, leading to increased ADMA production [26]. Could a defect in activity of the DDAH enzyme and/or increased methyltransferase activity provide additional risk factors for an increased progression of CRD?

Most likely, multiple factors determine whether NO deficiency will occur in a given patient with CRD. Recently, it was reported that some patients with renal diseases have an eNOS gene polymorphism that could be related to the renal function deterioration [9]. Studies in animal models of CRD indicate reduced expression of the eNOS enzyme within the vasculature and kidney [27]. The hyperparathyroidism of CRD apparently contributes to the depression of NOS activity since parathyroidectomy and Ca channel blockade reverse these abnormalities in vascular NO [27]. Another reason for reduced NOS activity in CRD relates to increased oxidant stress in which oxygen free radicals inactivate newly formed NO and prevent the normal vasodilatory response [28]. Over time, oxidative stress leads to nonenzymatic glycation and oxidation and the accumulation of advanced glycosylated end products (AGEs) [29]. AGEs modify the vascular wall so as to "quench" NO and thus reduce the vasodilatory action of NO (and other agonists) [29]. AGEs also down-regulate eNOS in cultured vascular endothelial cells [30]. In addition to contributing to hypertension and atherosclerosis, NO deficiency exacerbates underlying renal disease [31] and produces "de novo" renal disease in rats [32].

In summary, plasma from CRD patients has a variable effect on eNOS activity in vitro, depending on the ADMA concentration. We suggest that this reflects the in vivo situation. High plasma ADMA early in the course of CRD may be a bad prognosticator of a rapid rate of progression to ESRD and/or increased morbidity caused by cardiovascular complications such as hypertension and atherosclerosis. Why some CRD patients have high plasma ADMA levels is not clear at this time, but is not exclusively related to loss of renal clearance.

ACKNOWLEDGMENTS

These studies were supported by a Beatrice A. Madera grant from the School of Medicine, WVU, Office of the Dean and National Institutes of Health grant #R01 DK 45517. We are grateful to Ms. Jennifer Domico, Mr. Kevin Engels, Ms. Marilyn Howton, Mr. Glenn Kuenzig, and Ms. Lennie Samsell for technical assistance.

Appendix

APPENDIX

Abbreviations used in this article are: ADMA, asymmetric dimethyl L-arginine; BUN, blood urea nitrogen; CIN, chronic interstitial nephritis; CRD, chronic renal disease; DDAH, dimethylarginine dimethylaminohydrolase; eNOS, endothelial nitric oxide synthase; ESRD, end-stage renal disease; HDMEC, human dermal microvascular endothelial cells; iNOS, inducible nitric oxide synthase; mAb, monoclonal antibody; NFM, nonfat milk; NO, nitric oxide; NOS, nitric oxide synthase; ON, obstructive nephropathy; P_{Cr}, plasma creatinine; RT, room temperature; SDMA, symmetric dimethyl L-arginine.

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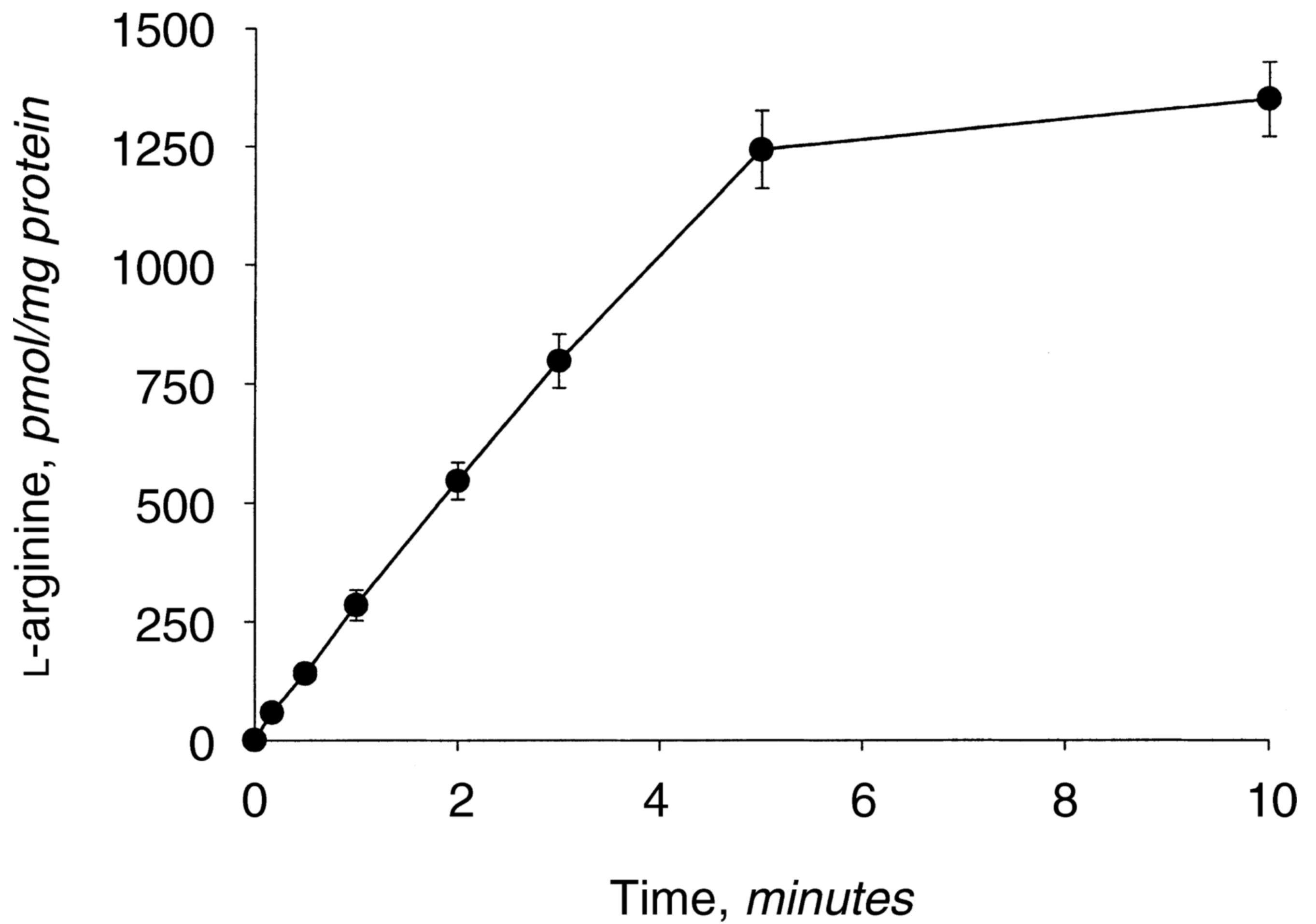

Fig. 1. Time course of L-arginine transport in human dermal microvascular endothelial cells (HDMECs)

Transport was initiated by addition of 50 μmol/L L-arginine/1 μCi L-^{3}H-arginine to the cells incubated with Kreb-HEPES buffer, and specific uptake was determined at indicated times.

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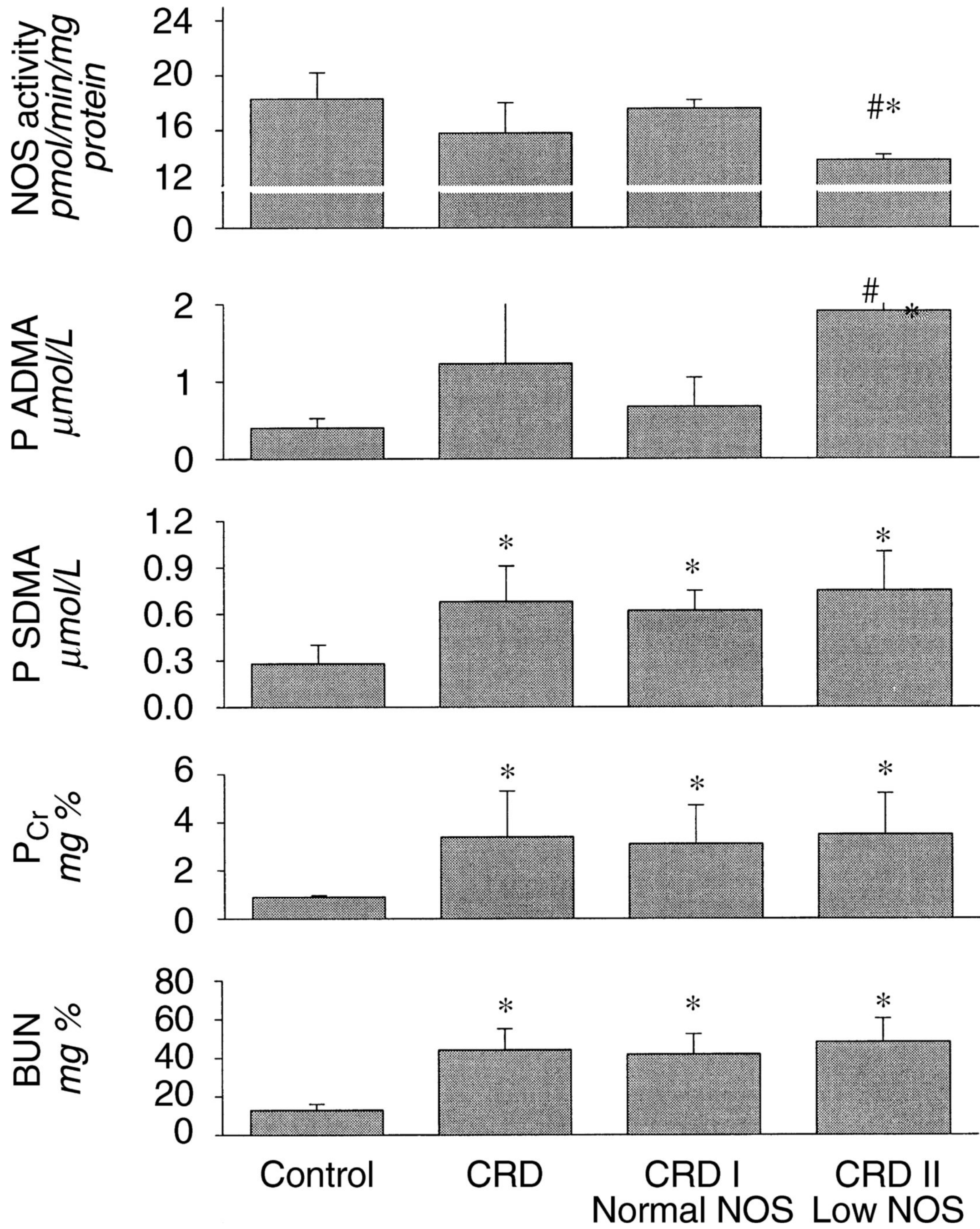

Fig. 2.

Relationship between nitric oxide synthase (NOS) activity of plasma, plasma concentrations of asymmetric and symmetric dimethyl L-arginine (ADMA and SDMA), as well as creatinine (Cr) and blood urea nitrogen (BUN) as indices of the level of renal function. *$P < 0.05$ compared with control; #$P < 0.05$ compared with CRD I patients with normal NOS activity in plasma.

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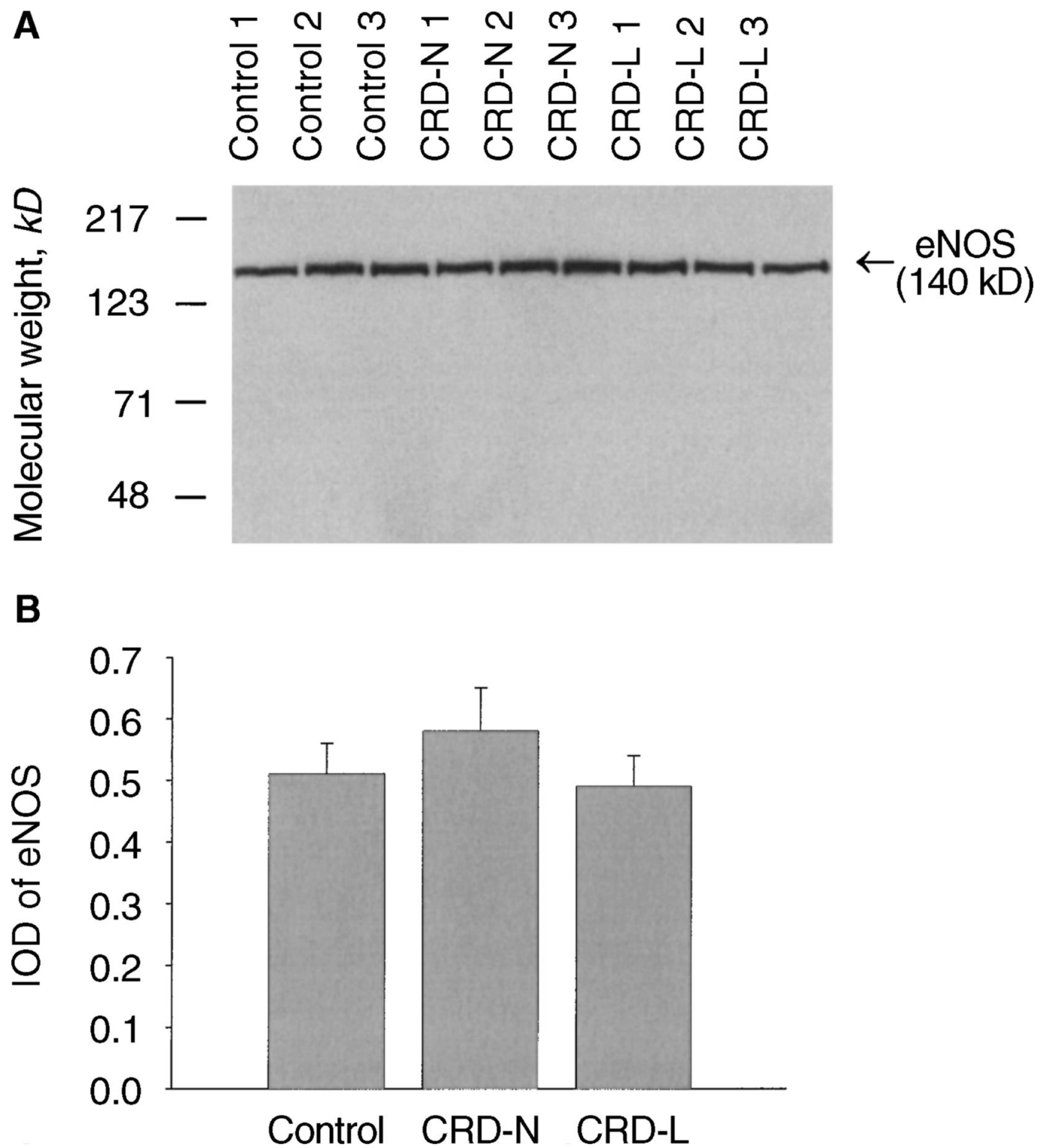

Fig. 3.

A) Western blot of the endothelial nitric oxide synthase (eNOS) protein density in HDMECs after incubation for six hours in 20% control plasma (lanes 1 through 3), 20% plasma from three chronic renal disease (CRD) patients with normal NOS activity (CRD-N, lanes 4 through 6) and 20% plasma from three CRD patients with low plasma NOS activity (CRD-L, lanes 7 through 9). (*B*) Data are expressed as average integrated optical density (IOD) in arbitrary units.

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Table 1

Demographic and clinical characteristics of the study population

CRD	Age	Sex	BSA	Disease	Medication[a]
1	29	M	1.67	Sol kid	ACEI
2	51	M	2.22	Wegener's	CCB
3	56	M	1.75	CIN	CCB, D
4	32	F	2.30	ON	ACEI
5	62	F	2.59	DM	BB, D
6	63	F	2.60	DM	BB
7	65	M	2.10	FSGS	CCB.D
8	78	M	1.92	DM	D
9	59	M	1.84	DM	ACEI
10	67	M	2.24	IgA + Sol kid	D
11	67	M	2.11	ON	BB
Average (*N* = 11)	57 ± 4	M8/F3	2.12 ± 0.09		
Control average (*N* = 5)	58 ± 6	M4/F1	2.09 ± 0.10	None	None

Abbreviations are: D, diuretic; CCB, calcium channel blocker; ACEI, angiotensin-converting enzyme inhibitor; BB, beta blocker; BSA, body surface area (m^2); Sol kid, solitary kidney; CIN, chronic interstitial nephritis; ON, obstructive nephropathy; DM, diabetes mellitus; FSGS, focal segmental glomularsclerosis.

[a] CRD patients were on one or more antihypertensive agents

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Table 2

Blood pressure, renal function in CRD patients and controls

	Blood pressure *mm Hg*		BUN	P_{Cr}	C_{Cr} *mL/min/m² BSA*
	Systolic	Diastolic	mg/dL		
CRD	148 ± 8[a]	80 ± 3	46.3 ± 1.1[a]	4.0 ± 1.5[a]	33 ± 6[a]
Control	117 ± 6	72 ± 4	10.0 ± 3.1	0.8 ± 0.2	119 ± 22[a]

Abbreviations are: BUN, blood urea nitrogen; P_{Cr}, plasma concentration of creatinine; C_{Cr}, plasma creatinine clearance.

[a]Different vs. control

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Table 3

Effects of plasma from patients with chronic renal diseases on NOS activity in human dermal microvascular endothelial cells

Type of CRD	NOS activity *pmol/min/mg · protein*		Type of CRD	NOS activity *pmol/min/mg · protein*	
	Baseline	**Dex**		**Baseline**	**Dex**
Control average	18.3 ± 1.9	18.4 ± 2.1			
CRD individual data			6 (IgA)	15.1 ± 0.5[a]	14.3 ± 1.1[a]
1 (Wegner's)	13.2 ± 0.9[a]	13.8 ± 1.1[a]	7 (DM)	19.5 ± 1.3	19.0 ± 2.5
2 (DM)	18.5 ± 0.8	19.0 ± 1.3	8 (GN)	15.9 ± 2.5	16.1 ± 2.3
3 (DM)	12.6 ± 1.8[a]	14.1 ± 1.1[a]	9 (FSGS)	13.9 ± 1.2[a]	14.1 ± 1.1[a]
4 (CIN)	16.8 ± 1.9	15.6 ± 1.5	10 (DM)	14.0 ± 1.3[a]	13.9 ± 1.4[a]
5 (GN)	18.2 ± 1.0	18.7 ± 0.9	11 (OBN)	16.4 ± 1.9	15.5 ± 2.1

Abbreviations are in Table 1.

[a] $P < 0.05$, compared to the control