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An Enzyme Hydrolyzing Methylated Inhibitors of Nitric Oxide Synthase is Present in Circulating Human Red Blood Cells

ELLEN S. KANG[a,b,*], TAYLOR B. CATES[a], DYETTE N. HARPER[c], THOMAS M. CHIANG[d,e], LINDA K. MYERS[a], SERGIO R. ACCHIARDO[d] and MASUMI KIMOTO[f]

[a]*Department of Pediatrics, University of Tennessee, Memphis, TN, USA;* [b]*Department of Pharmacology, University of Tennessee, Memphis, TN, USA;* [c]*Lane College, Jackson, TN, USA;* [d]*Department of Medicine, University of Tennessee, Memphis, TN, USA;* [e]*Department of Biochemistry, University of Tennessee, Memphis, TN, USA;* [f]*Department of Nutritional Science, Okayama Prefectural University, Okayama, Japan*

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N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine or ADMA) and N^G-monomethyl-L-arginine (L-NMMA) are post-translationally synthesized amino acids of nuclear proteins. Upon release during protein turnover, they are not used in protein synthesis, but are excreted or metabolized by dimethylarginine dimethylaminohydrolase (DDAH) found in many tissues. DDAH is present in monocytic and polynuclear cells of blood, but no report has appeared of its presence in red blood cells (RBCs). Because methylated arginines can inhibit nitric oxide synthase (NOS) and elevations are reported in several diseases, we explored whether RBCs express this enzyme. DDAH is present in RBCs as supported by hydrolysis of both ADMA and L-NMMA, but not symmetric dimethylarginine, and by immunoprecipitation/Western blot using a specific monoclonal antibody to human DDAH. In a pilot study of end-stage renal disease (ESRD) patients, RBC DDAH activity with ADMA as substrate correlated inversely with age ($p = 0.005$) and enzyme activities were higher in patients with greater diastolic blood pressure drops during hemodialysis ($p = 0.02$). Similar correlations were found with white cell DDAH activity. Thus, human RBCs can hydrolyze methylated arginines. These findings indicate the RBC could be used to assess the status of DDAH in various disease states.

Keywords: Dimethylarginine dimethylaminohydrolase; Methylated inhibitors of NOS; Red blood cells; Citrulline; Dimethylamine; End-stage renal disease or ESRD

INTRODUCTION

Accumulation of compounds such as asymmetric dimethylarginine (ADMA) and N^G-monomethyl-L-arginine (L-NMMA) have been

*Corresponding author. Address: Box 172311, Memphis, TN, 38187-2311, USA. Tel.: +1-901-683-4974. Fax: +1-901-448-7265. E-mail: eskang@pol.net

reported in a number of clinical disorders including chronic renal failure,[1] essential hypertension,[2] congestive heart failure,[3] arteriosclerosis,[4] hypercholesterolemia,[5,6] multiple sclerosis,[7] and schizophrenia.[8] The importance of these compounds is that high concentrations can inhibit nitric oxide synthase (NOS), as observed in chronic renal failure.[1] Nitric oxide (NO) is an important signaling agent in the regulation of physiological processes such as the maintenance of vascular tone, vascular permeability, and tissue perfusion, cellular immune defense mechanisms, platelet function, and neuronal transmission.[9,10] Several of these physiological processes are abnormal in the disorders in which the free methylated arginine levels in the body have been reported to be elevated.

ADMA and L-NMMA (see Fig. 1) are endogenously synthesized by dimethylation and monomethylation, respectively, at the guanidino site of selective L-arginine residues already incorporated into proteins that function as RNA-binding proteins in the nuclei of cells.[11,13] During turnover of these nuclear proteins, the methylated arginine residues are released and are not incorporated into protein. Thus, increased nuclear protein catabolism or regeneration of tissues would enhance the release of ADMA and L-NMMA.

How are these amino acids processed? In rats, after administration of ADMA labeled with ^{14}C at each of the carbons making up the backbone of the molecule, 13% of the load is excreted in the urine within 12 h as ADMA, the corresponding alpha ketoacids, decarboxylated products of the ketoacids, and the acetyl conjugate of ADMA.[14,15] More than 80% of the load is found in the tissues, mainly as L-citrulline, L-ornithine, L-arginine and guanidinoacetic acid. The presence of label from ADMA in these compounds is the result of the action of dimethylarginine dimethylaminohydrolase (DDAH, EC 3.5.3.18), an enzyme which is ubiquitous, requires no cofactors, and which irreversibly

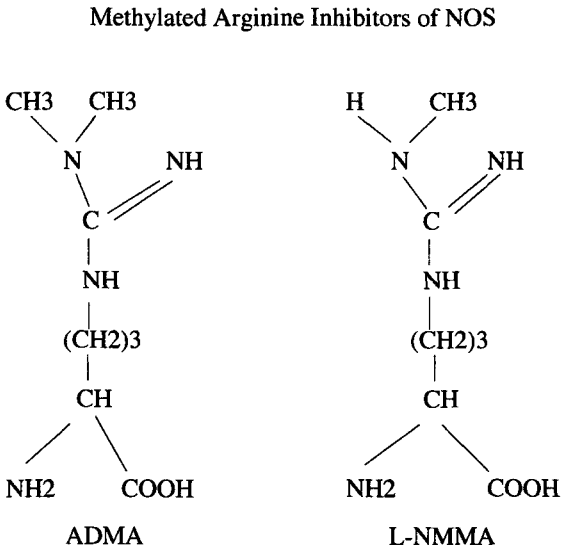

FIGURE 1 Methylated arginine inhibitors of NOS.

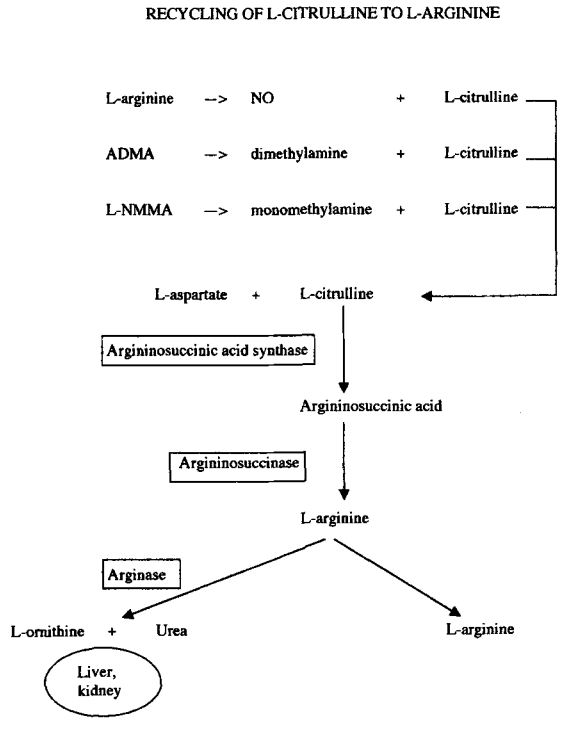

FIGURE 2 Recycling of L-citrulline to L-arginine. Enzymes enclosed in rectangles.

converts ADMA to L-citrulline and dimethylamine.[16] The latter is a precursor of the carcinogen, dimethylnitrosoamine, formed after nitrosation. The L-citrulline produced can enter the cytosolic steps of the urea cycle located primarily in the liver, but also in the kidney and other tissues,[17] accounting for the subsequent recovery of label as L-arginine, and L-ornithine (Fig. 2). The L-arginine produced in the kidney from ADMA can then be utilized as substrate by NOS for NO production,[18–23] for protein synthesis, or for conversion to guanidinoacetic acid.[14] Thus, most of the ADMA released during turnover of nuclear proteins (>80%) appears to be metabolized by DDAH, and only that amount escaping enzymatic hydrolysis would remain for renal excretion.

The availability of monoclonal antibodies to DDAH has facilitated detection of the enzyme in animal and human tissues.[24] Its distribution appears to correlate with the presence of some forms of NOS, and free ADMA and L-NMMA, both substrates for DDAH.[25] Study of the possibility that diminished DDAH activity might contribute to increase in the plasma levels of ADMA and L-NMMA, perhaps leading to inhibition of NOS activity, would require biopsy of a tissue expressing DDAH activity, such as the liver, kidney, heart, lung, brain, or the withdrawal of a sufficient quantity of blood to collect the white blood cells (WBC). Should this enzyme be present in the red blood cells (RBC), access to tissue would be greatly facilitated, especially in small children and anemic individuals. Therefore, whether the RBC expresses DDAH activity was explored, using cells obtained from normal adults.

Herein, we report the presence of both DDAH activity and protein expression in the human RBC by enzyme studies and immunochemical techniques using a specific monoclonal antibody prepared with a purified human DDAH enzyme.[26] Differences in the K_m and V_{max} values were found for ADMA and L-NMMA as substrates. A pilot study of RBC DDAH activities in hemodialyzed patients with end-stage renal disease (ESRD) was also done. Clinical correlations were found with RBC DDAH activity that were similar to correlations obtained with WBC DDAH activity. These findings indicate that the RBCs represent another reservoir for the enzyme DDAH.

MATERIALS AND METHODS

Human Samples

Samples of blood were collected in the morning by venipuncture after informed consent had been obtained, as approved by the Institutional Review Board at this University, from six healthy laboratory workers (three males and three females), who were not fasting. These samples were used to establish the presence, or not, of DDAH activity in the circulating red cells.

Ethylenediamine tetraacetic acid (EDTA) was used as the anticoagulant. Plasma was collected by centrifugation and the cellular layer was further processed by the method of Boyum[27] to separate the monocytes (MN), polymorphonuclear cells (PMN), and the RBCs by density gradient using a commercially available sodium metrizoate and Dextran 500 preparation (Accurate Chem. & Scientific, Westbury, NY). Afterwards, the cells were washed twice with 100 mM phosphate buffer, pH 6.5 and then homogenized by sonication (Branson, three 10 s bursts, setting 5) at 4°C. The homogenates were then centrifuged at 15,000 *g* (Beckman) for 30 min at 4°C. The supernatant fractions were collected, aliquoted and frozen at −72°C until analyzed.

PMN and MN cells were also sonicated, and processed as above, retaining the supernatant fractions for analysis of samples from patients with ESRD.

Protein Determination

Protein concentrations were determined by the Lowry method[28] with bovine serum albumin (BSA) as the standard.

DDAH Assay

DDAH activity was measured according to Ogawa *et al.*[29] DDAH activity can be quantitated by recovery of either of the two products that are produced in equimolar amounts, L-citrulline, which is common to both ADMA and L-NMMA as substrates, and either dimethylamine or monomethylamine with ADMA or L-NMMA as the substrates, respectively. In tissues containing the cytosolic enzymes of the urea cycle, the L-citrulline formed would be cycled through these enzymes after linkage with aspartate to produce argininosuccinate followed by L-arginine[17] for use by various enzymes including NOS[18,19,21–23] as depicted in Fig. 2. Therefore, depending on the substrate used and the tissue in which DDAH activity is measured, selection of a product that would not be processed further is important. However, since the human RBC does not contain the two enzymes that would convert L-citrulline to L-arginine, namely, argininosuccinate synthase and argininosuccinase or the lyase,[17] either of the equimolar products could be used to measure DDAH activity in this cell.

Prior to analysis of the RBC supernatant fractions for DDAH activity as described by Ogawa *et al.*,[29] the sensitivity of the two methods that could be used with ADMA as substrate was examined. An equimolar range of L-citrulline and dimethylamine standards were processed through the respective methods for their analysis. Citrulline was determined by the method of Prescott and Jones[30] and dimethylamine was determined by the method of Beal and Bryan.[31] A significant difference was found as shown in Fig. 3 and discussed under results, which led us to select L-citrulline recovery to measure enzyme activity.

RBC supernatants were analyzed using both ADMA and L-NMMA as substrates, recovering L-citrulline as the product. Incubation was for varying periods of time and quantitation of the citrulline recovered was determined by the method of Prescott and Jones[30] by spectrophotometry (Gilford) at 466 nm. Tissue L-citrulline did not pose a problem, as values from blanks were subtracted.

Symmetrical dimethylarginine (SDMA) used as a possible substrate failed to result in the recovery of either L-citrulline or dimethylamine.

Chemical Effects

L-citrulline, EDTA, norvaline and metallic ions (ferric, magnesium, zinc, calcium, and manganese) prepared as the chlorides in phosphate-buffered saline (PBS) were added to RBC supernatant preparations prior to the addition of L-NMMA as the substrate. RBC supernatants without these additions were simultaneously analyzed for DDAH activity for determination of percent change in activity.

Immunoprecipitation and Western Blot Analysis

Cellular supernatants were immunoprecipitated overnight at 4°C with 250 µl of a 10 µg/ml dilution of the anti-DDAH antibody (a monoclonal antibody or mab (IgG2b) against the recombinant human DDAH that had been purified by protein A affinity column from M. Kimoto). At the end of incubation, an aliquot of protein *G*-agarose (50 µg) was added to each tube followed by incubation for 1 h at room temperature (*T*). The precipitate was collected, washed five times with PBS, solubilized with SDS-PAGE sample buffer and subjected to 10% SDS-PAGE.[32] Protein bands were electrophoretically transferred onto a nitrocellulose sheet,

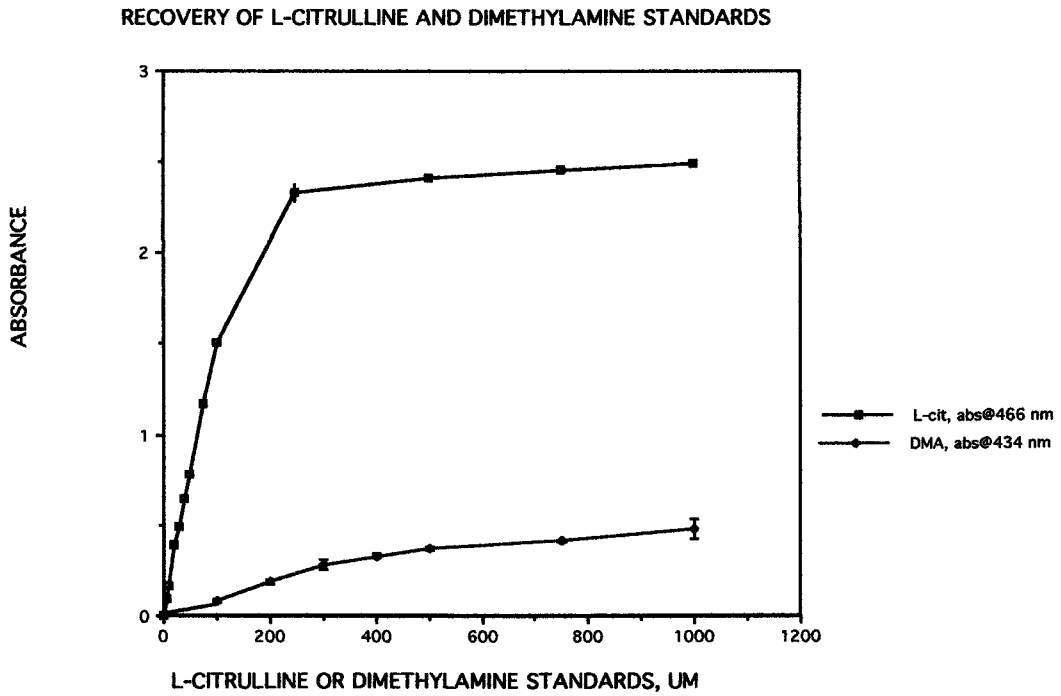

FIGURE 3 Recovery of L-citrulline and dimethylamine standards. Means±sem of samples of citrulline and dimethylamine standards recovered from buffer and quantitated by the Prescott and Jones[30] and the Beal and Bryant[31] methods by spectrophotometry at 466 and 434 nm, respectively. (Bars depicting sem ranges within the limits of the squares used to designate the mean values for L-citrulline).

followed by treatment with 3% dried milk in 20 mM Tris–500 mM NaCl–0.05% Tween 20, pH 7.4 (TBS) to eliminate non-specific binding. The nitrocellulose sheet was then washed 3 times with TBS and incubated with the antibody (10 μg/ml) overnight at 4°C. After washing 3 times in TBS, the nitrocellulose sheet was incubated with a second antibody (peroxidase conjugated goat anti-mouse IgG, 1/2000) for 2 h at room *T*. After 3 washings with TBS, the nitrocellulose sheet was developed with ECL solution as instructed by the manufacturer (Amersham, Arlington Heights, IL).

Reagent Sources

N^Gmonomethyl-L-arginine (L-NMMA) from Sigma (St. Louis, MO) was used for most of the studies until samples of ADMA that were recognized by the enzyme in rodent tissues became available through Cyclopss (Salt Lake City, UT). ADMA samples from other US companies did not result in the recovery of L-citrulline from rodent tissue samples, unlike the product obtained from Cyclopss in Salt Lake City, UT, which has continuously been recognized by both rodent tissue and the human RBC fractions. A limited amount of ADMA synthesized by one of us (M. Kimoto) was also used in some experiments and to validate the presence or absence of enzyme activity in various tissue fractions.

Pilot Study in ESRD

After establishing baseline conditions for measuring DDAH activity in the red cells, blood samples were collected from 13 non-

fasting ESRD patients on maintenance hemodialysis after informed consent had been obtained, before the initiation of the next dialysis. White and red cells were processed as above and analyzed for DDAH activity. Clinical data routinely monitored during hemodialysis were collected for statistical correlative analysis.

Analysis of Data

Mean values, standard deviations, and standard errors of the means, comparisons that were made by the unpaired and paired *t*-tests, and regression analysis were done using STATVIEW 512 + software (Brainpower, Calabasas, CA).

RESULTS

With ADMA as substrate, L-citrulline and dimethylamine would be produced in equimolar amounts by DDAH. Therefore, a comparison was made of the two methods selected for measuring these hydrolytic products. Absorbances at two different optimal wavelengths for measuring L-citrulline and dimethylamine are shown in Fig. 3 for various concentrations of standards that were added to buffer and analyzed by the methods indicated. Recovery of L-citrulline standards by the Prescott and Jones method with absorbance at 466 nm was far more sensitive than the recovery of equimolar amounts of dimethylamine standard by the Bryan and Beal method with absorbance at 434 nm (Fig. 3). Unlike tissues with the two argininosuccinate enzymes that recycle L-citrulline, which could pose a problem as outlined in Fig. 2,[18] the human RBC lacks argininosuccinate synthase and lyase activities.[17] Therefore, the selection of L-citrulline as the product to measure was preferred, allowing two different substrates, ADMA and L-NMMA, to be tested.

Using RBC supernatants from normal subjects as a source of DDAH with L-NMMA or ADMA as substrates, recovery of L-citrulline was found to increase rapidly within a short period of time with incubation at 37°C. An optimum duration was determined to be between 1–4 min, as shown for L-NMMA in Fig. 4. The optimum amount of protein for linearity was between 75 and 100 μg. Increase in substrate concentration also led to increase in L-citrulline recovered, as shown in Fig. 5 for L-NMMA. Similar findings were obtained with ADMA. Specific activities for this enzyme in RBCs were measured using 100 μg of RBC supernatant protein, 3 min incubation with 400 μM L-NMMA or ADMA. The mean specific activity for normal RBCs with L-NMMA was similar to that obtained with ADMA (4.40 + sem 0.961 and 5.65 + 1.190 nmoles/mg protein/min, respectively, P=NS). Michaelis–Menten constants derived by the Lineweaver–Burk or the Eadie–Hofstee methods resulted in similar results, as shown for one control RBC sample in Fig. 6, panels A and B. Kinetic constants for RBC DDAH with L-NMMA and ADMA as substrates were derived by the Lineweaver–Burk method and are presented in Table I. The K_m for the RBC DDAH was nearly two-fold higher with ADMA as the substrate compared to L-NMMA and the V_{max} was nearly four-times faster for ADMA than L-NMMA.

SDMA did not serve as a substrate for the enzyme in the red cells, in agreement with reports on the tissue enzymes of the rat,[29] cultured human endothelial cells,[33] and the bovine brain.[34]

The effect of L-citrulline, one of the two products, was examined as DDAH activity is said to be inhibited by L-citrulline.[29,33] L-citrulline inhibited DDAH activity by 50% at concentrations of 0.1 and 0.4 mM (Table III). Since the red cell does not contain partial urea cycle enzymes that could convert L-citrulline to arginine,[17] the effect of L-citrulline appears to be inhibitory to the hydrolytic activity of the

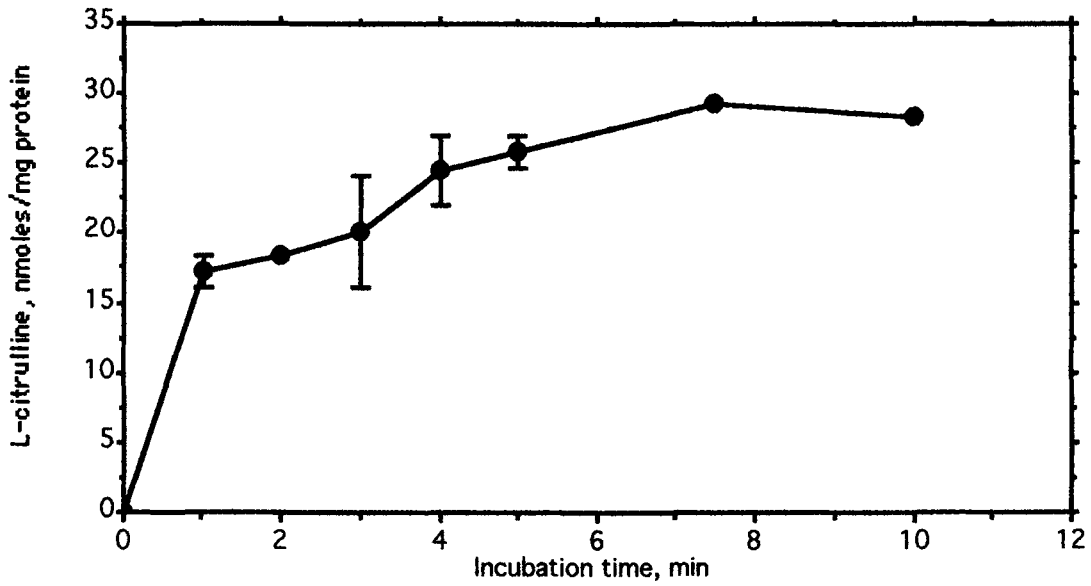

FIGURE 4 RBC DDAH activity determined by L-citrulline recovery as a function of incubation time. RBC supernatant (100 μg protein) was incubated with 600 μM L-NMMA in 100 mM phosphate buffer, pH 6.5 for the times indicated. Recovery of citrulline was by the method of Prescott and Jones.[30]

enzyme. $FeCl_3$, $MgCl_2$, and $ZnCl_2$, also inhibited RBC DDAH. Norvaline, an inhibitor of arginase activity, had no effect while EDTA inhibited DDAH, suggestive that a metallic ion could play a role in the hydrolytic capacity of the enzyme in the red cell. $CaCl_2$ as well as $MnCl_2$ at 0.1 and 0.4 mM had no apparent effect on the RBC enzyme.

Thus, the circulating human red cell can hydrolyze both ADMA and L-NMMA to L-citrulline, but does not act on SDMA. This hydrolytic activity is inhibited 50% by 0.1 mM L-citrulline and is inhibited to varying degrees by iron, magnesium, and zinc ions, and by EDTA, but not by calcium and manganese ions nor by norvaline.

TABLE I Kinetic constants for dimethylarginine dimethylaminohydrolase of normal adult human red blood cells. Michaelis–Menten constants from Lineweaver–Burk plots

Substrate	K_m (mM)	V_{max} (nmoles/mg protein/min)
Red blood cells		
N^G-monomethyl-L-arginine	0.305 ± 0.07	27.34 ± 3.139*
Asymmetric dimethylarginine	0.494 ± 0.006	121.4 ± 3.65*

* Difference significant by unpaired t-test, $p = 0.0003$.

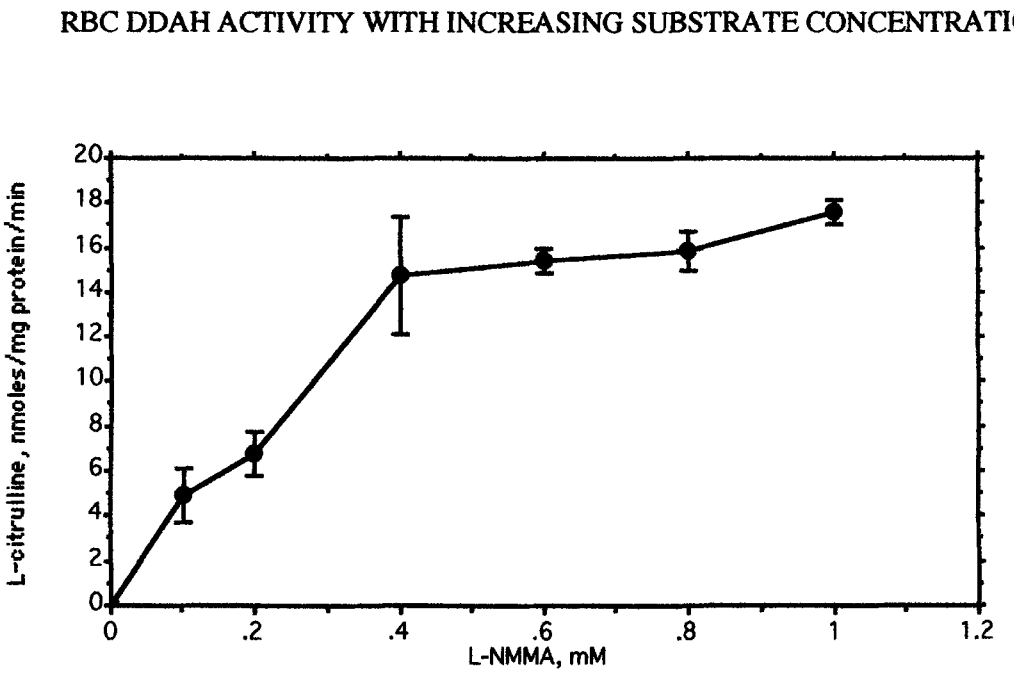

FIGURE 5 RBC DDAH activity with increasing substrate concentrations. RBC supernatant (100 μg protein) was incubated for 3 min with increasing concentrations of L-NMMA in 100 mM phosphate buffer, pH 6.5 with recovery of citrulline by the Prescott and Jones method.[30] Mean±sem from $n = 3$ are shown.

A monoclonal antibody (mab) to the human DDAH prepared by one of us (M. Kimotof[25,26] was used to search for the protein in human RBC supernatants. After immunoprecipitation with the mab, the proteins were subjected to SDS-gel electrophoresis followed by immunoblotting with the mab. An immunoreactive protein was recovered with an M_r of 32 kDa as shown for two separate samples of RBC supernatants in Fig. 7. There was wide variability in the recovery of the 32 kDa protein, which did not correlate with enzyme activity. Whether the discordance between the activity of the enzyme and the amount of enzyme protein recovered by immunoblot resulted from modification of the immunoreactive site(s) of DDAH by, for example, oxygen radicals such as peroxynitrite resulting in nitrotyrosylation[35] or another reaction, is unclear. These findings indicate that the human RBC, which exhibits DDAH catalytic activity also contains a 32 kDa protein that immunoreacts with a mab to human DDAH protein.

Pilot Study in Hemodialyzed ESRD Patients

In a cohort of hemodialyzed ESRD patients, predialysis blood was collected, the cells were isolated, and the RBCs, PMNs and MNs were analyzed for DDAH activity with ADMA or L-NMMA as the substrate. Mean specific activities for DDAH of the RBC, PMN and MN are presented in Table III. While the RBC enzyme activities with the two substrates used are slightly lower than the specific activities found in normal subjects (see above), the differences are not statistically significant. In these ESRD samples, slightly more citrulline was recovered per mg protein with L-NMMA as the substrate than ADMA, but these differences were also not significant. Far less variability in the amounts of

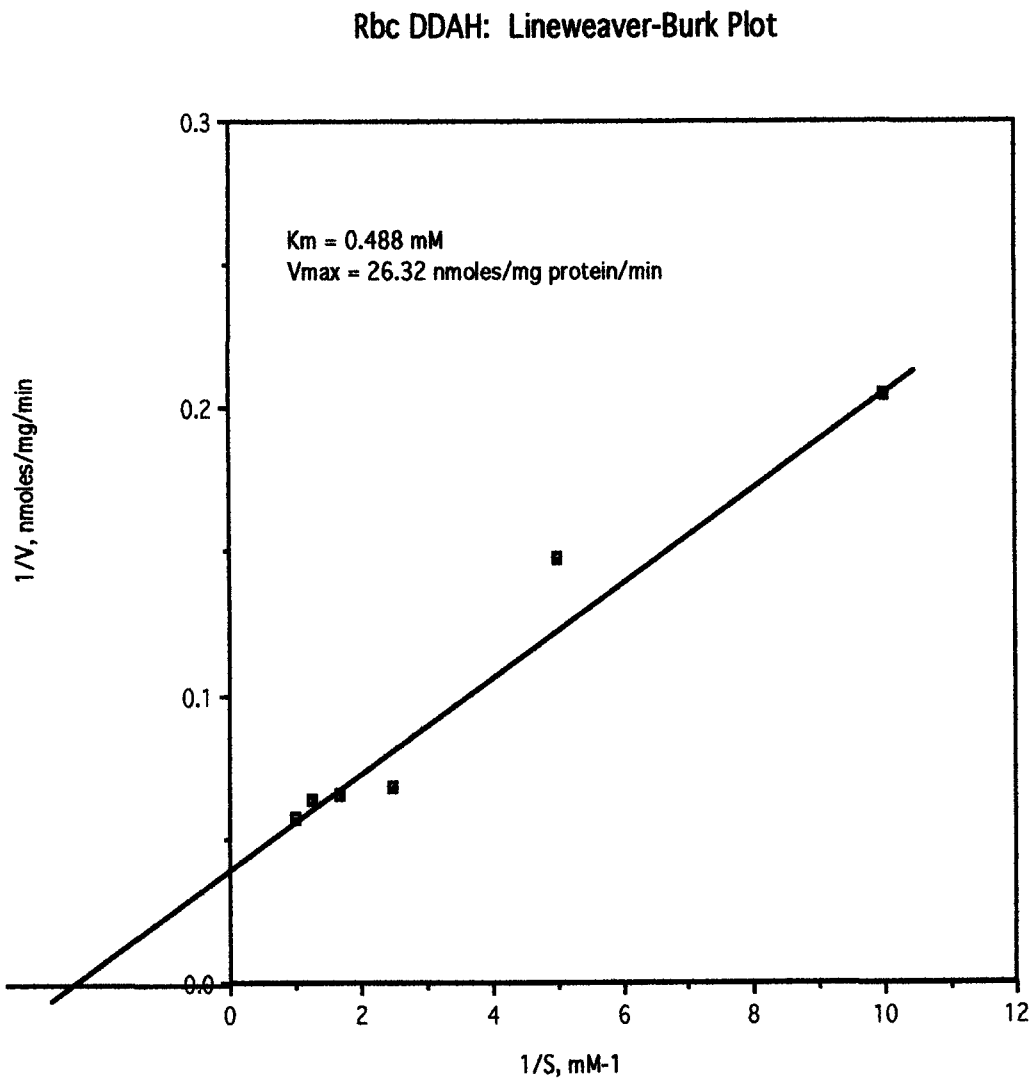

FIGURE 6. *Caption on next page.*

DDAH protein are seen with the MN than the PMN fractions (Fig. 8) and the amounts of protein seen in these blots failed to correlate with DDAH activities for the respective cell fractions (Table III).

Aliquots (100–200 μl) of plasma from these hemodialyzed ESRD patients were added to normal control RBC DDAH preparations for possible effects on normal RBC enzyme activity. Individual patient samples had varied effects on normal RBC DDAH activity, ranging from no measurable change with some, inhibition with others, and even stimulatory effects with several plasma samples compared to control plasmas, which had little effect (data not shown). While plasma concentrations of ADMA and L-NMMA were not measured in the patient samples, stimulation of control RBC DDAH activity is likely to be due to the presence of inhibitors of NOS, which accumulate to variable degrees in ESRD.[36] Inhibitory effects could be due to the accumulations of inhibitors as are listed in

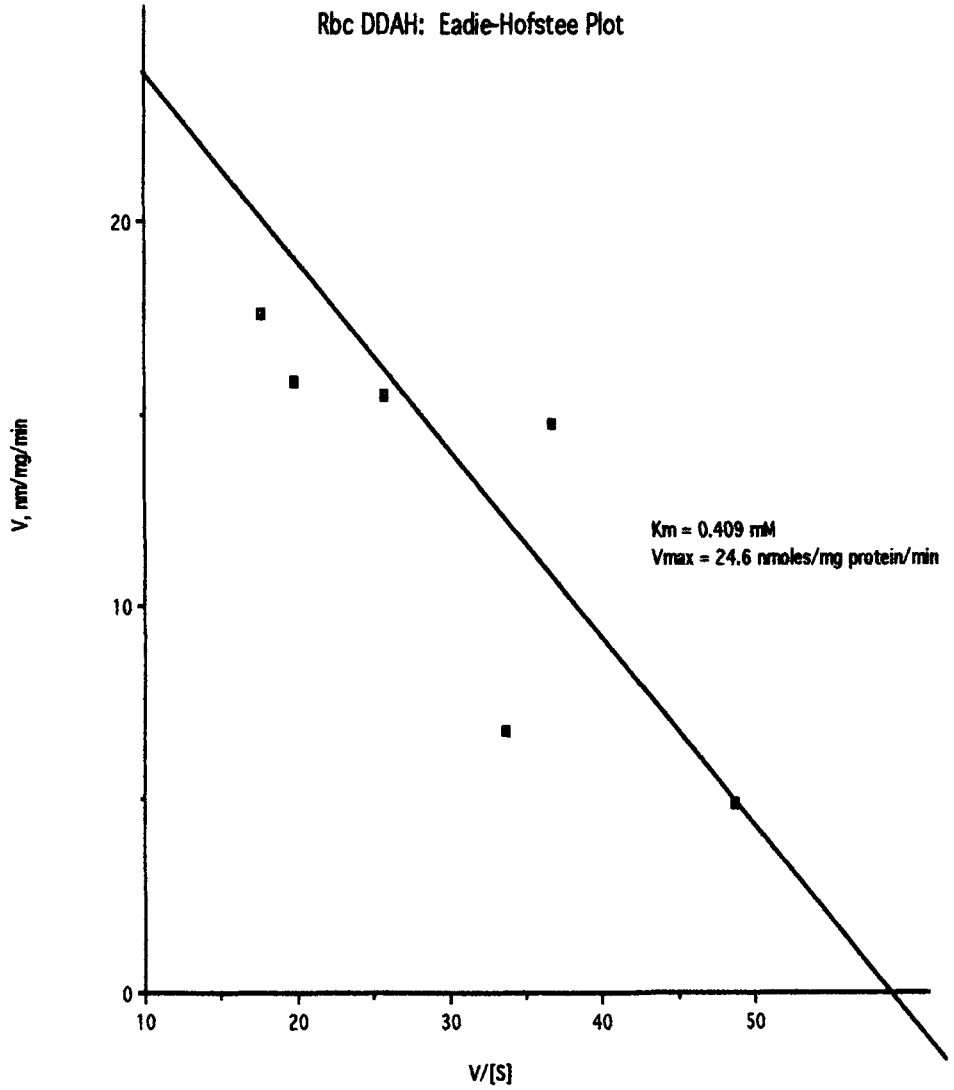

FIGURE 6 RBC DDAH kinetic constants. Panel A: RBC DDAH: Lineweaver–Burk plot. Panel B: RBC DDAH: Eadie–Hofstee plot. Substrate used was L-NMMA.

Table II, several of which are known to accumulate in ESRD.

In ESRD patients, RBC DDAH activities with ADMA as the substrate correlated inversely with age (Fig. 9), but correlation with age was not seen with L-NMMA as the substrate. In addition, RBC DDAH activities with ADMA as the substrate, but not with L-NMMA, correlated with the drop in diastolic blood pressures (BPS) that occurred during hemodialysis ($p = 0.02$, Table IV).

Patient PMN DDAH activities also correlated inversely with age, only with ADMA as a substrate and not with L-NMMA, similar to the RBC enzyme. Correlations were also found between BPS and DDAH activities of the PMN and MN cells. These findings indicated that ESRD patients with higher PMN DDAH activities with ADMA as a substrate had higher predialysis systolic BPS, greater reductions in BP at 60 min of hemodialysis and higher serum

TABLE II Effects of various compounds on RBC DDAH activity. Additives were prepared in PBS to give the concentrations indicated and were added prior to initiation of the assay with L-NMMA

	Added compound (mM)	Percent activity
RBC supernatant	none	100
	L-citrulline (0.1)	55
	L-citrulline (0.4)	46
	$FeCl_3$ (0.1)	64
	$FeCl_3$ (0.4)	21
	$CaCl_2$ (0.1)	105
	$CaCl_2$ (0.4)	109
	$MgCl_2$ (0.1)	42
	$MgCl_2$ (0.4)	42
	$MnCl_2$ (0.1 and 0.4)	100
	EDTA (0.1)	55
	EDTA (0.4)	55
	$ZnCl_2$ (0.1)	78
	$ZnCl_2$ (0.4)	25
	Norvaline (0.1 and 0.4)	100

TABLE IV Correlations between DDAH activity of predialysis blood cells and clinical findings during hemodialysis in ESRD patients

Parameters compared		P^*	R^*
RBC DDAH activity with ADMA as substrate			
Versus	Age	0.005	−0.752
	Drop in diastolic BP	0.02	0.645
Monocyte DDAH activity with L-NMMA as substrate			
Versus	PMN DDAH, same substrate	0.015	0.826
	Total NO_2	0.01	−0.726
Polymorphonuclear cell DDAH activity with L-NMMA as substrate			
Versus	90 min systolic BP	0.03	−0.718
	90 min diastolic BP	0.04	−0.703
	150 min pulse	0.02	−0.807
	180 min pulse	0.04	−0.700
	Postdialysis body temperature	0.04	−0.686
	Hemodialysis treatment time	0.02	−0.757
	Serum Ca^{++}	0.05	−0.664
Polymorphonuclear cell DDAH activity with ADMA as substrate			
Versus	Age	0.04	−0.832
	Predialysis systolic	0.05	0.822
	Change systolic BP at 60 min	0.03	0.852
	Change diastolic BP at 60 min	0.02	0.875
	Serum creatinine	0.01	0.941

* Data analyzed by regression analysis.

creatinine levels. Patients with higher PMN DDAH activities with L-NMMA as substrate had lower 90 min BPS, lower pulses late in hemodialysis, lower body temperatures at the end of dialysis, shorter dialysis durations, and lower serum Ca^{++} levels (see Table IV). Clinically, these data suggest that patients with higher PMN DDAH activities tended to have higher BPS prior to and earlier during hemodialysis with higher creatinines and lower serum Ca^{++}, but that later in dialysis, BPS and body temperatures are lower and the duration of dialysis is shorter than in patients with lower PMN DDAH activities.

In this group of hemodialyzed ESRD patients, the maximum drop in BP during hemodialysis correlated with the ability of the RBC DDAH to hydrolyze ADMA, with higher enzyme activities resulting in greater reductions in diastolic BPS during hemodialysis ($p = 0.02$, Table IV). PMN DDAH activity, also with ADMA as substrate and not with L-NMMA, correlated with the maximum drop in systolic BP ($p = 0.0245$; $R = 869$). Together, these DDAH correlations reflect some of the features in patients with higher plasma ADMA levels reported earlier.[37] Such patients with higher predialysis BPS,

TABLE III Specific activities for DDAH of the white and red blood cells in hemodialyzed ESRD patients. Differences between substrates for each cell type, not significant by paired *t*-test

Cell	Substrate	S.A. (nmoles/mg protein/min)
Red blood cells	N^G-monomethyl-L-arginine	3.70±0.438
	Asymmetric dimethylarginine	2.98±0.455
Polymorphonuclear	N^G-monomethyl-L-arginine	8.24±1.81
	Asymmetric dimethylarginine	8.56±0.908
Mononuclear	N^G-monomethyl-L-arginine	9.19±2.033
	Asymmetric dimethylarginine	6.19±1.742

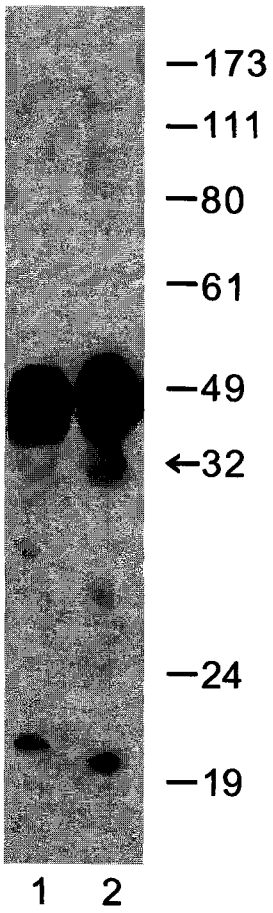

FIGURE 7 Immunochemical identification of dimethylarginine dimethylaminohydrolase protein in human RBC supernatants. Western blot of immunoprecipitated, SDS/PAGE separated RBC supernatants from: normal subjects in lanes 1,2 with the migration sites for the molecular weight markers designated in the right margin. DDAH is identified by the arrow as the 32 kDa component.

developed significant hypotension during dialysis, which could not be attributed to excessive ultrafiltration, but resulted from enhancement of NO generation secondary to the removal of a dialyzable inhibitor(s) of NOS.[36]

Finally, in these hemodialyzed ESRD patients, correlations between blood cell enzyme activities and clinical parameters showed an interesting degree of substrate-dependency, as seen in Table IV, the basis for which is not clear.

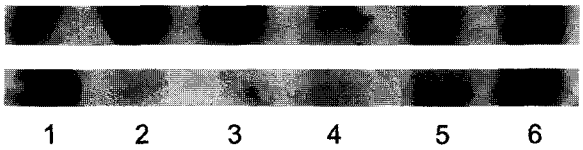

FIGURE 8 Dimethylarginine dimethylaminohydrolase protein in white blood cells from hemodialyzed ESRD patients by immunoblot. Upper row from MN and the bottom row from PMN samples from the same ESRD patients. Samples in lanes 2 and 3 are from males; others are all females. Immunochemical analysis as described under materials and methods.

DISCUSSION

DDAH is a recently discovered enzyme that is ubiquitously present in many organ tissues of rodents and humans, including the kidney, liver, lung, heart, pancreas, brain, endothelial cells of the arteries, and the white blood cells of the blood.[25,29] DDAH has also been reported in the bovine kidney and brain.[34] Now, the list can be extended to the largest cellular component of the blood in man, the circulating RBCs. The RBC itself would not be producing ADMA nor L-NMMA. Considering the size of this cellular pool and the fact that RBCs are continuously "sweeping" through the vascular compartments of all tissues, it undoubtedly represents a major ADMA and L-NMMA degrading "organ" in the human body.

Both ADMA and L-NMMA are naturally occurring[11–13] and are released by enzymatic degradation of the proteins that contained them. In the human plasma, concentrations of ADMA are said to be 10 times greater than that of L-NMMA.[1] On the other hand, tissue concentrations are said to be similar for free ADMA and L-NMMA. In rat brain and aorta, the concentrations of these methylated arginines are about 3–5 nmoles/gram wet weight while concentrations are about 1 nmol/100 million neutrophils and for the same number of macrophages.[37] According to Mendes Ribeiro *et al.* uremia is associated with plasma L-NMMA levels that are 8 μM, whereas L-NMMA is undetectable in controls.[38] Furthermore, whereas L-NMMA and L-arginine are normally

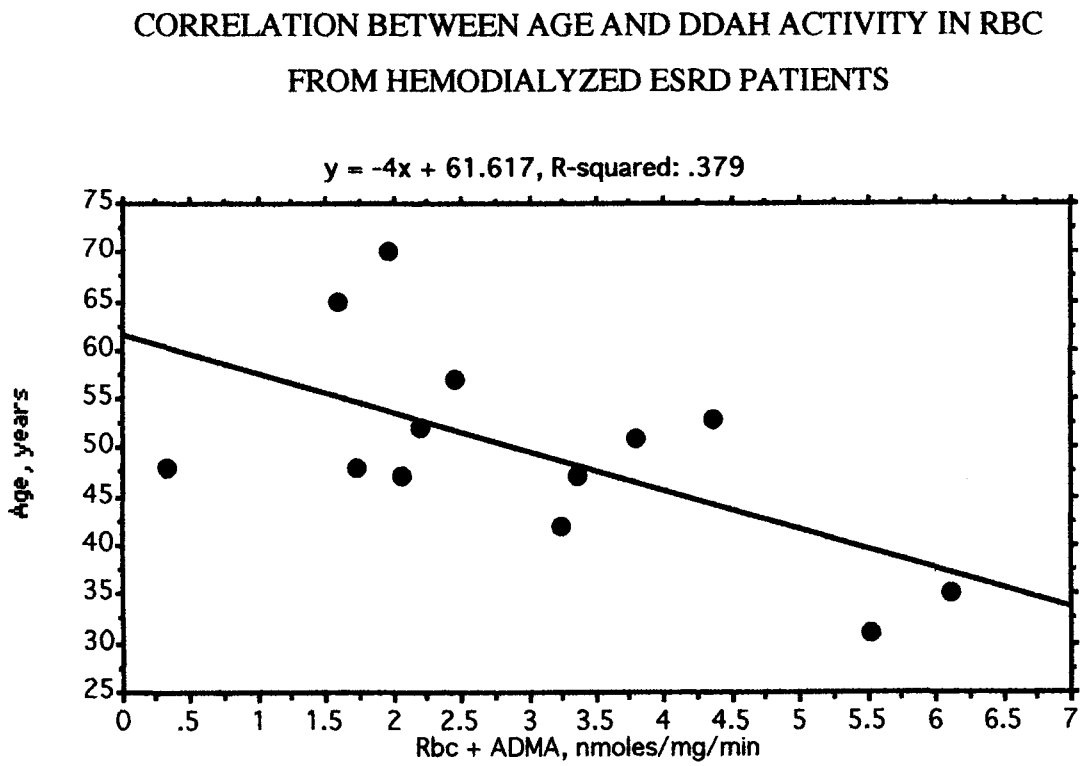

FIGURE 9 Correlation between age and DDAH activity in RBC from hemodialyzed ESRD patients. $p = 0.005$; $R = -0.752$ by regression analysis; ADMA was used as the substrate.

undetectable in control RBCs, both are present in uremic RBCs owing to increased transport capacities for both amino acids by different transport systems in RBCs.[38,39] Similar studies with ADMA have not been reported. An enhancement of L-NMMA transport into the RBC in uremia would tend to reduce the plasma concentration of one of several known inhibitors of NOS that accumulate in this condition due to the action of DDAH in the RBC. In the study reported here, addition of plasma from ESRD patients had stimulatory effects as well as inhibitory effects on control RBC DDAH activity. Stimulatory effects could result from high L-NMMA or/and ADMA levels in the plasma, while inhibitory effects could result from modulating the concentrations of the effective compounds present in the plasma such as L-citrulline, which is elevated in ESRD, or the effects of some of the inhibitory cations listed in Table II.

Kielstein *et al.* have reported that hemodialyzed ESRD patients show a range of elevated plasma ADMA levels compared to controls or peritoneally dialyzed ESRD patients.[40] We also reported elevated ADMA levels in hemodialyzed ESRD patients that ranged widely, correlating directly with age.[36] The finding made in the present study where age correlated inversely with RBC DDAH activity with ADMA as a substrate explains the previous report. Since older patients have lower DDAH activities, their ADMA levels would be expected to be higher, especially if the other route of disposal of the inhibitor, namely renal excretion, was jeopardized by disease.

Plasma levels of ADMA have been reported to be elevated in hypercholesterolemic individuals

and elderly patients with peripheral arterial occlusive disease and generalized atherosclerosis.[4–6,41] Hypothesizing that reduced degradation of ADMA might play a role in the accumulation of ADMA in these individuals, Ito *et al.*[41] studied the effects of oxidized low-density lipoproteins and tumor necrosis factor alpha on ADMA accumulation in transformed human umbilical vein cells. DDAH activity, but not protein expression, was reported to be reduced. Other disorders where plasma ADMA levels are reported to be elevated without loss of renal function include essential hypertension,[2] congestive heart failure,[3] multiple sclerosis,[7] and schizophrenia.[8] In experimental animals, hypercholesterolemia and alloxan diabetes raise ADMA levels and the degree of elevation correlates with intimal and medial thicknesses of the carotid arteries.[42]

In view of the size of the RBC pool with its capacity to degrade these potential inhibitors of NOS, the possibility that compromised RBC DDAH activity could be the basis for the elevation of ADMA in the clinical disorders reported above merits exploration, especially as the enzyme is present and appears to be quite active in the RBC, as indicated by our findings.

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