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Evaluation of endogenous nitric oxide synthesis in congenital urea cycle enzyme defects

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Abstract

Nitric oxide (NO) is synthesized from arginine and O_2 by nitric oxide synthase (NOS). Citrulline, which is formed as a by-product of the NOS reaction, can be recycled to arginine by the 2 enzymes acting in the urea cycle: argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). Although the complete urea cycle is expressed only in the liver, ASS and ASL are expressed in other organs including the kidney and vascular endothelium. To examine possible alterations of the NO pathway in urea cycle defects, we measured plasma concentrations of arginine and citrulline and serum concentrations of nitrite/nitrate (NOx^-, stable NO metabolites) and asymmetric dimethylarginine (ADMA, an endogenous NOS inhibitor) in patients with congenital urea cycle disorders of 3 types: ornithine transcarbamylase (OTC) deficiency, ASS deficiency, and ASL deficiency. All were receiving oral arginine replacement at the time of this study. The same parameters were also measured in healthy subjects, who participated as controls. The OTC-deficient patients had significantly high NOx^- and nonsignificantly high ADMA concentrations. Their NOx^- was significantly positively correlated with arginine. The ASS-deficient patients had significantly low NOx^- and significantly high ADMA concentrations. The ASL-deficient patients had normal NOx^- and nonsignificantly high ADMA concentrations. In ASS-deficient and ASL-deficient patients, the NOx^- was significantly inversely correlated with citrulline. These results suggest that NO synthesis is enhanced in OTC-deficient patients while receiving arginine but that NO synthesis remains low in ASS-deficient patients despite receiving arginine. They also suggest that endogenous NO synthesis is negatively affected by citrulline and ADMA in ASS-deficient and ASL-deficient patients. Although the molecular mechanisms remain poorly understood, we infer that the NO pathway might play a role in the pathophysiology related to congenital urea cycle disorders.

1. Introduction

Identification of nitric oxide (NO) with endothelium-derived relaxing factor and discovery of its synthesis from the amino acid arginine led to the realization that the NO pathway is widespread and plays various physiologic roles. These include the maintenance of vascular tone, neurotransmitter function, and mediation of cellular defense. Moreover, NO interacts with mitochondrial systems to regulate cell respiration and to augment the generation of reactive oxygen species, thereby triggering mechanisms of cell survival or death [1,2]. Nitric oxide is synthesized by nitric oxide synthase (NOS), which converts arginine and O_2 to citrulline and NO. Three isoforms of NOS, encoded by different genes, are present in various cell types throughout the body. Neural NOS (NOS1) and endothelial NOS (NOS3) are, respectively, expressed constitutively in neuronal cells and vascular endothelial cells. The inducible isoform (NOS2) is not normally expressed in resting cells; however, under

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inflammation, it is induced in macrophages, endothelial cells, smooth muscle cells, and other cell types.

The NOS activity is controlled by endogenous inhibitors, with asymmetric dimethylarginine (ADMA), an arginine analogue, being the most important [3]. Asymmetric dimethylarginine is produced by methylation of protein-associated arginine via *N*-methyl protein transferases and subsequent protein turnover. Although excreted in the urine, ADMA is metabolized mainly by the enzyme dimethylarginine dimethylaminohydrolase, which hydrolyzes ADMA to citrulline and dimethylamine [4,5]. Results of recent clinical and experimental studies indicate that ADMA is both a strong marker and a mediator of many aspects of endothelial dysfunction syndrome, including cardiovascular and renal diseases [6,7].

The major site of arginine metabolism in ureotelic animals is the liver. Arginine is synthesized from citrulline by successive actions of the 2 enzymes acting in the urea cycle: argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) [1,8,9]. Arginine is converted to urea and ornithine by arginase, and the cycle is completed with the transformation of ornithine to citrulline by ornithine transcarbamylase (OTC). Another major site of arginine metabolism is the kidney and vascular endothelium, where arginine is synthesized from citrulline by ASS and ASL and released into the blood. Although the complete urea cycle is expressed only in the liver, ASS and ASL are expressed in many other tissues and cells; ASS and sometimes ASL are coinduced together with NOS [1].

Inborn errors of urea synthesis are characterized by hyperammonemia and are often fatal. Patients with congenital deficiencies of urea cycle enzymes exhibit unusual blood arginine and citrulline concentrations that vary according to the defects and pharmaceutical treatments [10]. At present, however, little information is available about the alterations of endogenous NO synthesis in congenital urea cycle disorders. Our group previously showed that 5 patients with OTC deficiency of late-onset type, who often developed migraine-like headaches or vomiting, had low blood and urine concentrations of nitrite/nitrate (NOx^-), which are stable metabolites of NO [11]. Another group recently showed that 8 patients with ASS deficiency had high blood concentrations of ADMA [12].

Understanding the NO pathway in these disorders might be of clinical importance; such knowledge might identify new therapeutic interventions modifying NO-related dysfunction and NO-dependent oxidative stress in the patients. Therefore, this study was intended to examine NO and ADMA formation in patients with congenital urea cycle disorders of 3 types−−OTC deficiency (MIM 311250) presenting low plasma citrulline concentrations, ASS deficiency (MIM 215700), and ASL deficiency (MIM 207900)−−inherently showing hyperammoninemia and hypoargininemia, both presenting high plasma citrulline concentrations.

2. Subjects and methods

2.1. Subjects and sample collection

Seven patients with OTC deficiency of late-onset type aged 1 to 7 years (male/female, 2/5), 5 patients with ASS deficiency aged 9 to 19 years (male/female, 1/4), and 3 patients with ASL deficiency aged 5 to 8 years (male/female, 1/2) were included in this study (Table 1).

The OTC-deficient patients developed seizures and unconsciousness or vomiting at ages ranging from 3 weeks to 3 years and were found to have hyperammonemia together with low plasma arginine and citrulline concentrations. Under suspicion of urea cycle enzyme defects such as OTC deficiency, sodium benzoate (200-250 mg/[kg d]) and L-arginine (150-250 mg/[kg d]) were administered.

Table 1
Background of enrolled patients

Case	Sex (M/F)	Deficiency	Age at diagnosis	Enzyme activity (%)	Plasma concentrations of arginine/citrulline (μmol/L) at diagnosis	Medication[a]
1	M	OTC	3 wk	9	15/2.1	Arg + SB
2	M	OTC	2 mo	6	17/2.3	Arg + SB
3	F	OTC	12 mo	11	22/6.6	Arg + SB
4	F	OTC	6 mo	18	26/5.5	Arg + SB
5	F	OTC	12 mo	15	25/8.2	Arg + SB
6	F	OTC	3 y	10	18/6.7	Arg + SB
7	F	OTC	1 mo	21	29/8.9	Arg + SB
8	F	ASS	1 mo	6	28/1399	Arg
9	F	ASS	3 wk	8	21/1436	Arg
10	F	ASS	2 d	3	19/956	Arg
11	F	ASS	2 mo	12	23/1211	Arg
12	M	ASS	1 wk	4	9/1726	Arg
13	M	ASL	2 y	5	11/478	Arg
14	F	ASL	3 y	12	10/321	Arg
15	F	ASL	3 y	7	13/396	Arg

Arg indicates arginine; SB, sodium benzoate.

[a] Medication was started soon after the onset of hyperammonemia in each patient.

L-Arginine was administered as the L-arginine HCl/L-arginine mixture (1: 1, mol/mol) produced by Ajinomoto (Tokyo, Japan). Thereafter, the patients continued to have episodic attacks of hyperammonemia 1 to 3 times per year.

The ASS-deficient patients developed seizures and unconsciousness because of hyperammonemia at ages ranging from 2 days to 2 months and were found to have extremely high plasma citrulline together with low plasma arginine. Based on the putative diagnosis of ASS deficiency, arginine (350-550 mg/[kg d]) was administered. Thereafter, the incidence of episodic hyperammonemia was 0 to 2 times per year.

The ASL-deficient patients were found to have abnormally high urinary argininosuccinate when they received precise examination for developmental delay and mental retardation at the ages of 2 to 3 years. L-Arginine (350–550 mg/[kg d]) was administered. Thereafter, no patient developed an apparent attack of hyperammonemia.

Clinical and laboratory profiles of the patients before arginine replacement initiation are shown in Table 1. The diagnosis of urea cycle enzyme defects was established by demonstration of impaired activities of the respective enzymes in liver biopsy samples obtained from the patients and of mutated genes for the enzymes [10].

Plasma concentrations of arginine and citrulline and serum concentrations of NOx^- and ADMA inhibiting NO synthesis were determined for these patients under oral arginine administration to examine possible alterations of the NO pathway in urea cycle defects. For comparison, blood samples were collected from 36 healthy subjects (male/female, 18/18) who were 1.1 to 19.9 years old (mean ± SD, 7.9 ± 3.8 years). All blood samples were centrifuged; the supernatants were stored at −30°C until analysis. The methods and purpose of the study were explained to the parents. Their informed consent was obtained before enrollment. Approval of the project was obtained from the institutional medical ethics committee.

2.2. Determination of plasma arginine and citrulline and serum NOx^- and ADMA

Plasma arginine and citrulline were determined using routine ion-exchange chromatography. Serum NOx^- was measured using the Griess method (Nitrate/Nitrite Colorimetric Assay; Cayman Chemical, Ann Arbor, MI) [13]. Serum ADMA was determined using a recently developed enzyme-linked-immunosorbent assay method (ADMA-ELISA; DLD Diagnostics, Hamburg, Germany) [14]. Competitive ADMA-ELISA uses the microtiter plate format. Briefly, serum samples, as well as standards and kit controls, are acetylated in 96-well plates. The acetylated samples, standards, and kit controls are pipetted into the respective wells of the ADMA-coated microtiter strips and incubated with a polyclonal antibody (rabbit anti–*N*-acetyl-ADMA). After incubation, the antiserum solution is discharged; and the wells are washed with washing buffer. A peroxidase-conjugated secondary antibody is added, and then all wells are washed and incubated with tetramethylbenzidine solution as the substrate for peroxidase. The enzymatic reaction is stopped using an acidic stop solution; the absorbance is then measured using a microplate reader at 450 nm. The amount of antibody bound to the solid-phase ADMA is inversely proportional to the ADMA of the sample concentration. This methodology was validated by Schulze and coworkers [15,16]; the validation data have been published elsewhere.

All analyses were performed in duplicate. The examiner was blinded to clinical and laboratory results. Intraassay and interassay coefficients of variation were less than 10% for each measurement.

2.3. Statistical analyses

Data are presented as mean ± SD and range. Differences between groups were examined for statistical significance using an unpaired *t* test. Correlations between variables were assessed using Pearson correlation test. A *P* value of less than .05 was inferred as statistically significant.

3. Results

Table 2 shows plasma arginine and citrulline concentrations, and serum NOx^- and ADMA concentrations together with the ratios of ADMA to NOx^-. Arginine concentrations of OTC-deficient patients receiving arginine

Table 2
Concentrations of plasma arginine and citrulline and serum NOx^- and ADMA

Deficiency (age at present)	Arginine (μmol/L)	Citrulline (μmol/L)	NOx^- (μmol/L)	ADMA (μmol/L)	ADMA/NOx^-
OTC, n = 7	185 ± 45‡	8.4 ± 2.1‡	67 ± 23*	0.860 ± 0.271	0.015 ± 0.007
(1.7-7.8 y)	(129-278)	(5.9-9.6)	(35.3-108)	(0.367-1.556)	(0.003-0.027)
ASS, n = 5	229 ± 19$^{‡, §}$	2867 ± 864‡	22 ± 7†	1.422 ± 0.257†	0.061 ± 0.021$^{‡, ‖}$
(9.3-17.1 y)	(204-256)	(1768-3870)	(13.8-32.1)	(1.090-1.872)	(0.034-0.082)
ASL, n = 3	259 ± 14$^{‡, §}$	562 ± 84‡	49 ± 2	1.125 ± 0.064	0.024 ± 0.002
(5.6-8.8 y)	(235-276)	(457-688)	(45.1-50.8)	(1.032-1.225)	(0.024-0.027)
Controls, n = 36	99 ± 19	36 ± 8	39 ± 16	0.513 ± 0.116	0.019 ± 0.008
(1.1-19.9 y)	(66-156)	(16-56)	(17.9-80.1)	(0.199-0.875)	(0.006-0.036)

Data are presented as mean ± SD and range.
*$P < .05$, $^{†}P < .01$, $^{‡}P < .001$ vs controls; $^{§}P < .05$ vs OTC deficiency; $^{‖}P < .01$ vs OTC deficiency and ASL deficiency. In the control group, no significant correlation was found between the age and the variables.

Table 3
Correlation (r values) in pairs of variables

Deficiency	Arginine vs NOx^-	Arginine vs ADMA	Citrulline vs NOx^-	Citrulline vs ADMA	NOx^- vs ADMA
OTC	0.99†	−0.47	0.08	−0.14	−0.39
ASS	−0.75	0.55	−0.97*	0.93*	−0.96*
ASL	0.67	−0.75	−0.98*	0.99*	−0.75

*$P < .05$, †$P < .01$ (Pearson correlation test).

were significantly higher than those of the control subjects. Those of ASS-deficient and ASL-deficient patients receiving arginine were significantly higher than those of the control levels; both levels were also significantly higher than those of OTC-deficient patients. No significant difference was found in arginine concentrations between ASS-deficient and ASL-deficient patients. Citrulline concentrations of OTC-deficient patients were significantly lower, although those of ASS-deficient and ASL-deficient patients were significantly higher than the control levels. Citrulline concentrations of ASS-deficient patients were significantly higher than those of ASL-deficient patients; their respective mean concentrations were about 80 times and 15 times as high as those of the controls.

The NOx^- concentrations of OTC-deficient patients receiving arginine were significantly higher than those of the control subjects, although those of ASS-deficient patients receiving arginine were significantly lower. The NOx^- concentrations of ASL-deficient patients were comparable with the control levels. The ADMA concentrations of ASS-deficient patients were significantly higher than the control levels. Those of ASL-deficient patients appeared to be higher than the control levels, but the difference was not statistically significant. The ratios of ADMA to NOx^- of ASS-deficient patients were significantly higher than those of the control subjects and those of OTC-deficient and ASL-deficient patients.

Table 3 presents correlations between pairs of variables. In OTC-deficient patients, arginine concentrations were significantly and positively correlated with NOx^- concentrations, but not with ADMA concentrations. Citrulline concentrations were not significantly correlated with either NOx^- or ADMA concentrations. No significant correlation was found between their NOx^- and ADMA concentrations. In ASS-deficient and ASL-deficient patients, arginine concentrations were not significantly correlated with either NOx^- or ADMA concentrations. In those patients, the citrulline was significantly inversely correlated with NOx^- and significantly positively correlated with ADMA. In ASS patients, the NOx^- was significantly and inversely correlated with ADMA.

4. Discussion

Nitric oxide is synthesized from arginine by NOS. The availability of arginine is a rate-limiting factor in cellular NO production. Citrulline, a by-product of the NOS reaction, is recycled to arginine by successive actions of ASS and ASL, forming the citrulline-NO cycle [1,2]. The citrulline-NO cycle operates mainly in the kidney and vascular endothelium. Of note, ASS and ASL are colocalized with NOS3 in the caveola, a subcompartment of the plasma membrane, in endothelial cells [17,18]. The NOS3-ASS-ASL complex in the caveola might play a key role in endothelial NO production. In various cells including activated macrophages, vascular endothelial cells, and smooth muscle cells, ASS and sometimes ASL are coinduced with NOS. Arginine is hydrolyzed to ornithine and urea by arginase. The arginase pathway is operating mainly in the liver. Therefore, whole-body NO production might be modulated by the recycling and degradation of arginine.

Prompted by the possible connection of the urea cycle to the NO pathway, we sought to examine NO metabolism in congenital urea cycle enzyme defects inherently showing hyperammoninemia and hypoargininemia.

The results of our study demonstrated that the blood concentrations of NOx^- and ADMA inhibiting NO synthesis, along with those of arginine and citrulline, varied considerably according to the type of defect.

Amino acid profiles of OTC-deficient patients receiving arginine were characterized by significantly high arginine and significantly low citrulline concentrations. The NOx^- concentrations of those patients were significantly higher than those of the control levels. Their ADMA concentrations were also increased, maintaining ADMA/NOx^- ratios comparable with the control levels. Their NOx^- concentrations were significantly and positively correlated with arginine concentrations. These findings suggest that arginine replacement contributes substantially to NO production in OTC-deficient patients and that blood arginine concentration might affect NO synthesis in these patients.

On the other hand, ASS-deficient and ASL-deficient patients receiving arginine presented with prominent hypercitrullinemia accompanying high plasma arginine concentrations. The ASS-deficient patients showed low NOx^- and high ADMA concentrations, resulting in the high ratios of ADMA to NOx^-. Lucke et al [12] also found significantly higher ADMA concentrations in ASS-deficient patients than in their controls. In our patients, the NOx^- was significantly and inversely correlated with citrulline but not with arginine. Moreover, their ADMA concentrations were increased in parallel with the citrulline concentrations. The ASL-deficient patients also presented with prominent hypercitrullinemia, but the citrulline concentrations were less severe in magnitude than those of ASS-deficient patients. The ASL-deficient patients had NOx^- concentrations that were comparable with the control levels, different from ASS-deficient patients. Their ADMA concentrations and ADMA/NOx^- ratios also remained almost comparable with the respective control levels. However, their NOx^- concentrations were significantly and inversely correlated with the citrulline concentrations, but not with the arginine

concentrations. Based on these results, we speculated that the extremely high blood concentration of citrulline suppressed NO synthesis in ASS-deficient and ASL-deficient patients.

Our previous study raised a possibility that clinical presentations of OTC deficiency before arginine replacement were, in part, attributable to the low NO synthesis resulting from low plasma arginine concentration [11]. The results described herein suggest that arginine replacement is effective in maintaining NOx^- at normal or supranormal levels in OTC and ASL-deficient patients. Despite keeping arginine at high concentrations by arginine replacement, however, the NOx^- concentrations remained low in ASS-deficient patients. That finding indicates that oral arginine administration does not enhance NO synthesis sufficiently in ASS-deficient patients.

Suppressed NO synthesis in ASS deficiency might be attributable to high plasma ADMA concentrations [3]. Considering that the blood ADMA concentrations were increased in parallel with the citrulline concentrations in ASS-deficient and ASL-deficient patients, ADMA production in these 2 disorders might be influenced by blood citrulline concentration. Further study of ADMA metabolism, including dimethylarginine dimethylaminohydrolase that hydrolyzes ADMA to citrulline and dimethylamine, should be required to elucidate the mechanisms leading to increased ADMA in association with hypercitrullinemia [4,5].

The pathophysiology of congenital urea cycle disorders remains to be clarified precisely. Hyperammonemia and glutamine accumulation do not appear to be the only cause of brain dysfunction and damage in patients [10]. Our data raised a possibility that inappropriate NO synthesis would also be associated with the pathophysiology and clinical presentations of urea cycle defects.

The present study showed that endogenous NO syntheses in patients with urea cycle disorders receiving arginine replacement were associated with the plasma arginine or citrulline concentrations. These results suggest that more attention should be devoted to the NO pathway in the management of congenital urea cycle disorders.

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