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Letter to the Editor

Assessment of nitric oxide biosynthesis and peroxynitrite formation within the central nervous system by measuring L-citrulline in the cerebrospinal fluid?

L-Arginine is a semi-essential amino acid. This basic, a guanidino group (N^G) containing amino acid is involved in many vital physiological processes. In the urea cycle, L-arginine is hydrolyzed to L-ornithine, which is further converted to L-citrulline. A relatively minor part of L-arginine is decarboxylated to form the biogenic amine agmatine. L-Arginine is also the substrate for the enzyme nitric oxide synthase (NOS; EC 1.14.13.39), which converts L-arginine to nitric oxide (NO) and L-citrulline (Fig. 1). It is estimated that in humans, much less than 1% of L-arginine is involved in the L-arginine/NO pathway [1]. Nevertheless, this pathway actively participates in the regulation of numerous physiological processes including vasodilation, inhibition of platelet aggregation and neurotransmission. These biological functions are attributed to NO and are mediated largely by cGMP.

From the quantitative point of view, the major part of L-arginine is utilized in protein synthesis. Thus, human serum albumin contains 24 molecules of L-arginine. The guanidino group of L-arginine moieties in tissue proteins is methylated by protein–arginine methyltransferases to produce N^G-mono- and N^G-dimethylated L-arginine moieties. Ordinary proteolysis leads to formation of the free amino acids N^G-monomethyl-L-arginine (MMA) and N^G-dimethyl-L-arginine which exists in two isomeric forms, i.e. as N^G,N^G-dimethyl-L-arginine or asymmetric dimethylarginine (ADMA) and N^G,N'^G-dimethyl-L-arginine or symmetric dimethylarginine (SDMA). MMA, ADMA and SDMA are inhibitors of NOS, with ADMA being considered to mainly control NOS activity [2]. On the other hand, the enzyme dimethylarginine dimethylaminohydrolase (DDAH) controls the inhibitory action of ADMA by regulating its concentration intracellularly [2]. DDAH hydrolyzes ADMA to form L-citrulline and dimethylamine (DMA) (Fig. 1). ADMA and DMA are primarily eliminated by the kidney. The daily whole-body production rate of ADMA in healthy humans is estimated to be approximately 300 μmol, of which 250 μmol is renally eliminated as DMA and 50 μmol is eliminated as ADMA [3]. Thus, the daily whole-body production rate of L-citrulline from ADMA by DDAH is estimated to be approximately 250 μmol. On the basis of the 24-h urinary excretion of nitrate in healthy humans with standardized low-nitrate diet, the whole-body NO production rate is estimated to be 600 μmol per day [4], which corresponds to a daily production rate of 600 μmol of L-citrulline from L-arginine via NOS. Therefore, NOS as well as DDAH contribute to L-citrulline by the same order of magnitude which is several times smaller than the daily contribution of the urea cycle to L-citrulline. Certainly, at present the respective extent of contribution of a particular organ and metabolic pathway to whole-body production of NO and L-citrulline is unknown.

In physiological conditions, the L-arginine/NO pathway is balanced. On a biochemical basis, the status of the L-arginine/NO pathway can be quantitatively characterized by determining NOS activity and the concentration of major determinants of this pathway in the circulation, notably L-arginine and ADMA. In principle, NOS activity can be determined by measuring NO or L-citrulline. Because of its very short half-life, NO itself is not accessible to accurate analytical determination. Instead of NO, the concentration of its oxidative metabolites, i.e. nitrite and nitrate, represents a reliable method to assess NOS activity [4]. However, this approach may be limited by the fact that nitrite and nitrate are ubiquitous laboratory chemicals and components of foods and drinking water. Nevertheless, this limitation can be overcome by taking appropriate measures such as standardized low-nitrite/nitrate diet in clinical studies. In theory, measuring L-citrulline may be considered as a method of assessing NOS activity. In practice, however, this approach is limited to in vitro conditions in which isolated NOS preparations are used and/or other sources of L-citrulline are excluded.

In an article, recently published in the journal, Pérez-Neri et al. [5] reported on a method to estimate NO biosynthesis in the central nervous system (CNS) by measuring the concentration of L-citrulline in the cerebrospinal fluid (CSF) of humans by means of a standard HPLC method with precolumn derivatization using *o*-phthaldehyde/2-mercaptoethanol. By this method, other amino acids were also measured in the CSF, but only L-arginine concentrations were reported. Pérez-Neri et al. proposed that the concentration of L-citrulline in the CSF could be used as a biomarker of NO biosynthesis in CNS. This proposal has been justified mainly by the finding that L-citrulline concentration in the CSF from the patients with in-

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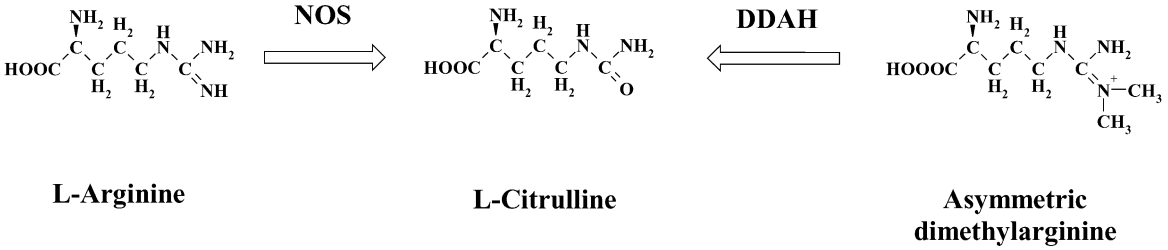

Fig. 1. Contribution of two metabolic pathways to L-citrulline. Left: the enzyme nitric oxide synthase (NOS) oxidizes L-arginine to L-citrulline and nitric oxide (NO). Right: the enzyme dimethylarginine dimethylaminohydrolase (DDAH) hydrolyzes asymmetric dimethylarginine (ADMA) to form L-citrulline and dimethylamine [$(CH_3)_2NH$; DMA]. For simplicity, the reaction products NO and DMA as well as other participants are not shown. ADMA is formed by proteolysis of asymmetrically dimethylated L-arginine moieties in proteins and is an inhibitor of NOS.

flammatory and/or infectious processes was by 8 μM higher than in the patients without inflammation and infection in the CNS who served as control. The authors' assumption, that the increase in L-citrulline concentration in the CSF cannot be attributed to an inflammation-induced/infection-induced elevated DDAH activity, is entirely unfounded. I would like to discuss this important issue.

DDAH is highly expressed in the brain, and brain tissue is highly rich in proteins containing mono- and dimethylated L-arginine, e.g. 1–2 μmol of ADMA per gram of protein [6]. It has been reported that the levels of MMA and ADMA in the brain are at least 10 times lower than those of L-arginine [7]. The molar ratio of ADMA to L-arginine in the brain seems to be even higher than that in the circulation, which amounts to approximately 1:150 [8]. We may, therefore, reasonably assume that L-citrulline will be abundantly present in the brain and, moreover, L-citrulline may be derived mainly from ADMA by the catalytic action of DDAH rather than from L-arginine via NOS. Indeed, the relatively high concentrations of L-citrulline of the order of 24 μM in the CSF of the control group, which are even higher than those of L-arginine (18 μM), support a high DDAH activity in the brain. Considering an ADMA concentration of 50 nM in the CSF in controls [9], the results by Pérez-Neri et al. [5] suggest that the ADMA-to-L-arginine molar ratio in CSF is 1:360, i.e. two times lower than that in plasma. Such a ratio would be supportive of a high rather than a low DDAH activity in the brain. Unfortunately, the authors did not determine the neuronal DDAH activity in the patients by measuring DMA in the CSF nor that of NOS by measuring CSF nitrite and/or nitrate concentrations. We have shown that ADMA is a potent (IC_{50} 1.5 μM), non-competitive inhibitor (K_i 0.4 μM; K_{ii} 1.6 μM) of neuronal NOS [8]. We may, therefore, assume that neuronal NOS activity will be considerably inhibited under basal conditions.

Pérez-Neri et al. [5] reported a concomitant increase in the CSF concentration of L-arginine by approximately 5 μM, which, however, did not achieve statistical significance ($P = 0.115$) most likely because of the small patient group ($n = 5$) investigated. The concomitant increases in the L-citrulline and L-arginine CSF concentration, and perhaps also in that of other amino acids measured, do not convincingly support the idea of an inflammation/infection-mediated elevation in NOS activity. Several sources leading to elevated amino acid concentrations in the CSF in inflammatory/infectious patients may exist. Elevated NOS and DDAH activity may explain, at least in part, the increased concentrations of L-citrulline in the CSF. Further potential reasons may include enhanced permeability of the blood–brain barrier (BBB) for amino acids including L-citrulline and L-arginine as well as elevated proteolysis as a result of inflammation and/or viral or non-viral infection. The authors' finding of clearly elevated protein concentration in the CSF in the inflammatory group is supportive of these considerations.

Furthermore, Pérez-Neri et al. [5] suggest that citrulline concentration could be taken as complimentary to the detection of other markers of NO biosynthesis, and a reduction in nitrite and/or nitrate levels combined with an increase in citrulline concentration might be considered suggestive of peroxynitrite formation. This suggestion is in contradiction to literature data available so far. It is well established that the major metabolic fate of peroxynitrite, which is formed by the reaction of NO and superoxide ($O_2^{\bullet -}$), involves isomerization/decomposition to nitrate and nitrite and that only a very minor part of peroxynitrite reacts with biomolecules to form other reaction products, notably 3-nitro-L-tyrosine and *S*-nitrosothiols. This is impressively reflected in the concentration levels of nitrite, nitrate and 3-nitro-L-tyrosine in the circulation, which are of the order of 1 μM, 40 μM, and 1 nM, respectively [10]. Thus, the best way to show and quantify peroxynitrite formation is to measure directly a footprint left by peroxynitrite, notably 3-nitro-L-tyrosine. In this context, it should also be emphasized that inhibition of DDAH activity based on *S*-nitrosylation of the cysteine-249 of DDAH by peroxynitrite should be considered possible [11], but in vivo most likely negligible.

In conclusion, in vivo in humans NO biosynthesis is best assessed by measuring the concentration of its major metabolites nitrite and nitrate in biological fluids including the CSF under fasting conditions or standardized low-nitrite diet/low-nitrate diet. Estimation of NO biosynthesis by measuring L-citrulline concentration is by far much more problematic because of considerable contribution to L-citrulline by the urea cycle and the DDAH pathway in particular in the CNS. The citrulline concentration cannot at all be taken as complimentary to detect peroxynitrite.

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