Reply to comment on: "Assessment of nitric oxide biosynthesis and peroxynitrite formation within the central nervous system by measuring L-citrulline in the cerebrospinal fluid?"

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Reply to comment on: "Assessment of nitric oxide biosynthesis and peroxynitrite formation within the central nervous system by measuring L-citrulline in the cerebrospinal fluid?"

In a study performed by our group and published recently in this journal, we reported on a chromatographic method that may be complementary to the study of nitric oxide (NO) biosynthesis in the central nervous system (CNS), based on the measurement of cerebrospinal fluid (CSF) concentration of citrulline [1]. Even though we have discussed in that paper why citrulline accumulation during infection/inflammation in the CNS should be considered as a product of NO synthase (NOS, EC 1.14.13.39) activity and less likely a result from other metabolic pathways, Dr. Dimitrios Tsikas discussed our conclusions arguing that citrulline accumulation is more likely to be produced by dimethylargininase (dimethylarginine dimethylaminohydrolase, EC 3.5.3.18) activity. We thank Dr. D. Tsikas for his valuable comments and consider proper to clarify some important issues regarding our results.

In our opinion, dimethylargininase activity, although a possible source of L-citrulline, is not the most likely explanation for the rise in CSF citrulline concentration found in patients with infectious and/or inflammatory processes within the CNS. This enzyme has been identified in the rodent brain tissue [2], although it has not been found in hippocampal tissue from normal human controls by some authors [3].

Furthermore, inflammation/infection-induced elevation of dimethylargininase activity remains controversial. Interleukin-1β-induced dimethylargininase expression found by Ueda et al. in smooth muscle cells [4] has not been found, however, in endothelial cells, using tumor necrosis factor-α (TNF-α) as an inducer [5]; methodological differences may account for these discrepancies. Both cytokines may be secreted during an immune response and, to the best of our knowledge, it has not been already reported which of them develops the predominating effect over enzyme expression in vivo. Even though our results may be debated arguing changes in dimethylargininase activity, studies that have found an inhibitory effect of NOS II activity [6], viral infection [7] and cytokines such as TNF-α [5] on the activity of this enzyme should not be underestimated. These studies, although in vitro, are consistent with findings on models of endothelial injury in vivo [8]. However, direct measurement of enzyme activity during an immune response in vivo awaits further investigation, and the hypothesis that dimethylargininase activity is responsible for citrulline accumulation during infection should be supported on experimental data.

On the other hand, the concentration of asymmetric dimethylarginine (ADMA) in the nervous system has been estimated to be at least 10 times lower than that of arginine [8]. In this regard, it seems contradictory to consider ADMA as the main source of citrulline biosynthesis, while arginine is the most available substrate. In fact, if dimethylargininase activity may be the main source for citrulline biosynthesis, citrulline immunoreactivity would not be expected to be lacking in nNOS knockout animals as it has been reported by Keilhoff et al. [10]. It is true that the low ADMA concentration combined with the accumulation of citrulline is suggestive of a high rate of dimethylargininase activity, but it is also consistent with a decreased enzyme activity in the absence of enough substrate concentration.

Also, it is not the ratio of ADMA to the total pool of citrulline what would reflect dimethylargininase activity, but the ratio of ADMA to only the amount of citrulline generated by this enzyme, since it is not the only enzyme that regulates citrulline concentration in the CNS [9]. We think that only enzyme activity assays would resolve this issue and, to the best of our knowledge, a comparative study between NOS and dimethylargininase activities in brain tissue has not been reported so far. To our point of view, we do not need to determine CSF nitrate concentration in our study [1] to estimate NOS activity; NOS II expression and activity during infection/inflammation is a well-known event [9] and CSF nitrate concentration during CNS infection has already been reported [11]; in our opinion, these results do not need to be replicated.

The elevation of CSF citrulline concentration in infectious disorders reported recently [1] is not likely to be due to increased blood–brain barrier (BBB) permeability, since the expected correlation between CSF protein and citrulline

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concentrations was not found. Also, although CSF protein was significantly elevated, the slight increase in arginine concentration in the same samples was not statistically significant [1]. These results do not seem to be due to sample size since this was not a problem to achieve statistical significance in citrulline and protein concentrations, independently. Even though BBB permeability may be altered, this is not sufficient to explain our results. Also, the increase in the concentration of some amino acids in the CSF of patients with CNS infection/inflammation is not likely to be due to infection-induced proteolysis since the only amino acid whose accumulation reached statistical significance is a non-proteogenic amino acid, i.e. citrulline [1].

We propose that citrulline determination should be considered complementary to the analysis of other markers of NO biosynthesis, including nitrite and nitrate. This should be especially important in the context of non-enzymatic NO synthesis that has been reported [12]. We agreed that the best way to analyze peroxynitrite formation is nitrotyrosine quantitation, although it should be evaluated carefully because HPLC artifacts have been extensively reported [13,14]; for that reason elevations in nitrotyrosine concentration should be taken carefully.

In our opinion, there is no reason to consider that nitrosylation inhibition of dimethylargininase during NOS II activity is negligible in vivo. An assessment like this, is contradictory to some studies [6], again in vitro, that should not be underestimated. In this regard, to discard a demonstrated inhibitory mechanism should be accompanied by in vivo experimental results.

Finally, we agreed with the fact that citrulline accumulation should be analyzed carefully under normal conditions where both dimethylargininase and argininosuccinate synthetase participate in the regulation of the concentration of this amino acid.

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