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Cerebrospinal Fluid Secretory Ca²⁺-Dependent Phospholipase A2 Activity: A Biomarker of Blood-Cerebrospinal Fluid Barrier Permeability

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Abstract

The blood-brain barrier, the blood-cerebrospinal fluid barrier (BCB) and other specialized brain barriers are increasingly recognized as a major obstacle to the treatment of most brain disorders. The impairment of these barriers has been implicated in neuropathology of several diseases, such as autism, ischemia, multiple sclerosis and Alzheimer disease. This dual function of the blood-neural barriers points out the importance and need for the development of techniques that can evaluate the nature and level of their integrity. Here we report the discovery of CSF secretory Ca²⁺-dependent phospholipase A2 (sPLA2) activity as a measure of BCB permeability. Lumbar CSF from BCB-impaired ($n=26$), multiple sclerosis ($n=18$) and healthy control ($n=32$) cases was analyzed using both a newly developed continuous fluorescence assay for CSF sPLA2 activity and CSF/Serum albumin ratio (Q_{Alb}), the most common and established method to evaluate BCB permeability. While both measurements showed no significant differences between multiple sclerosis and age-matched normal healthy cases, they were highly correlated. Though the CSF sPLA2 activity and Q_{Alb} had over 95 % agreement, the former was found to be more sensitive than the latter in measuring low levels of BCB impairment.

Keywords

Albumin; Blood-brain barrier; Blood-cerebrospinal fluid barrier; Cerebrospinal fluid; Phospholipase A2

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Introduction

Barriers between the peripheral circulation and neural tissues are collectively referred to as blood-neural barriers (BNB) and include among others, the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCB). While the BBB is diffusely distributed throughout the brain capillaries, the BCB is found at the choroid plexus [5]. BBB and BCB both are involved in regulation of transport of substances into and out of the brain and the cerebrospinal fluid (CSF), respectively. This essential role makes their integrity, which is in part achieved by the well-known tight junctions, essential in order to maintain brain homeostasis [5].

A disruption of the tight junction architecture and dysregulation of transporters increase the permeability of the BNB and *de facto* contribute to the pathophysiology of many neurodevelopmental diseases (e.g., autism, schizophrenia, epilepsy), neurological lesions (e.g., stroke) and neurodegenerative disorders (e.g., Alzheimer disease, Parkinson disease). Paradoxically, the high level of integrity of these BNB provides obstacles for successful delivery of therapeutical neuro-pharmacologic drugs. This contradiction points out the importance of evaluating the BNB integrity in physiological as well as pathophysiological conditions. The secretory Ca^{2+} -dependent phospholipases A2 (sPLA2) belong to the phospholipase A2 (PLA2) family and catalyze the hydrolysis of the *sn*-2 ester bond of glycerophospholipids, resulting in the production of free fatty acids (e.g. arachidonic acid, docosahexaenoic acid) and lysophospholipids, both of which are precursors for the synthesis of proinflammatory mediators such as eicosanoids [8]. While the contribution of sPLA2 isozymes to various aspects of inflammation and their expression in the central nervous system are well-documented [8,29], their involvement in BBB and/or BCB function has not been reported.

In the present study, using a newly developed continuous fluorescence assay for CSF sPLA2 as well as CSF/Serum albumin ratio (Q_{Alb}), the most common and established method to evaluate BCB permeability, we report: 1) the discovery of CSF sPLA2 as a sensitive new technique for the measurement of BCB permeability and 2) an apparent lack of BCB impairment in multiple sclerosis (MS).

Materials and Methods

Chemicals

Bis-BODIPY[®] FL C₁₁-PC (1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine) was purchased from Invitrogen (Eugene, OR, USA). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and other chemical products were from Sigma-Aldrich (St Louis, MO, USA).

Study Participants

The study population was retrospectively collected lumbar CSFs and sera available for research purpose from healthy individuals, patients with MS and cases with BCB impairment. The clinical features of the subjects involved in this study are summarized in Tables 1 and 2. The healthy control group (n = 32) consisted of subjects that were actively recruited to serve as controls and for which no cognitive disturbance was observed. The MS group (n = 18) consisted of three patients with primary progressive MS and 15 patients with relapsing-remitting MS. No patient was under immunomodulatory treatment. Patients with BCB impairment (n = 26) were seen on the clinical suspicion of a neurological disorder but no structured follow-up regarding final diagnoses was available.

The study was approved by the ethics committees of Eastern Norway and the University of Gothenburg and by the Institutional Review Board of the New York State Institute for Basic

Research in Developmental Disabilities. Informed consent was obtained from all participants in accordance with the provisions of the Helsinki Declaration.

CSF/Serum sampling and biochemical analyses

While venous puncture was performed to collect serum, CSF was obtained by lumbar puncture from the L3/L4 or L4/L5 intervertebral space and the first 12 mL of CSF were collected, centrifuged at $2000 \times g$ for 10 min at 4 °C and then aliquoted in 1 ml polypropylene tubes. All samples were sent in dry ice from Sahlgrenska University Hospital to New York State Institute for Basic Research, and were aliquoted once again to minimize freeze/thaw steps and finally kept at -80 °C until used.

Biochemical analyses were performed as described previously. In summary, Q_{Alb} determination and oligoclonal IgG-bands identification were used to evaluate the BCB integrity [31] and as an indicator in the diagnosis of multiple sclerosis, respectively. Quantitative determination of both parameters was performed by common approach as previously described [11]. Thus, while Q_{Alb} was calculated as CSF albumin (mg/L)/serum albumin (g/L), identification of CSF-enriched oligoclonal IgG-bands was based on a cutoff level of two or more IgG bands.

sPLA2 activity was measured using a newly developed continuous fluorescent assay [4]. In a 96-well microplate, 5 μ L lumbar CSF was diluted in 90 μ L sPLA2 assay buffer (10 mM Tris-HCl, pH 7.4; 100 mM KCl; 5 mM $CaCl_2$; 1 mM DTT). Then, 5 μ L liposomes made from 0.4 mg/mL 100 % DOPC and labeled with 100 μ M Bis-BODIPY[®] FL C₁₁-PC were added to each well and the microplate was immediately placed in a temperature controlled (30 °C) cytofluor multi-well plate reader series 4000 (PerSeptive Biosystems, Foster City, CA, USA). The fluorescence intensity was recorded over 90 min (91 cycles of 60 sec each) at 485 nm excitation and 530 nm emission. Finally, sPLA2 activity was evaluated using linear curve fitting with Graph Prism 3.0 (GraphPad, San Diego, CA, USA).

Statistical analyses

Statistical analyses were performed using Statgraphics Centurion XV (StatPoint, Herndon, VA, USA) and Graph Prism 3.0. A failure in the normal distribution of a variable was considered when values of Skewness and Kurtosis were outside of the range -2 to +2. If a variable was not normally distributed, a logarithmic transformation followed by a parametric test or a non-parametric test was performed. Frequency distributions were compared with Fisher's test. Differences between two means were assessed with unpaired, two-tailed Student's *t*-test or Mann-Whitney's test. Correlations were analyzed statistically using Pearson's correlation test or Spearman's rank correlation test. Bland-Altman curves [2] and Sotgia curves [28] were constructed for comparison between Q_{Alb} and sPLA2 activity assays. While Bland-Altman analysis allows to calculate limits of agreement and systematic errors, Sotgia approach allows to visualize graphically the difference in magnitude between two analytical methods. To minimize the unit difference and to perform both analyses, variables were normalized to the average of all values of the corresponding parameters and converted to percentage. Receiver operating characteristic (ROC) analysis was used to calculate sensitivity, specificity and cutoff values of considered biomarkers in selected groups. The optimal cutoff value was defined at the optimal combination of sensitivity and specificity. The level of significance was defined as $P < 0.05$.

Results

Correlation of CSF sPLA2 activity to Q_{Alb}

Secretory PLA2s are known to be associated with systemic inflammatory, autoimmune or allergic diseases [22,32] and to play a key role in neuroinflammation [23]. Recently, we have shown that the CSF sPLA2 activity is significantly increased in Alzheimer disease, a well known neuroinflammatory disorder [4]. In order to assess the relevance of CSF sPLA2 activity measurement in an ongoing neuroinflammatory process, we investigated its level in MS, an inflammatory disease of the central nervous system characterized by destruction of myelin sheaths and axonal loss [27]. While no significant difference ($t_{(df=40)} = 0.607$, $P = 0.548$) of sPLA2 activity between MS and age-matched healthy controls was found (Table 1), a significant and positive correlation between sPLA2 activity and Q_{Alb} in age-matched healthy control ($r = 0.590$; $P < 0.005$; 95% CI: 0.212–0.814) as well as in MS ($r = 0.870$; $P < 0.0001$; 95% CI: 0.670–0.953) cases was observed (Table 1). Moreover, neither a significant difference in Q_{Alb} between multiple sclerosis and age-matched healthy controls (Table 1), nor a significant correlation between age and sPLA2 activity or Q_{Alb} were observed (*data not shown*). These results suggest a strong correlation of the CSF sPLA2 activity with the degree of BCB function as measured by Q_{Alb} .

According to the upper reference limit for Q_{Alb} (6.8 for individuals under 45 years of age and 10.2 for individuals over 45 years of age [3]), only 2 out of the 42 cases (5 %) independent of their clinical diagnoses were found to have a BCB dysfunction. Even when these two cases are removed from data analysis, the positive correlation between CSF sPLA2 activity and Q_{Alb} remains in age-matched healthy controls ($r = 0.464$; $P = 0.039$; 95% CI: 0.027–0.752) and in MS ($r = 0.859$; $P < 0.0001$; 95% CI: 0.633–0.950) as well as in total population ($r = 0.602$; $P < 0.0001$; 95% CI: 0.341–0.777). Thus, independent of the clinical diagnoses and when no BCB impairment is observed, sPLA2 activity is strongly and positively correlated to Q_{Alb} .

CSF sPLA2 activity as a measure of blood-CSF barrier permeability

To determine if the positive correlation between sPLA2 activity and Q_{Alb} is also applicable in BCB impaired cases, we investigated this relation in a set of patients with and without BCB dysfunction (Table 2). As expected, significant differences in both sPLA2 activity and Q_{Alb} between BCB impaired and age-matched BCB normal groups were found (Table 2). While a significant and positive correlation between sPLA2 activity and Q_{Alb} in BCB impaired ($r = 0.710$; $P = 0.0001$; 95% CI: 0.429–0.865) and age-matched BCB normal ($r = 0.516$; $P = 0.0099$; 95% CI: 0.142–0.761) and groups as well as in the total ($r = 0.877$; $P < 0.0001$; 95% CI: 0.790–0.930) cases was observed (Fig. 1A), no significant correlation between sPLA2 activity or Q_{Alb} with age was noticed (*data not shown*). Thus, independent of the age and of the BCB condition, sPLA2 activity is strongly correlated to Q_{Alb} . These results suggest that like Q_{Alb} , sPLA2 activity measurement can evaluate BCB impairment.

We evaluated the strength of this new method of measuring BCB impairment by assessing the agreement between the two measurements, i.e. Q_{Alb} and sPLA2 activity, using the Bland-Altman method [2] and the Sotgia method [28] which allow to calculate limits of agreement as well as systematic errors and to visualize graphically the difference in magnitude between two analytical methods, respectively. The Bland-Altman plot is shown in Figure 1B. The difference between % Q_{Alb} and % sPLA2 activity measurement was plotted against the % mean from both methods and for each sample measured. The systematic difference between the two assay methods as calculated by the Bland-Altman analysis was <0.001 . The two methods agreed fairly well for Q_{Alb} values <24.9 and only 4.2 % of the patients had values that differed by >2 SD. Since agreement between two methods is generally acceptable if <5 % of values differ by >2 SD, we can consider that the two approaches are equivalent in evaluating

BCB impairment. Moreover, the Sotgia analysis shows that the values from sPLA2 activity measurement are higher and lower than the values from Q_{Alb} at low and high levels of Q_{Alb} , respectively (Fig. 1C). To evaluate the discrimination power of sPLA2 activity measurement, we performed a ROC analysis (Fig. 1D). This analysis indicates that sPLA2 activity efficiently discriminates normal BCB from impaired BCB (AUC = 0.9809; 95% CI: 0.95–1.01). Furthermore with a cutoff value of 5.146 $\Delta FI/min$, the sensitivity and specificity are 91.67 % (95% CI: 73.00–98.97) and 95.83 % (95% CI: 78.88–99.89), respectively. Thus, sPLA2 activity measurement in CSF provides a sensitive and simple new approach to evaluate the BCB integrity.

Discussion

In this study we have discovered that BCB function can be evaluated by measurement of CSF sPLA2 activity. From the Q_{Alb} and CSF sPLA2 activity analyses on individuals with and without BCB dysfunction, we have found that both approaches are equivalent in evaluating BCB impairment as shown by an agreement of over 95 % between the two methods. Moreover, using the Sotgia method [28] we observed that the measurement of sPLA2 activity in human CSF was a more sensitive technique than the determination of Q_{Alb} . Alterations of BNB architecture contribute to the pathophysiology of several brain disorders and their high level of integrity can be obstacles for successful delivery of potentially beneficial neuro-pharmaceutical agents. The discovery of CSF sPLA2 as a measure of BNB permeability described in the present study can help, among others to 1) identify BNB impairment in various neurological conditions; 2) increase the specificity of differential diagnosis of neurodegenerative disorders; 3) monitor time-dependent response to therapies that target BNB disruption as well as disease progression and 4) identify potential therapeutic windows in order to increase drug efficacy.

To date, several techniques other than CSF sPLA2 activity measurement are available to assess blood-neural barriers permeability but their safety, specificity or sensitivity have been recently reconsidered. For instance, imaging approaches, in particular Gadolinium enhancement in T1-weighted magnetic resonance imaging (MRI) scans, are considered as the most robust to assess specifically BBB impairment [10,20]. But, this MRI analysis requires 1) intravenous administration of Gadolinium (Gd), a contrast agent for which a possible toxic effect has been recently reported [30] and 2) highly specialized equipment and expertise, which make medical imaging less suitable for routine clinical labs compared to microplate assays of CSF sPLA2 activity. Since disruption in blood-neural barrier integrity allows protein leakage in both directions, the other strategies currently in use consist in blood and/or CSF evaluation of either a blood-specific protein or a brain-specific protein such as albumin and S100 β , respectively. However, due to the lack of specificity to the brain attributed lately to S100 β [13], only CSF/Serum albumin ratio (Q_{Alb}) remains extensively used and widely accepted to assess BCB and/or BBB function [3,9,31].

Although the CSF sPLA2 assay is advantageous over existing methods of measurement of BNB in its ease of use, the nature of BNB measured remains unknown. But, based on the strong correlation between Q_{Alb} and CSF sPLA2 activity, it seems that the latter mainly measures the assessment of BCB impairment instead of BBB. Indeed, even if it is sometimes assumed that an increase of albumin concentration in CSF is an estimation of a BBB permeability, it seems more accurate to associate such assessment to a BCB breakdown. The reason for such a conclusion is due to the fact that a Q_{Alb} increase more likely and directly reflects albumin release across the BCB than a transport from blood to brain across an impaired BBB followed by diffusion across the permeable barrier between brain and CSF. In this context we also considered the possibility that changes in CSF sPLA2 activity reflect mainly changes in brain metabolism rather than altered BCB function. It is conceivable that this increase in CSF sPLA2

activity is due to a transport from the brain interstitial fluid, which readily communicates (by relative unrestricted diffusion) with the CSF. But our results that showed a lack of increase in Q_{Alb} and CSF sPLA2 activity in MS patients indicated to the contrary.

MS is a chronic inflammatory demyelinating autoimmune disease of the central nervous system [27]. Studies from MS patients as well as from experimental autoimmune encephalomyelitis, a well-known animal model for multiple sclerosis [16,17], have shown a key role for both cytosolic Ca^{2+} -dependent PLA2 [14,17,21] and sPLA2 [7,24] in the pathophysiology of this disorder. In contrast, in the present study, we found a lack of alteration in the activity of CSF sPLA2 in MS. Together, these data do not reflect conflicting outcomes but instead assume that CSF and brain sPLA2 isozyme(s) are structurally and functionally distinct.

Because most of the CSF emanates from the choroid plexus, and because the bulk flow of the secreted CSF through the ventriculo-subarachnoid spaces is an order of magnitude greater than that of the brain interstitial fluid [15], it is likely that increase of CSF sPLA2 activity reflects phenomena taking place at the BCB interface. Thus, a transchoroidal transport of sPLA2 from the blood after a BCB breakdown as it is for albumin can be considered. But we have recently shown that CSF sPLA2 is of central nervous system-origin [4]. Another possibility is an increase of sPLA2 synthesis/secretion from the choroidal epithelium which in turn is involved in the BCB breakdown process. Corroborating this hypothesis, Hampel H. *et al.* proposed that an altered BCB function in neurodegenerative disorders may result from immune mediated events initiated, for example by increased levels of circulating inflammatory mediators [12]. In return, these inflammatory cytokines can induce the secretory PLA2 expression [26] followed by an increase of arachidonic acid synthesis. Then, this polyunsaturated fatty acid may increase the blood-neural barrier permeability [1]. This postulate suggests different roles of CSF sPLA2 and Q_{Alb} in the BCB dysfunction pathway. While CSF sPLA2 seems to be involved early in the BCB impairment process, Q_{Alb} increase results from it. The fact that the values from sPLA2 activity measurement were higher and lower than the values from Q_{Alb} at low and high levels of Q_{Alb} , respectively, the Sotgia analysis of these data in the present study corroborated this assumption.

Our findings of no significant differences in Q_{Alb} or CSF sPLA2 activity in MS patients compared to age-matched controls, allow us to confirm an absence of BCB impairment in this chronic inflammatory demyelinating disease of the central nervous system. Similar findings were reported using Q_{Alb} , previously [6,18]. Pohl D. *et al.* [25] reported a blood-CSF barrier dysfunction in less than 15 % of both early- and adult-onset MS, but it is important to note that such conclusion was based on Q_{Alb} cutoff values of only 5 and 7, respectively. Thus, the BNB dysfunction considered as a major hallmark of MS seems strictly limited to a BBB impairment as shown previously by MRI scans [10,19,20]. Interestingly, we found a BCB impairment both by Q_{Alb} and by CSF sPLA2 measurements in Alzheimer disease cases previously [4]. These findings, taken together with a lack of any detectable changes in Q_{Alb} and CSF sPLA2 activity in MS in the present study, indicate a different nature of the BNB in MS vs. Alzheimer disease, and further support that Q_{Alb} and CSF sPLA2 probably mainly measure BCB integrity.

In conclusion, the present study shows that the CSF secretory (extracellular) Ca^{2+} -dependent PLA2 activity in humans measures the degree of BCB integrity with a higher sensitivity than Q_{Alb} . This finding, among others, may facilitate further studies on 1) understanding the cause of BCB impairment, 2) identifying neurologic disorders with any BCB dysfunction and 3) monitoring time-dependent response to therapies that target BCB disruption as well as disease progression.

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Fig 1. Comparison of sPLA2 activity with Q_{Alb} in patients with normal or impaired BCB

(A) Positive correlations between sPLA2 activity and Q_{Alb} (CSF/serum albumin ratio) in BCB Normal ($r = 0.516$; $P = 0.0099$; 95% CI: 0.142–0.761) and BCB Impaired ($r = 0.710$; $P = 0.0001$; 95% CI: 0.429–0.865) cases as well as in total population ($r = 0.877$; $P < 0.0001$; 95% CI: 0.790–0.930). Pearson's correlation was assessed after a logarithmic transformation of both variables; (B) Bland-Altman analysis showing the agreement between Q_{Alb} and sPLA2 activity for the measurement of BCB impairment. The mean difference ± 2 SDs are represented by horizontal solid and dotted lines, respectively; (C) Sotgia plot showing the distribution distance of individual % Q_{Alb} or % sPLA2 activity from each other and around the line of best fit (average versus average); (D) ROC analysis of diagnostic properties of sPLA2 activity for the differentiation of impaired BCB from normal BCB. The optimal cutoff level was 5.146 $\Delta FI/min$, resulting in a sensitivity of 91.67 % (95% CI: 73.00–98.97) and a specificity of 95.83 % (95% CI: 78.88–99.89), with an area under the curve (AUC) of 0.9809 (95% CI: 0.95–1.01).

Table 1Characteristics of patients with Multiple Sclerosis (MS) and age-matched healthy controls ^a

	Healthy Control ^b (n=24)	Multiple Sclerosis (n=18)	Statistical Analysis	
			Statistical value	P
Demographic, clinical and biochemical features				
Gender (Male)	10 (42%)	2 (11%)	NA	0.041
Age (years)	46.1 ± 12.3	41.6 ± 8.9	z=-1.868	0.063
Oligoclonal bands	0 (0%)	18 (100%)	NA	<0.0001
Q _{Alb}	4.7 ± 2.2	4.5 ± 1.4	t _(df=36) =0.096	0.924
sPLA2 activity (ΔFI/min)	3.7 ± 1.0	3.8 ± 0.8	t _(df=40) =0.607	0.548
Pearson Correlation between Q _{Alb} and sPLA2 activity				
Pearson coefficient	0.590	0.870		
P	<0.005	<0.0001		
95% CI	0.212 – 0.814	0.670 – 0.953		

^a Categorical data (*gender and oligoclonal bands*) are expressed as number of subject (%) and differences between MS and age-matched healthy control were assessed using Fisher's test. Continuous variables are expressed as mean ± SD and differences between MS and age-matched healthy control group were assessed using Mann-Whitney's test (*age*) or Student's *t*-test after logarithmic transformation (Q_{Alb} and sPLA2 activity). Pearson's correlation coefficient was assessed after a logarithmic transformation of both variables (Q_{Alb} and sPLA2 activity);

^b 24 out of the 32 healthy controls were selected to match age of MS group.

NA, not applicable.

Table 2Characteristics of patients with or without BCB impairment ^a

	BCB Normal ^b (n=24)	BCB Impaired ^c (n=24)	Statistical Analysis	
			Statistical Value	P
Gender (Male)	11 (46%)	16 (67%)	NA	0.244
Age (years)	57.6 ± 11.5	63.7 ± 17.5	$z=-1.876$	0.062
Q_{Alb}	5.0 ± 1.9	16.7 ± 6.6	$t_{(df=46)}=11.46$	<0.0001
sPLA2 activity ($\Delta FI/min$)	3.8 ± 0.8	6.7 ± 1.5	$t_{(df=46)}=9.261$	<0.0001

^a Categorical data (*gender*) are expressed as number of subject (%) and differences between BCB normal and BCB impaired cases were assessed using Fisher's test. Continuous variables are expressed as mean ± SD and differences between BCB normal and BCB impaired cases were assessed using Mann-Whitney's test (*age*) or Student's t-test after logarithmic transformation (Q_{Alb} and *sPLA2 activity*);

^b BCB Normal group was composed of healthy controls without BCB impairment and 24 out of the 32 healthy controls were selected to age-match with BCB Impaired group;

^c BCB Impaired group was composed of cases with $Q_{Alb}>10.2$ and for which no structured follow-up regarding final diagnoses was performed; Following a Grubbs' Test analysis, 2 outliers out of the 26 patients with BCB impaired were identified and were not considered in this analysis.

NA, not applicable.