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Lipidomics Detection of Brain Cardiolipins in Plasma Is Associated with Outcome after Cardiac Arrest

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Abstract

Objective: Brain mitochondrial dysfunction limits neurological recovery after cardiac arrest (CA). Brain polyunsaturated cardiolipins (CLs), mitochondria-unique and functionally essential phospholipids, have unprecedented diversification. Since brain CLs are not present in plasma normally, we hypothesized their appearance would correlate with brain injury severity early after CA and return of spontaneous circulation (ROSC).

Design: Observational case-control study

Setting: Two medical centers within one city

Participants (subjects): We enrolled 41 adult cardiac arrest patients in whom blood could be obtained within 6h of resuscitation. Two subjects were excluded following outlier analysis. Ten healthy subjects were controls. Sprague-Dawley rats were used in asphyxial CA studies.

Interventions(s): None

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Measurements and main results: We developed a high-resolution liquid chromatography/mass spectrometry method and determined CLs speciation in human brain, heart and plasma within 6h of (ROSC) from 39 patients with CA, 5 with myocardial infarction and 10 healthy controls. Cerebral score (C-score) was derived from brain-specific CLs identified in plasma of patients with varying neurological injury and outcome. Using a rat model of CA, CLs were quantified in plasma, brain and heart. Human brain exhibited a highly diverse cardiolipinome compared to heart that allowed the identification of brain-specific CLs. Nine of 26 brain-specific CLs were detected in plasma and correlated with brain injury. The C-score correlated with early neurologic injury and predicted discharge neurologic/ functional outcome. CL(70:5) emerged as a potential point-of-care marker predicting injury severity and outcome. In rat CA, a significant reduction in hippocampal CLs corresponded to their release from the brain into systemic circulation. C-score was significantly increased in 10min vs. 5min no-flow CA and naïve controls.

Conclusions: Brain-specific CLs accumulate in plasma early after ROSC and proportional to neurological injury representing a promising novel biomarker.

Keywords

Lipidomics; Cardiac Arrest; Plasma cardiolipin; Prognostic Biomarker; Mitochondrial dysfunction; Neurological injury; global ischemia reperfusion; lipid mediators

Introduction

The essential requirement of lipids as building blocks of membranes, bio-energetic fuel, and signaling molecules has prompted the use of lipidomic analysis for discovering biomarkers of human health and disease (1). Early attempts were encouraging revealing “prognostic” markers for common neurodegenerative diseases (2–4). Despite this general success, the application of lipidomics to identify tissue-specific injury markers under conditions of combined or sequential interrelated organ injuries has yet to be performed. Mitochondrial dysfunction is a critical mechanism underlying the pathogenesis of cerebral and myocardial injury after CA (6, 7). Cardiolipin (CL) is a mitochondria-specific phospholipid central in maintaining and regulating function. Precise regulation of molecular species of CL is necessary to facilitate diverse mitochondrial functions, including integration of cellular bioenergetics, apoptosis, mitophagy, membrane fusion and fission (7).

CLs exhibit the greatest structural diversity of any phospholipid class given their tetra-acylated structure. These diverse acyl chains can be converted into a homo-acylated structure (tetralinoleic CL [TLCL]) through post-synthetic remodeling (8). Different tissues exhibit varying extents of CL remodeling resulting in organ-specific CL spectra (9) (Figure S1). Heart and brain lie at opposite ends of the CL remodeling spectrum: heart expresses high levels of remodeling resulting in a homogenous CL population, whereas brain exhibits little CL remodeling resulting in a highly diverse CL population. Based on the remarkable diversity of brain CLs, we hypothesized that brain-specific CL released into the circulation could be an early, specific marker of brain mitochondrial dysfunction and hence clinically significant brain injury. We further hypothesized that plasma CL would be associated with clinical patient centered outcomes such as discharge survival and neurological function. We report our clinical and experimental studies in human and rat CA which provide the first

evidence of the primacy within the plasma phospholipidome of CL as a marker of brain injury with strong associations to clinical outcome.

Methods

Complete methods in supplemental materials.

Design and Setting—This study was approved by the Institutional Review Board (IRB) at the University of Pittsburgh with blood obtained under an initial waiver of informed consent with subsequent full consent obtained. Plasma was obtained within 6h of ROSC following in- or out-of-hospital CA in 41 subjects. Exclusion criteria were traumatic CA, hemoglobin<8.0 and high likelihood of death in <48h. Two patients were excluded following Iglewicz and Hoaglin's outlier analysis (-3.5 modified Z-score 3.5) based on total phospholipid content in the plasma. Healthy subjects and patients with myocardial infarction (MI) without apparent neurological injury were enrolled as controls after obtaining informed consent.

Data Collection—We prospectively collected patient demographics and CA details. An experienced member of the Pittsburgh Post-Cardiac Arrest Service assigned brain injury severity scores within 6 h post-ROSC. Outcomes were assigned based on discharge disposition (primary), cerebral performance category (CPC) and modified Rankin scale (mRS) (10) using a standardized template and confirmed by two reviewers. Healthy controls were assigned favorable outcomes.

Human tissue—Discarded surgical human heart and postmortem human brain tissue are collected in a deidentified tissue bank at the University of Pittsburgh and were used in this study consistent with their IRB approved use.

Rat cardiac arrest (CA) model—Experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. An established rat asphyxial CA model (6) was used. Rats underwent no experimental manipulations (naïve control), mild (5 min) or severe (10 min) no-flow ischemia before resuscitation (100% ROSC). At euthanasia, plasma, cortex and hippocampus were snap-frozen for lipidomics.

Lipid extraction, mass spectrometry, identification and quantitation—Lipids were extracted from plasma, heart and brain. Total phospholipid content was quantified. Lipid extracts were dried and re-suspended. Samples were injected in duplicate into the liquid chromatography tandem-mass spectrometry (LC-MS/MS) system. Identification and quantitation of the lipid species were performed with an optimized work flow developed for lipid analysis using SIEVE™ software (ThermoFisher Scientific, San Jose, CA). Lipid identifications were manually confirmed by verifying the fragmentation pattern of representative lipids of the same class. Absolute quantity was calculated using a calibration curve generated from reference standards provided in Table S1.

Statistical analysis and data presentation—Most statistical analyses were performed using SPSS software 25 (IBM Corporation, Armonk, NY). Cerebral CL score (C-score) was derived using a backward step-wise linear regression where the 6h Full Outline of

UnResponsiveness (FOUR) score was the dependent and the brain-specific CL species present in plasma (Table S2) were the independent variables. Association of C-score with outcomes was evaluated using univariate logistic regression with the dependent being discharge disposition, CPC or mRS (10). Data are presented as mean $\pm$ standard error. The z-score $[(x-\text{mean})/SD]$ was used to develop the heat map.

Results

Differential molecular speciation of CL in heart and brain.

To characterize tissue specific CL diversity, we initiated high-resolution LC-MS/MS analysis of CL content in human heart and brain (Figure 1). Heart contained homogenous, highly remodeled homo-acylated CL species whereas brain contained heterogeneous, minimally remodeled diverse CL species. The human CL spectra (Figure 1a) and quantitation (Figure 1b) revealed that 26 out of 51 CL species were exclusively found in brain not in heart. Only 2 were present in relatively equal quantities in heart and brain (Table S2). Thus, the brain cardirolipinome is distinct from that of the heart based on number of CL species, acyl chain lengths and units of unsaturation. We confirmed in rats that the brain CL profile is distinct from all other tissues (Figure S1) and that large differences exist between rat heart and brain which mirror those results seen in humans (Figure S2).

Plasma cardirolipinome differentiates CA patients from healthy controls

Using high-resolution MS, we analyzed the plasma phospholipidome from 10 healthy controls and 39 CA patients within 6h of ROSC. The demographic characteristics of the CA patients are shown in Table1 (healthy subjects previously reported (11). We quantified 343 phospholipid species in human plasma belonging to all major classes. (Figure S3b). Identities of the phospholipid species were confirmed by fragmentation of representative high-abundance lipids within the same retention time (RT) range (Figure S3a). Orthogonal partial least squares discriminant analysis (OPLS-DA) of the phospholipidome revealed significant differences between control and CA samples [$R^2(\text{cum})=0.63$ and $Q^2(\text{cum})=0.42$] (Figure 1c). Analysis of variable importance within the OPLS-DA model showed 90% of the top 20 plasma species which differentiated CA from control were CL (Figure 1d). Thus, the early appearance of CL in plasma following CA differentiates these patients from healthy controls.

Plasma CLs are associated with increased brain injury

To determine whether plasma CL levels were correlated with brain injury after CA, we utilized three validated clinical brain injury scores: Pittsburgh Cardiac Arrest Category (PCAC) (12), FOUR score (13), and Glasgow Coma Scale (GCS) (14). Hierarchical clustering analysis with these scores revealed two distinct injury groups: severe injury (Figure 2, **left side**) and mild injury (Figure 2, **middle**). In the severe group, 28/33 plasma CL species were significantly increased compared to the mild CA group ($p<0.05$). Both groups differed substantially from healthy controls (Figure 2, **right side**). Based on this analysis we concluded that the plasma cardirolipinome may detect brain injury in a dose-dependent manner after CA.

Brain-specific CL levels correlate with the early neurological injury and predict outcome

We derived a parsimonious plasma CL cerebral score (C-Score) model using non-collinear brain-specific CLs (Figure 1) which were detected in plasma (Table S2). Out of 26 CL species found in brain not heart, only 9 could be quantified in plasma (Table S2, bold). The C-score incorporated three of these species measured in units of pmol/ml (Figure 3a, **underlined**) weighted using standardized model coefficients (model $R^2=0.299$; $p=0.001$):

$$\text{C-score} = 0.009 * \text{CL}(70:3) + 0.431 * \text{CL}(72:5) + 0.138 * \text{CL}(74:7)$$

C-score correlated well with all three early clinical measures of neurologic injury: FOUR score ($r=-0.55$; $p<0.001$), GCS ($r=-0.50$; $p<0.001$) and PCAC score ($r=0.52$; $p<0.001$; Figure 3b). Consistent with brain injury specificity, C-score was not correlated with CPR time (low flow time not incorporating CPR quality) or initial or 6h maximum troponin. C-score was significantly higher in subjects with poor neurological/functional outcome (Figure 3c) and predicted poor discharge disposition (Figure 3d; area under receiver operator curve [AUROC]=0.80; $p=0.001$), CPC (AUROC=0.75; $p=0.003$) and mRS (AUROC=0.79; $p=0.001$). C-score was associated with favorable discharge disposition (OR=1.73; 95% CI: 1.20–2.50), CPC (OR=1.49; 95% CI: 1.08–1.97) and mRS (OR=1.64; 95% CI: 1.15–2.32).

CL(70:5) can be used as a simplified brain injury marker following CA

Hoping to identify a single CL species amenable to future point of care testing, we further analyzed CL(70:5) which had the strongest correlation with FOUR score ($r=-0.41$; $p=0.004$). CL(70:5) also correlated with GCS ($r=-0.39$; $p=0.006$) and PCAC score ($r=0.43$; $p=0.002$; Figure 3b) and distinguished good vs. poor neurologic outcome (Figure 3c). CL(70:5) predicted discharge disposition AUROC=0.84 ($p<0.001$; Figure 3e). Using 0.93 pmol/ml as the cutoff, plasma CL(70:5) was 83% sensitive and 90% specific for predicting poor neurologic outcome and was associated with good outcome (OR=2.00; 95% CI 1.11–3.62). When compared to the validated PCAC score which strongly predicts discharge outcome(12), C-score and CL(70:5) performed similarly and provided a small though not significant increase in AUROC (Table S3). C-score and CL(70:5) were not elevated in critically ill myocardial infarction patients without neurologic injury (Figure S4).

CL appearance in plasma correlates with disappearance from brain tissue

We hypothesized that the appearance of brain-type CLs in plasma resulted from loss from injured brain (mitochondria) after CA. Since we were unable to simultaneously assess plasma and brain CL in humans after CA, we tested this hypothesis in a rat CA model where injury severity (no-flow time) could be controlled and CL simultaneously assessed in brain and plasma. The rat brain and heart cardiolipinome differ slightly from that seen in humans (Figure S2, Table S4) yet we observed the same pattern of high brain CL diversity whereas heart consisted predominantly of the homo-acylated species TLCL. Plasma C-Score increased stepwise based on the severity of experimentally induced brain injury (naïve: 0.57 ± 0.07 ; 5min CA: 2.08 ± 1.00 ; 10min CA: 6.35 ± 1.44 pmol/ml; $p<0.001$; Figure 4a). Each C-score CL component and CL(70:5) were significantly increased after 1min no-flow CA vs. naïve (Figure 4b) with no overlap between groups (AUROC=1.0). Since asphyxial CA produces significant hippocampal damage (15), we examined whether appearance of

increased brain type CL in plasma corresponded with hippocampal loss of these same species. All 8 brain-specific CL species present in rat plasma (Table S4) significantly decreased within hippocampus after 10min CA vs naïve (all $p < 0.05$; Figure 4b).

Discussion

We provide the first demonstration that CL is distinct among the phospholipidome in its diversity within brain compared to other organs. Shortly after reperfusion (ROSC) from global ischemia (CA), some of these distinct CLs are expressed in plasma and quantify brain injury in close correspondence with early clinical exam (PCAC, FOUR, GCS). Furthermore, loss of brain CL into the plasma permits early prediction of discharge neurologic and functional outcome. Thus, the unique diversity of brain CL can be exploited to develop a specific plasma marker of early brain injury which corresponds to early neurologic injury and subsequent outcome.

Brain injury from cardiac arrest affects over 500,000 people annually in the US alone (16). Only 10–12% survive out-of-hospital CA(16) with most deaths resulting from brain injury (17). The lifelong cost for continued care of disabled post-CA patients approached \$33 billion annually (18). In this setting development of early markers of brain injury is desperately needed to permit early risk stratification, a first step to targeted therapy and resource allocation, and to provide early prognostic and theragnostic quantification. CA results in global ischemia-reperfusion injury making specific detection of brain injury challenging. Delayed neuronal death has been strongly linked to mitochondrial dysfunction after ROSC providing one strategy for specific early neurological injury detection.

Early and accurate neurological prognostication after CA is challenging as summarized in the recent European guidelines (19). Early prognosis is best established with a combination of neurological exam, imaging and EEG(20). However, these modalities are not always readily available, and the exam is often limited by sedation, neuromuscular blockade and lack of objectivity. In this setting a quantitative marker of brain injury is highly desirable. Neuron-specific enolase (NSE) (21), S100B (22), and glial fibrillary acidic protein (GFAP) (23) are the most commonly used biomarkers of neurological injury post-CA, but their predictive power within 24h of ROSC is insufficient for prognostication (19). On the other hand, mitochondrial dysfunction in brain following ischemic injury occurs as early as 30min post-insult (6, 24). We therefore hypothesized that CL, a unique and critical component of brain mitochondria, would be released into systemic circulation early after CA preceding elevations in the traditionally utilized biomarkers which correspond to neuronal death. Our findings provide the first evidence that this hypothesis may be substantiated and deserves further study.

The identification of brain-type CL in systemic circulation as an indicator of neurological injury offers two clear advantages. First, plasma CL may not function solely as an injury marker but may indicate an underlying pathologic process that could serve as a target for therapeutic intervention. CL is localized to the inner mitochondrial membrane (25), and its externalization stimulates the repair or elimination of injured mitochondria at the intracellular or intercellular levels (26). Because of the tight regulation of CL, it is likely that

the release of CL into circulation occurs through a specific pathological event(s). Second, CL exhibits extraordinary tissue-specific differences which are relatively unaltered by exogenous lipid sources such as nutrition (27). Our results demonstrate that the C-score and more simply CL(70:5), can aid in the prediction of brain injury even within the setting of complex multi-organ injury which occurs after CA. The predictive capacity of CL(70:5) as a potent focused biomarker makes it amenable to point-of-care testing which could greatly increase the utility of our findings.

The appearance of plasma CL may serve important intracellular signaling functions with the ability to modulate organ injury and clinical outcome. Translocation of CL from the inner to outer mitochondrial membrane and its subsequent oxidation through cytochrome C/CL complex results in the release of pro-apoptotic signals into the cytoplasm (28). Membrane externalization of CL alone can act as an “eat-me” signal via activation of the autophagy protein, microtubule-associated-protein-1-light chain-3 (29). Both pathways are implicated in the pathogenesis of post-CA syndrome though importantly we have not identified any oxidized CL products in the plasma. Activation of non-apoptotic regulated cell death pathways may result in the release of mitochondria-derived particles (31) acting as damage-associated molecular patterns (DAMPs) (32). Plasma CL may play important roles in inflammation and immunity (33) and coagulation (34) which have been associated with outcome after CA(35).

This study is the first demonstration of brain-specific release of CL into plasma after CA and naturally has limitations. First, the limited sample size of this study precluded the normalization for other demographic parameters and utilization of multivariable analysis. Despite the small size of our cohort, we were able to observe significant differences in brain-type CL species, correlation of these species to injury severity, and ability of these species to predict neurological outcome. Second, since non- erythrocyte blood cells possess CL, they may contaminate plasma CL measurements. Our identification of brain-specific CL species however permits us to distinguish brain injury. Third, we do not have long-term follow-up on these patients beyond hospital discharge. Despite this limitation, brain CL correlated with discharge location which is highly predictive of long-term outcome (36). While our data are promising additional work is required for clinical translation. To validate that CL has significant additive value in early neuroprognostication after CA will require a larger observational study with comparison made to clinical prediction tools such as the OHCA(37) or PCAC(12) scores, other early prognostic modalities such as CT(38) and commonly used biomarkers, which are not presently recommended for use in this setting(19). Although the clinical lab application of LC-MS is rapidly increasing(39), detection of CL species still represent technical challenges. Our goal in characterizing a single CL species (70:5) with strong association to outcomes was to prepare a more rapid, perhaps even point of care, test which would permit broad clinical application.

Conclusions

Brain-type CL is released into the systemic circulation early after resuscitation from both human and rat CA commensurate with the degree of brain injury. The C-Score derived from brain-specific CL correlates with neurological injury severity and predicts discharge

outcome. CL(70:5) performs similar to C-score and may have potential utility as an early point-of-care test for brain injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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variable importance for the prediction of the OPLS-DA model (data described in Fig. 1c) against its rank, indicating that CL species (red) are the major PL class which distinguish CA from healthy controls (all other PL represented in blue).

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Figure 2. Changes in cardiolipinome associated with injury severity following CA.

Various clinical brain injury scores (PCAC, FOUR score, and GCS) were used to cluster the patients by severity of brain injury. The hierarchical centroid clustering resulted in the separation of CA patients into two groups: severe injury (left) and mild injury (middle). Healthy controls are shown on the right. Heat map (z-scores) of plasma CL in these patients showed a progressive increase in plasma CL between uninjured, mild, and severe injury groups. * $p < 0.05$, Kruskal-Wallis H test.

Figure 3. C-score and CL(70:5) correlate with brain injury and predict outcome.

a) Bar graph showing levels of each of the brain-specific CL present in plasma trend to be higher in patients who have poor neurologic outcome. Only CL(70:5) is significantly different. The components of the C-score are underlined. The mean C-score and CL(70:5) score increase with greater neurologic injury based on b) PCAC score and c) discharge disposition (outcome). Both d) C-score and e) CL(70:5) are associated with outcome with area under the curve ~0.80. Data are Mean $\pm$ SEM, * p <0.01 vs. good outcome.

Adult rats underwent asphyxial cardiac arrest (ACA) of varying no-flow time (5 min and 10 min). a) Bar graph showing an increased C-Score with in-creasing no-flow time. b) Quantitation of CL species brain-enriched CL species showed an increase in plasma CL (top) and decrease in hippocampal CL (bot-tom) after CA, indicating that the CL species identified in plasma are released from the brain after CA (Data are Mean + SEM, * $p < 0.05$ vs naive, # $p < 0.05$ vs ACA 10 min, one-way ANOVA, Fisher LSD post-hoc analysis, N=4-7). All of the CL species shown here had an AUC of 1.00 for determination of injury severity with ROC analysis.

Table 1.

Characteristics of cardiac arrest patients.

Characteristic	Data available (n)	n (%), mean $\pm$ SD or median [IQR]
Out-of-hospital cardiac arrest	39	20 (53%)
Witnessed	39	28 (72%)
Bystander CPR	38	34 (89%)
Initial rhythm	39	
VT/VF		13 (33%)
PEA		15 (38%)
Asystole		10 (26%)
Unknown		1 (3%)
Cause of Arrest	39	
Myocardial infarction		7 (18%)
Primary cardiac arrhythmia		7 (18%)
Medical illness leading to respiratory arrest		16 (41%)
Cardiomyopathy/cardiogenic shock		2 (5%)
Other shock		2 (5%)
No flow time (min)	28	0 [0, 1.5]
Low flow time (min)	37	10 [5, 19]
Baseline Glasgow Coma Score	38	6.7 $\pm$ 4.3
Baseline FOUR score	38	7.1 $\pm$ 5.4
Baseline Pittsburgh Cardiac Arrest Category (PCAC)	38	
PCAC 1		9 (23%)
PCAC 2		6 (15%)
PCAC 3		7 (18%)
PCAC 4		16 (41%)
Discharge destination	39	
Home or inpatient rehabilitation		10 (26%)
Other (LTAC, SNF, hospice, morgue)		29 (74%)
Discharge modified Rankin scale (mRS)	39	
mRS 0 – 3		10 (26%)
mRS 4 – 6		29 (74%)
Discharge Cerebral Performance Category (CPC)	39	
CPC 1 and 2		13 (33%)
CPC 3 – 5		26 (67%)

Abbreviations: SD = standard deviation; IQR, interquartile range; VT/VF, ventricular fibrillation and tachycardia; PEA, pulseless electrical activity; FOUR, Full Outline or UnResponsiveness; LTAC, long term acute care; SNF, skilled nursing facility.