Supplementary to

Phospholipid screening post-cardiac arrest detects decreased plasma lysophosphatidylcholine: Supplementation as a new therapeutic approach

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Supplementary Method

Materials and reagents

Reagent-grade chemicals and HPLC-grade solvents were purchased from major commercial suppliers (Fisher Scientific and Sigma Aldrich). 1-Oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(18:1)), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(18:0)), 1-(10Z-heptadecenoyl)-2-hydroxy-sn-glycero-3-phosphocholine (LPC(17:1)), 1-(10Z-heptadecenoyl)-sn-glycero-3-phosphoethanolamine (LPE(17:1)), and 1-(10Zheptadecenoyl)-2-hydroxy-sn-glycero-3-phospho-(1'-myo-inositol) (LPI(17:1)) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Dipalmitoyl-sn-glycero-3-phospho-N-methylethanolamine (PME) was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). 1-Docosahexaenoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(22:6)) was custom-synthesized and purchased from Avanti Polar Lipids.

Asphyxia-induced rat cardiac arrest model

This study was conducted in accordance with policies of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institutes for Medical Research (IACUC protocol number 2017-033). Adult male Sprague-Dawley rats (400–500 g, ~ 3 months of age, Charles River Laboratories, MA, USA) were used for this study. The procedures for asphyxia-induced CA and CPR were conducted as published previously (1). Rats were anesthetized with 4% isoflurane (Isosthesia, Butler-Schein AHS) and intubated with a 14-gauge plastic catheter (Surflo, Terumo Medical Corporation, NJ, USA). Animals were mechanically ventilated with a fraction of inspired O_2 (F_IO_2) of 0.3 and anesthesia was maintained with 2% isoflurane. The left

femoral artery and vein were cannulated for monitoring arterial pressure and infusing medications, respectively. After the administration of heparin (300 U) and vecuronium bromide (2 mg/kg), asphyxia was induced by switching off the ventilator. CA, defined as a mean arterial pressure below 20 mmHg, was achieved within 3 min after the induction of asphyxia. After 10 or 14 min of asphyxia, mechanical ventilation was restarted at an F_IO_2 of 1.0 and chest compressions were performed at a rate of approximately 300 per min. At 30 seconds after beginning of chest compressions, a 20 μg/kg bolus of epinephrine was administered, and chest compressions continued. Typically, rats achieve ROSC within 1 min after the initiation of chest compressions. Body temperature was controlled between 36 to 37°C and rats were then extubated 2 h after achieving ROSC. Although heparin has shown to activate phospholipase A2 in plasma (2), we did not find a significant change in the content of total and individual LPC species, showing a minimal interference of heparin for our LPC analysis.

Administration of LPC

LPC injection solutions were prepared by solubilizing LPC in 0.5% (w/v) bovine serum albumin (BSA) in PBS buffer by sonication (10 s, 5 cycles) on ice. LPC(18:0) solution was prepared and stored at room temperature because it precipitated at low temperature. LPC(18:0) was injected with 10 min of preparation. The concentration of LPC was adjusted to give desired doses in 0.5 ml solution. The LPC-BSA (0.5 ml) solution was injected via the femoral vein cannula 1 min after ROSC over 1 min. Animals in the vehicle group were injected with 0.5 ml of 0.5% BSA in PBS following the same method. For the survival analysis with LPC(18:1), animals were randomized into 2 groups: (1) vehicle iv group (n=12): (2) LPC(18:1) iv group (n=12). For the survival analyses with LPC(18:0) and LPC(22:6), animals were randomized into 3 groups: (1)

vehicle iv group (n=12): (2) LPC(18:0) iv group (n=12): (3) LPC(22:6) iv group (n=12).

HPLC-MS analysis

Phospholipids were extracted from the plasma samples according to the previously published method (3, 4). Briefly, 50 μL of frozen plasma was extracted with 750 μL of methanol in the presence of 0.04 nmol of PME, 0.85 nmol of LPC(17:1), 0.15 nmol of LPE(17:1), 0.1 nmol of LPI(17:1) as internal standards. The mixture was vortexed for 2 min, incubated for 10 min at 4 °C, and centrifuged for 10 min at 22,000×g. The supernatant was evaporated to dryness under N_2. The residue was reconstituted in a 200 μL of a solution containing isopropanol (IPA): t-butyl methyl ether (TBME): aqueous ammonium formate (94 mM) (34:17:5, v:v:v). Finally, 20 μL of this mixture was injected into the HPLC-MS for analysis.

The phospholipids were analyzed using normal-phase HPLC-MS (5). Eluent A was created using IPA: TBME: aqueous ammonium formate (94 mM, pH ~2.5) (34:17:5, v:v:v) with eluent B containing 100% MeOH. The gradients used for the 40 min chromatogram were as follows: 100% A for 18 min, 100% A to 20% A over 6 min, 20% A for 11 min, 20% A to 100% A over 1 min, and hold 100% A for 4 min. The flow rate was 0.3 mL/min, and the column temperature was 30°C. MS and MS/MS data were obtained with an LTQ XL spectrometer (Thermo Scientific, San Jose, CA) operated in the negative ion mode (6). Data was processed using Thermo Xcalibur software (version 2.2) (6, 7). Retention time, MS, and MS/MS data of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylinositol (PI), lysophosphatidylinositol (LPI), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), and SM, were compared to control species to identify the individual species. The concentrations of the PC, PE, and PI were calculated using standard curve (6). Sphingomyelin was calculated using the PC standard curve as

they share the same head group. The concentrations of LPC, LPE, and LPI species were calculated by comparing their peak areas to the areas of corresponding internal standards (3).

Brain histological analyses

For the histological experiments, we used rats that survived to 72 h from the survival studies. For Nissl staining, brain sections were stained by using 0.1% cresyl violet acetate (Fisher Scientific) followed by differentiation and dehydration with ethanol baths (50%, 75%, 95%, and 100%). The quantification of neurons was performed using a modified method from a previously published study (8). The number of neurons from a 200 □m section of the hippocampal CA1 stratum pyramidale was quantified on coronal sections of the dorsal part of CA1 region located between 3.5 mm and 4.5 mm posterior to bregma. This was assessed using BZ-X800 Fluorescence Microscope (Keyence, USA), and the number of neurons was counted by the application of Image J. For Fluoro-Jade B (FJB) staining, the brain sections were immersed in 100% ethyl alcohol, followed by 70% alcohol and distilled water (9). The slides were transferred to a solution of 0.06% potassium permanganate, rinsed in distilled water and exposed to a 0.0004% working solution of FJB (Millipore Sigma) (including 0.1% acetic acid) for 30 min. The number of FJB positive cells was counted among the medial part of cerebral cortex (0.16 mm^2). A mean value was obtained from bilateral measurements on two sections randomly selected from each brain sample. The counting was performed by the application of Image J.

Multivariable regression analyses of human plasma samples for phospholipid screening

In order to identify which phospholipid classes are associated with CA, we performed the least absolute shrinkage and selection operator (LASSO) logistic regression analysis followed by multivariable logistic regression analysis using 25 controls (absent) and 36 cases (present).

Coefficients of each phospholipid class were plotted against the log of the shrinkage parameter, λ. Using fivefold cross validation, the model with the λ that optimizes CA prediction within 1 SE of the deviance was chosen (10, 11). The selected variables from the LASSO regression analysis were tested for their association with CA using multivariable logistic regression analysis. The logistic regression analyses for phospholipids screening were performed using the "glmnet" package (http://www.jstatsoft.org/v33/i01/) in R (The R Foundation for Statistical Computing, Vienna, Austria).

Reference

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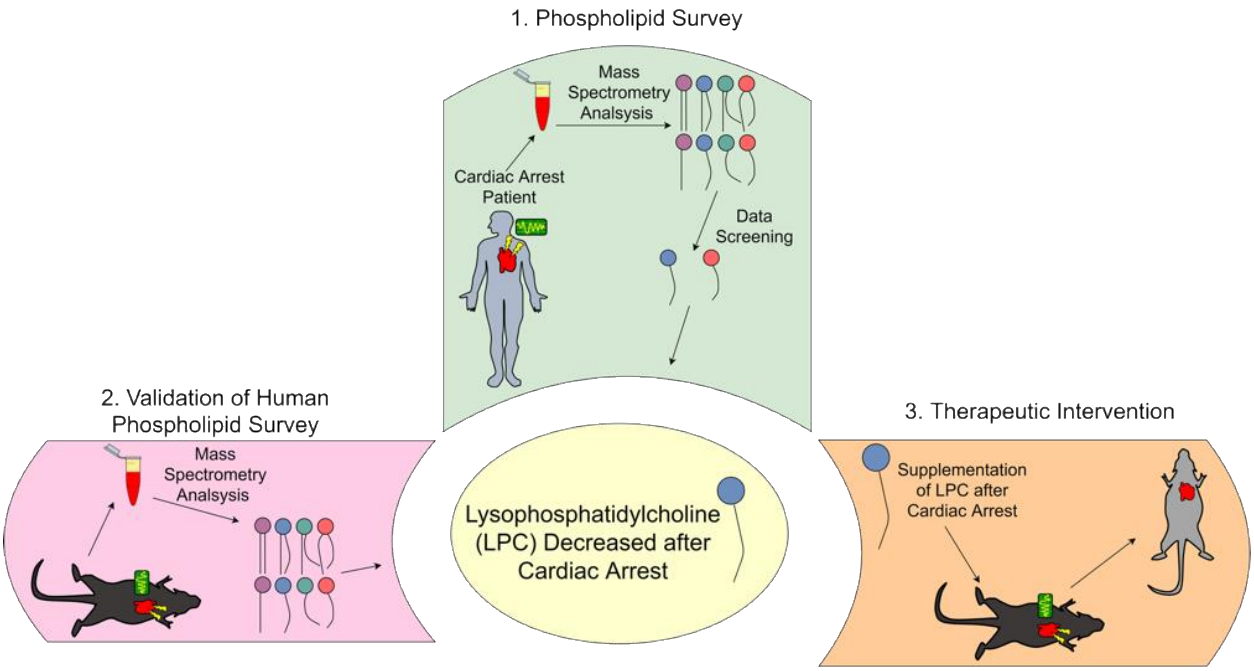
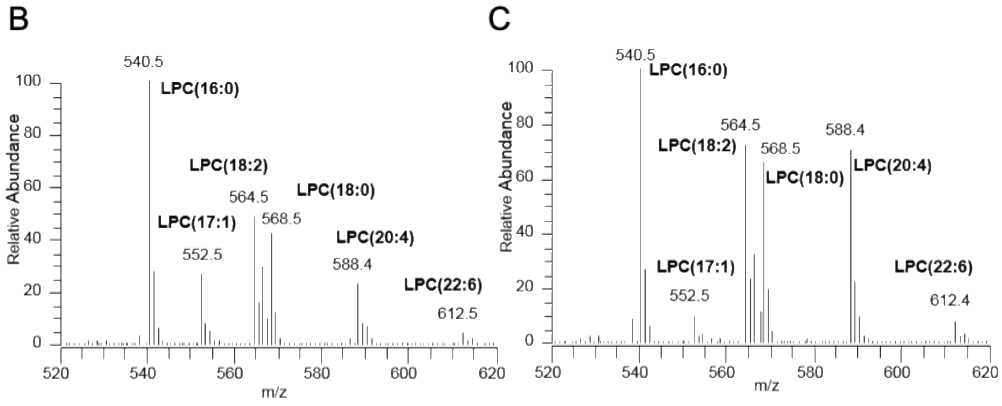
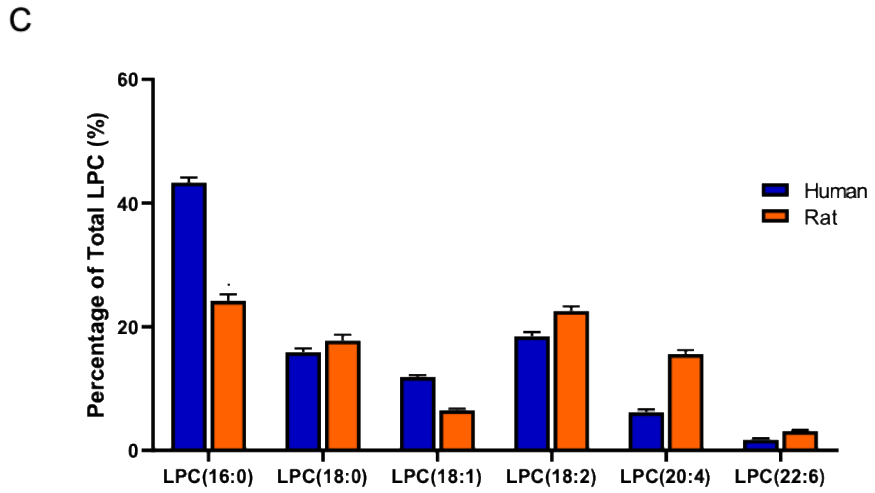


Supplementary Figure 1. Experimental flow of this study by using both samples of humans and rats. Our study was performed by both samples of humans and rats. The first part is screening phase by using human plasma samples to find out the specific phospholipid whose decrease contribute to CA pathology. The second and third part are rat study, which is the confirmation of the result of human data in our rat model of cardiac arrest and survival analysis to test the beneficial effect of supplementation of the specific phospholipid. PC, phosphatidylcholine; PE, phosphatidylethanolamine, PI, phosphatidylinositol; SM, sphingomyelin; LPI, lysophosphatidylinositol;; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine.

A

LPC species	Attached fatty acid	
LPC(16:0)	Palmitic acid	Saturated fatty acid
LPC(18:0)	Stearic acid	
LPC(18:1)	Oleic acid	Monounsaturated fatty acid
LPC(18:2)	Linoleic acid	Polyunsaturated fatty acid
LPC(20:4)	Arachidonic acid	
LPC(22:6)	Docosahexaenoic acid	





Supplementary Figure 2. Representative MS spectra of plasma lysophosphatidylcholine (LPC). (**A**) The list of six major LPC species containing different fatty acids. Representative MS spectra of plasma LPC in a (**B**) control human and (**C**) control rat and (**D**) relative content of individual LPC species in humans and rats. Data are displayed as mean ± SEM.

Supplemental Table 1. Baseline characteristics for survival analysis

LPC(18:1) vs vehicle

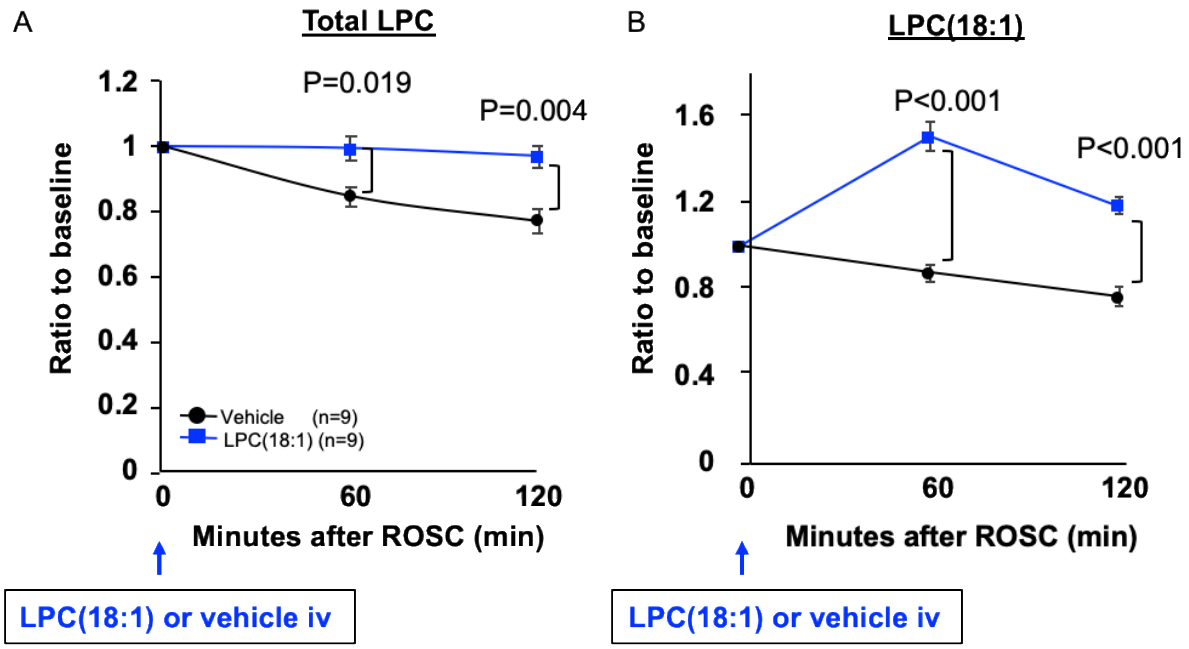
Variable	Vehicle (n = 12)	LPC(18:1) (n = 12)	P value
Body weight, g	465 ± 8.0	456 ± 7.0	0.40
Surgical procedure time, min	35.5 ± 1.2	34.3 ± 1.1	0.79
Before CA			
MAP, mmHg	78.4 ± 2.4	80.5 ± 4.4	0.66
HR, bpm	303 ± 10.2	277 ± 9.0	0.08
RR, times/min	44.8 ± 0.7	45.5 ± 0.3	0.56
BT, ºC	36.4 ± 0.1	36.4 ± 0.1	0.66
Until CA time, sec	147 ± 7.3	157 ± 8.0	0.37
CPR time, sec	54 ± 1.0	54 ± 0.7	0.40

Data are presented as mean ± standard error.

LPC(18:0) vs LPC(22:6) vs vehicle

Variable	Vehicle (n = 12)	LPC(18:0) (n = 12)	LPC(22:6) (n = 12)	P value
Body weight, g	461 ± 4.9	449 ± 8.5	450 ± 6.0	0.19
Surgical procedure time, min	31.7 ± 1.1	33.6 ± 1.2	31.8 ± 0.8	0.46
Before CA				
MAP, mmHg	85.9 ± 2.4	88.4 ± 3.7	88.3 ± 4.9	0.68
HR, bpm	292 ± 10.1	307 ± 8.2	289 ± 12.7	0.48
RR, times/min	45.3 ± 0.5	46.0 ± 0.2	45.8 ± 0.2	0.54
BT, ºC	36.5 ± 0.1	36.3 ± 0.1	36.3 ± 0.1	0.32
Until CA time, sec	174 ± 7.6	160 ± 6.8	170 ± 7.4	0.37
CPR time, sec	54 ± 0.7	56 ± 3.7	56 ± 1.3	0.55

Data are presented as mean ± standard error.



Supplementary Figure 3. Plasma lysophosphatidylcholine (LPC) levels after cardiac arrest (CA) following 6.0 mg/Kg LPC(18:1) injection. (**A**) Total LPC levels and (**B**) LPC(18:1) were measured at 1 h and 2 h after the administration of LPC(18:1) (n=9) or vehicle (n=9) after 10 min CA. This data indicates that injected LPC(18:1) attenuated the decrease of other endogenous LPC species post-resuscitation, preserving total LPC for 2 h. However, 0.6 or 1.2 mg/Kg was not enough to maintain LPC levels (data not shown). Data are displayed as mean ± standard error of mean. The difference between the two groups was compared using the Mann-Whitney U test.

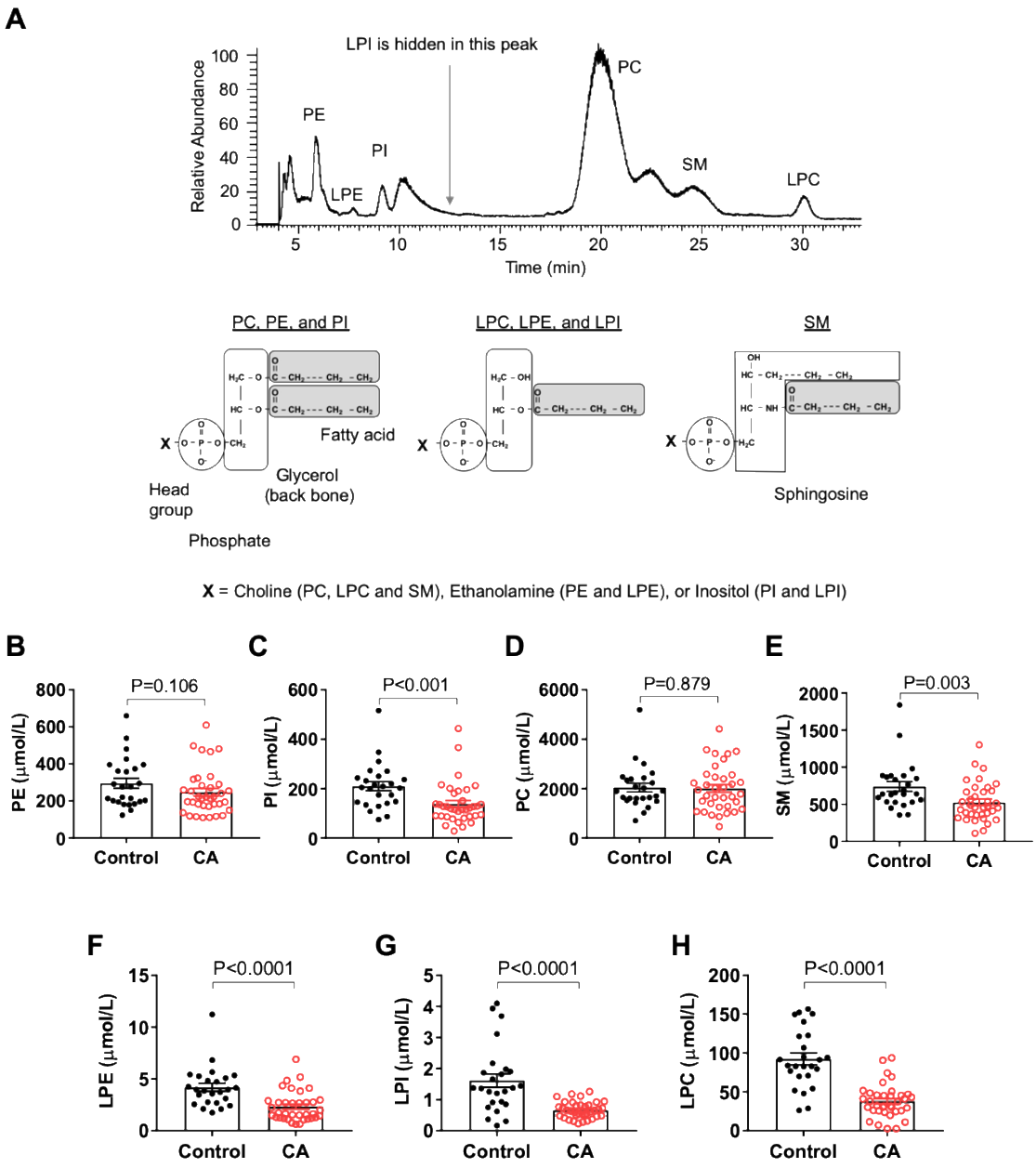
Supplemental Table 2. Modified neurological deficit score (mNDS)

mNDS			
1. General			
Consciousness	Unresponsive (0), depressed (50), normal (100)		
Respiration	Abnormal (<60 or >120) (0), normal (100)		
		Total points	/200
2. Cranial nerves			
Olfactory	Orient to smell: no (0), yes (20)		
Vision	Visual stimulus startle response: no (0), yes (20)		
Corneal reflex	Blink response to corneal stimulus: no (0), yes (20)		
Whisker movement	Spontaneous: no (0), yes (20)		
Hearing	Startle response to loud noise: no (0), yes (20)		
		Total points	/100
3. Motor			
Left forepaw	Spontaneous or withdraw from pain: no (0), yes (10)		
Right forepaw	Spontaneous or withdraw from pain: no (0), yes (10)		
Left hindpaw	Spontaneous or withdraw from pain: no (0), yes (10)		
Right hindpaw	Spontaneous or withdraw from pain: no (0), yes (10)		
Tail	Spontaneous or withdraw from pain: no (0), yes (10)		
		Total points	/50
4. Sensory			
Left forepaw	React to pain: no (0), yes (10)		
Right forepaw	React to pain: no (0), yes (10)		
Left hindpaw	React to pain: no (0), yes (10)		
Right hindpaw	React to pain: no (0), yes (10)		
Tail	React to pain: no (0), yes (10)		
		Total points	/50
5. Coordination			
Ledge traverse	no (0), yes (25)		
Righting reflex	no (0), yes (25)		
Placing test	no (0), yes (25)		
Stop at table edge	no (0), yes (25)		
		Total points	/100
		Total score	**/500**
		Percent NDS	**(0-100%)**

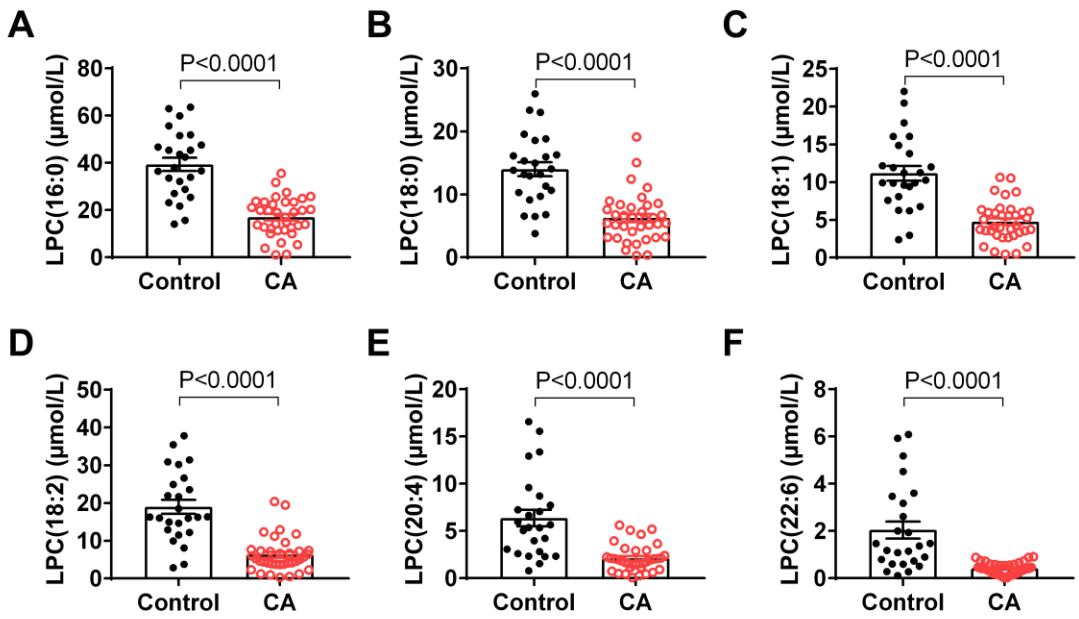
Supplemental Table 3. Baseline characteristics of 36 cardiac arrest patients

Variable	Survived (n=9)	Died (n=27)
Age, years, median (IQR)	68 (60-82)	76 (69-86)
Gender, male, *n* (%)	5 (56)	11 (41)
Comorbidity		
Hypertension, *n* (%)	6 (67)	12 (44)
Diabetes mellitus, *n* (%)	4 (44)	10 (37)
Ischemic heart disease, *n* (%)	2 (22)	12 (44)
Any other heart disease, *n* (%)	3 (33)	14 (52)
Any lung disease, *n* (%)	1 (11)	5 (19)

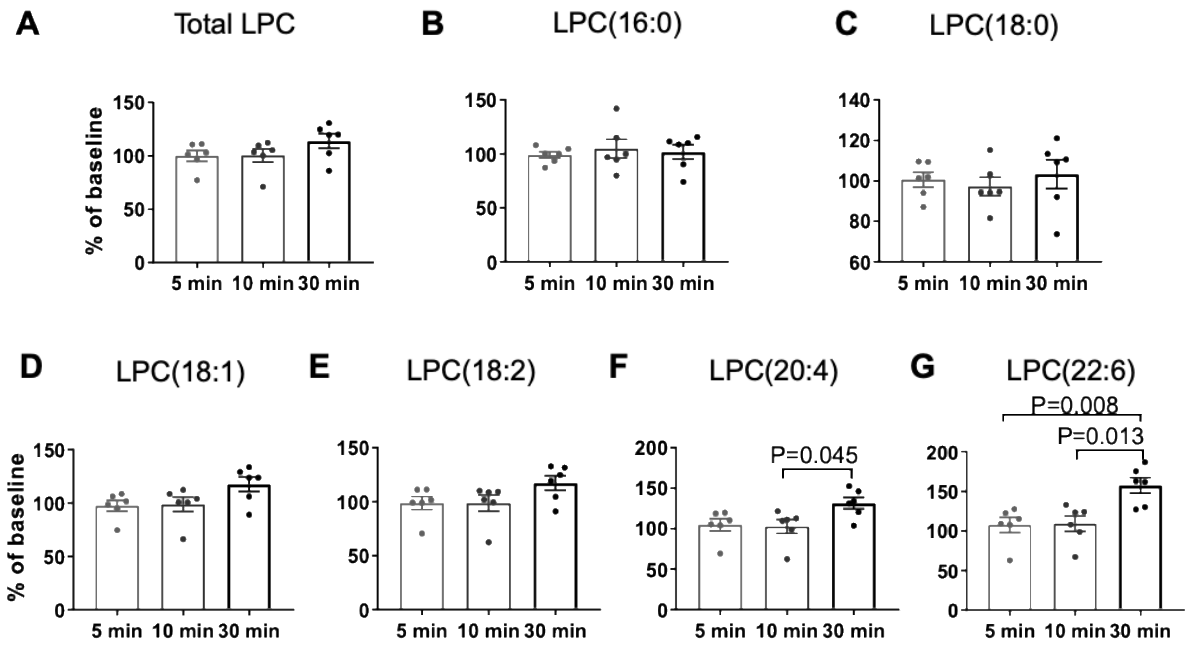
IQR = interquartile range



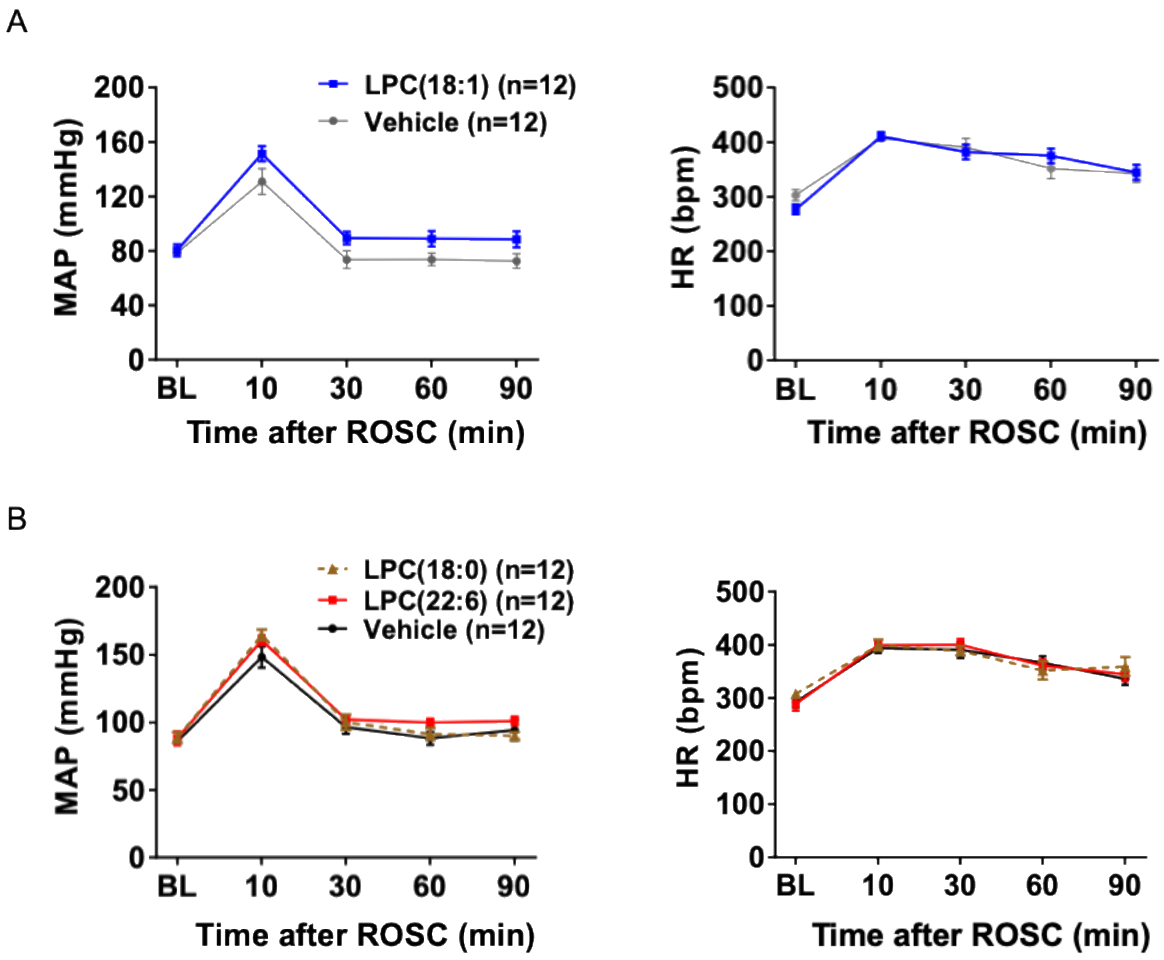
Supplementary Figure 4. Plasma phospholipid levels in control and cardiac arrest (CA) patients. (**A**) Representative ion chromatogram shows the retention times of 7 major phospholipid classes and structure of phospholipids, lysophospholipids, and sphingomyelin. (**B-H**) Plasma levels of 7 major phospholipids in control and CA patients. Data are presented as mean ± standard error of the mean. Statistical analyses for each metabolite were performed using the Mann-Whitney U test (n=25 for control and n=36 for CA patients).



Supplementary Figure 5. Comparison of the levels of individual plasma lysophosphatidylcholine (LPC) species between cardiac arrest (CA) patients and Control. (**A-F**) The levels of individual LPC species are significantly higher in Controls (n=25) than CA patients (n=36). Data are presented as mean ± standard error of the mean. The Mann-Whitney U test was used for the comparison.



Supplementary Figure 6. The comparison of plasma lysophosphatidylcholine (LPC) levels after 5, 10, and 30 min of untreated CA in rats. Box and whisker plots show the distribution of average percent changes of LPC levels post-CA compared to baseline (n=6 in each). The levels of total LPC and LPC species containing saturated fatty acids and MUFA were not significantly changed until 30 min of CA (A-D). However, the level of LPC(22:6) after 30 min CA was significantly increased by 58% compared to baseline (G). These results indicate that the decrease in LPC primarily begins after resuscitation, but not during the ischemic phase of CA.Data are displayed as mean ± standard error of mean. The one-way ANOVA with post-hoc Tukey HSD test was used.



Supplementary Figure 7. Vital parameters after cardiac arrest and cardiopulmonary resuscitation (CA/CPR) between the rats injected with lysophosphatidylcholine (LPC). (A) Changes in mean arterial pressure (MAP) and heart rate (HR) with LPC(18:1) injection. **(B)** Changes in MAP and HR with LPC(22:6) and LPC(18:0) injection. MAP and HR after CA between LPC and vehicle injected groups (n=12 each) measured at baseline and 10 min, 30 min, 60 min, and 90 min after resuscitation. The repeated measures analysis of variance (ANOVA) was used to compare more than three groups, and paired t-test was used to compare between two groups, as appropriate. *$p < 0.05$.