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Effects of methylprednisolone and aprotinin on phospholipase D activity of leukocytes in systemic inflammatory response induced by cardiopulmonary bypass[1]

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KEY WORDS cardiopulmonary bypass; inflammation; leukocytes; phospholipase D; methylprednisolone; aprotinin

ABSTRACT

AIM: To investigate the role of leukocyte phoshpolipase D (PLD) in systemic inflammatory response induced by cardiopulmonary bypass (CPB) and the effects of methylprednisolone and aprotinin on leukocyte PLD activity. **METHODS**: Forty-two patients who received CPB open heart surgery were divided into 3 groups: methylprednisolone group, aprotinin group, and control group. Arterial blood (10 mL) was collected for assay of leukocyte PLD activity, myeloperoxidase (MPO) activity, and CD11b expression at 8 different time points in perioperative period. Plasma IL-6, IL-8, and C-reactive protein levels were also determined. **RESULTS**: At the time point of ascending aorta declamped, leukocyte PLD activity for control group was (18 ± 8) nmol choline$\cdot h^{-1} \cdot mg^{-1}$, which was higher than that of pre-CPB ($P < 0.01$); the PLD activity for methylprednisolone group was (10 ± 6) nmol choline$\cdot h^{-1} \cdot mg^{-1}$ that was lower than control ($P < 0.05$), while it had no statistical difference compared with that of pre-CPB. In methylprednisolone group, PLD activity elevation was postponed to the time point of CPB stopped. There was no statistical difference in PLD activity between aprotinin group and control ($P > 0.05$). After administration of methylprednisolone or aprotinin, leukocyte CD11b expression, plasma IL-6, IL-8, C-reactive protein levels, and MPO activity decreased by different extent. **CONCLUSION**: Leukocyte PLD activity was elevated significantly in systemic inflammatory response induced by CPB and methylprednisolone partially blunted the CPB-induced inflammatory response by inhibiting PLD activity.

INTRODUCTION

Leukocyte activation plays an important role in the initiation and development of cardiopulmonary bypass (CPB)-induced systemic inflammatory response syndrome (SIRS). CPB rendered patients at a risk of early neutrophil-mediated tissue injury and delayed inflammatory complications, even multiple organ failure (MOF), via both early neutrophil priming (hyperresponsiveness) and late neutrophil malfunction[1]. It was demonstrated that phospholipase D (PLD) activity of primed or activated PMN was higher than that of quiescent PMN[2]. PLD catalyzes the hydrolysis of phosphatidylcholine (PC), and the direct product of PLD is phosphatidic acid (PA) and the indirect products of PLD are diacylglycerol (DAG) and lysophosphatidic acid (LPA). The PLD-catalyzed PC hydrolysis is an important mechanism of signal transduction in cells[3]. PLD-derived PA amplified the effect of CD11b by enhancing the affinity of the CD11b/CD18 integrin for its ligands[4]. Our former works demonstrated that PLD played a role in inflammation[5], specially at the steps of respiratory burst of neutrophil, inflammatory cell exocytosis, and the arachidonic acid release[6,7]. However, changes of leukocytes PLD activity in CPB-induced systemic inflammatory response have yet to be established and whether PLD plays a role in CPB-induced inflammation is still a puzzle.

The present study was carried out to explore the role of leukocyte PLD in systemic inflammatory response

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induced by CPB and evaluate whether blunting CPB-induced inflammatory response by methylprednisolone and aprotinin is related to the inhibition of PLD.

MATERIALS AND METHODS

Patients Body temperature, blood routine, C-reactive protein, and antistrepto-O were examined during preoperative period in patients who received CPB open heart surgery in June 2000 to January 2001. Among them, 42 patients (20 male and 22 female) were chose in this research and the others were ruled out because of the abnormal parameters. The age of patients were (28 ± 25) a varied from 15 to 66. The diagnosis were as following: 6 cases of atrial septal defect, 10 cases of ventricular septal defect, 3 cases of tetralogy of Fallot, 15 cases of mitral valvular disease, 8 cases of aortic valvular disease. Eighteen of the 23 cases of valvular disease were rheumatic, but nobody was having rheumatic fever before operation.

All the patients received routine anesthesia and CPB (moderate hypothermal 26 – 28 ℃, cold crystalloid cardioplegia, Bentley membrane oxygenator, and Cobe artificial heart-lung system). The aortic clamped duration was (56 ± 40) min and the total CPB duration was (82 ± 50) min. After the operation, all the patients conformed to the diagnosis standard of SIRS[8] and all the patients recovered from the surgery. The patients were divided into 3 groups, 26 cases in control group, 8 cases received intravenous administration of methylprednisolone 10 mg/kg in induction phase of anesthesia (methylprednisolone group) and 8 cases received aprotinin 10^5 kIU/kg (aprotinin group, half dose was intravenously administered in induction phase of anesthesia and half dose added into extracorporeal circulation machine).

There was no statistical difference in age, proportion of disease (congenital : acquired), CPB duration, and aortic clamped duration between the 3 groups ($P > 0.05$).

Drugs Phosphatidylcholine (C10:0), oleic acid (sodium salt), choline oxidase, choline hydrochloride, 4-AAT (Sigma), HEPES (E Merck), peroxidase (Shanghai Lizhu Dongfeng Biotechnology Co, Ltd) were dissolved in double-distilled water. Dextran T500 (Amersham Pharmacia Biotech) was dissolved in saline.

Leukocytes isolation[5,9] Arterial blood (10 mL) was obtained from patients and placed immediately into heparin-containing tubes at the following time points: pre-CPB, 30 min after initiation of CPB, ascending aorta declamped, CPB stopped, 1 h, 2 h, 24 h, and 72 h after the CPB stopped. Dextran 6 % was added to the whole blood (1 : 5, v/v), and these samples were inverted several times and sedimented at 25 ℃ for 1 h. The leukocyte-containing layer was harvested with syringe and centrifugation (450 × g, for 10 min at 4 ℃). Erythrocytes in the pellet were hypotonically lysed in 1 volume of distilled water for 40 s, followed addition of 1 volume of 1.8 % NaCl. Erythrocytes-free cell pellet was washed three times (450 × g, for 10 min at 4 ℃) and resuspended with 0.5 – 1 mL lysis buffer (pH 7.2, HEPES 50 mmol/L). The resuspended leukocytes were proved over 90 % viability as determined by trypan blue exclusion. The cells were lysed with ultrasound in ice-bath. The lysate was centrifuged to eliminate nuclei and unbroken cells, and measurement was carried out within 2 h after the ultrasonic lysis. Protein content in leukocyte lysate was determined by method of Bradford using bovine serum albumin as a standard.

Myeloperoxidase (MPO) activity assay MPO activity in the plasma was determined kinetically[5]. Briefly, 150 μL of plasma was incubated with 3 mL of assay reagent containing *O*-dianisidine dihydrochloride 0.167 g/L, hydrogen peroxide 0.0005% and potassium phosphate buffer 50 mmol/L (pH 6.0). Product formation was linear for 2.5 min and measured spectrophotometrically at 460 nm. MPO activity was expressed as absorbance change per min and per milligram protein.

PLD-catalyzed reaction[5,10] Reaction was carried out in eppendorf tube, the 360 μL of reaction system was composed of HEPES 25 mmol/L (pH 7.2), $MgCl_2$ 5 mmol/L, $CaCl_2$ 1 mmol/L, phosphatidylcholine 2 mmol/L, oleic acid 6 mmol/L, $(NH_4)_2SO_4$ 1.6 mol/L. Leukocyte lysate containing 300 – 800 μg protein was added to initiate the reaction. These eppendorf tubes were bathed at 37 ℃ for 60 min. The reaction was terminated by placing the tube in boiling water for 10 min. After cooling to room temperature, each sample was mixed with 360 μL of methanol and whirled for 1 min (2000 r/min, amplitude is 6 mm) before centrifugation (4000 × g, for 10 min). PLD activity assay mixture contained 200 μL of supernatant after the centrifugation and 800 μL of color reagent (containing Tris-HCl 45 mmol/L, pH 8.0, peroxidase 5 U, 4-aminoantipyrene 0.3 mg, phenol 0.2 mg, choline oxidase 1 U). The reaction was carried out in 37 ℃ water bath for 90 min and terminated by adding 1 mL of ice-cold Tris-HCl 50 mmol/L (pH 8.0). A standard

curve was constructed each day with fresh standard solution of choline chloride (10 – 120 nmol). The PLD activity was quantified by calculation of produced choline according to a standard curve. One unit of PLD activity was defined as 1 nmol choline produced by 1 mg of leukocyte lysate protein in 1 h at 37 ℃.

Determination of CD11b expression of neutrophils, cytokine level, and C-reactive protein level in plasma CD11b expression of neutrophils was examined on a flow cytometer (B-D FACScan). The CD11b-FITC monoclone antibody was purchased from Immunotech Co Ltd, France. IL-6 and IL-8 levels were determined by enzyme-linked immunosorbent assay (commercial test kits of Yes Biotech Laboratories Ltd, Canada). C-Reactive protein level was determined by scattered-turbidimetry in Beckman-Array-360 system.

Statistical analysis All data were expressed as $\bar{x} \pm s$. Difference among the means of more than two groups were analyzed by One-way ANOVA and Dunnett's test using a computer software (SigmaStat 1.01 for Windows 95, 1992, Jandel Corp, USA). Differences were accepted as significant at $P < 0.05$.

RESULTS

Phospholipase D activity of leukocytes At the time point of ascending aorta declamped, leukocyte PLD activity for control group was (18 ± 8) nmol choline $\cdot h^{-1} \cdot mg^{-1}$, which was significantly higher than that of pre-CPB (4 ± 5) nmol choline $\cdot h^{-1} \cdot mg^{-1}$ ($P < 0.01$). At the same time point, the PLD activity for methylprednisolone group (10 ± 6) nmol choline $\cdot h^{-1} \cdot mg^{-1}$ was significantly lower than that of control ($P < 0.05$), but did not reach statistical significance compared with that of pre-CPB ($P > 0.05$). So, the significant increment of PLD activity in methylprednisolone group ($P < 0.01$ *vs* pre-CPB) was delayed till CPB stopped. The PLD activity in 3 groups remained high level till 72 h after CPB stopped. The changes of leukocytes PLD activity in aprotinin group were coincided with that of control at all time points, which implied that aprotinin had hardly effect on PLD activity ($P > 0.05$ *vs* control, Tab 1).

IL-6 and IL-8 level in plasma In all groups, plasma IL-6 level increased significantly at the time point of CPB stopped ($P < 0.01$ *vs* pre-CPB). At 24 h after CPB stopped, plasma IL-6 level in both methylprednisonlone and aprotinin groups were lower than that of control ($P < 0.01$) and decreased to the level of preoperative at 72 h after CPB stopped (Tab 1). Plasma IL-8 level increased significantly at CPB stopped in both control and aprotinin group ($P < 0.01$ *vs* pre-CPB). In methylprednisonlone group, the time point that IL-8 level elevated significantly was postponed to 1 h after CPB stopped. The peak level of IL-8 in methylprednisonlone group was lower than that of control ($P < 0.05$). Compared with control, IL-8 level in aprotinin group was lower at 1 h, 2 h after CPB stopped, but there was no statistical difference ($P > 0.05$, Tab 1).

CD11b expression of neutrophils CD11b expression was up-regulated significantly at CPB stopped and reached peak level at 2 h after CPB stopped in both control and aprotinin groups. In methylprednisonlone group, CD11b expression of neutrophils increased significantly at 1 h after CPB stopped and reached peak level at 24 h after CPB stopped (Tab 1).

MPO activity and C-reactive protein level in plasma In all groups, MPO activity significantly increased at 1 h after CPB stopped ($P < 0.01$ *vs* pre-CPB) and decreased at 72 h after CPB stopped but remained above pre-CPB level ($P > 0.05$ *vs* pre-CPB). Plasma C-reactive protein level of all groups was increased significantly at 24 h after CPB stopped ($P < 0.01$ *vs* pre-CPB) and decreased at 72 h after CPB stopped. At 72 h after CPB stopped, the level of C-reactive protein in both methylprednisonlone and aprotinin groups was lower than that of control, but had no statistical difference ($P > 0.05$, Tab 1).

DISCUSSION

Our study showed that PLD activity of leukocytes was increased significantly at early phase of CPB (at the time point of ascending aorta declamped), while it was remained high level during the whole experimental period (till 72 h after CPB stopped). The alterations of other inflammation-related indicators, such as leukocyte CD11b, plasma IL-6, IL-8, and C-reactive protein, were later than that of PLD activity, and the levels of these substances were elevated in shorter duration and therefore, could not be responsible for the entire post-CPB systemic inflammatory response. These results suggested that the PLD activation in leukocytes is an early response in CPB-induced inflammation and the activation of PLD might be one of the causes for post-CPB inflammatory response. However, further investigations

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Tab 1. Effects of methylprednisolone(MPD) and aprotinin(APR) on phospholipase D activity of leukocytes and some inflammation-related factors in systemic inflammatory response induced by CPB. $\bar{x} \pm s$. $^cP < 0.01$ *vs* pre-CPB. $^eP < 0.05$, $^fP < 0.01$ *vs* Control.

Group	*n*	Pre-CPB	CPB 30 min	Ascending aorta declamped	CPB stopped	After the CPB stopped 1 h	2 h	24 h	72 h
PLD activity (nmol choline·h^{-1}·mg^{-1})									
Control	26	4±5	12±7	18±8^c	21±12^c	21±9^c	23±10^c	21±7^c	20±6^c
MPD	8	4±5	8±8	10±6^e	18±6^c	19±12^c	22±12^c	17±6^c	18±9^e
APR	8	6±7	11±4	19±6^c	20±9^c	18±10^c	24±7^c	21±6^c	19±12^c
IL-6($ng \cdot L^{-1}$)									
Control	26	4±4	6±5	27±46	693±234^c	906±171^c	866±181^c	273±237	16±18
MPD	8	3±4	10±5	31±36	793±227^c	902±183^c	816±193^c	41±29^f	6±5
APR	8	5±4	7±3	40±65	848±156^c	603±294^{ce}	916±111^c	34±4^f	8±7
IL-8($ng \cdot L^{-1}$)									
Control	26	8±11	26±22	28±22	164±172^c	1031±747^c	1118±703^c	71±73	42±61
MPD	8	6±5	48±40	64±69	75±91	301±111^{ce}	328±315^{ce}	80±60	32±33
APR	8	11±8	31±20	20±3	208±65^c	473±240^c	462±251^c	35±17	17±3
CD11b (mean flurescence intensity)									
Control	26	324±157	490±182	466±183	744±366^c	867±496^c	1034±518^c	957±531^c	532±169^c
MPD	8	267±82	398±121	327±104	324±115^c	486±156^c	471±124^{ce}	673±156^c	499±75^c
APR	8	221±70	362±108	384±126	541±239^c	752±388^c	842±522^c	620±288^c	504±230
MPO activity ($\triangle A \cdot min^{-1} \cdot mg^{-1}$)									
Control	26	0.47±0.22	0.5±0.4	0.4±0.6	0.6±0.6	1.3±1.1^c	1.7±1.5	0.7±0.4	0.9±0.5
MPD	8	0.38±0.23	0.23±0.18	0.29±0.16	0.4±0.5	1.0±0.5^c	0.8±0.6	0.52±0.27	0.59±0.28
APR	8	0.30±0.10	0.27±0.15	0.44±0.18	0.44±0.17	0.74±0.22^c	0.6±0.6^e	0.59±0.27	0.55±0.17
C-reactive protein ($ng \cdot L^{-1}$)									
Control	26	0.6±0.5	0.8±0.7	1.1±1.0	1.1±0.9	0.8±0.6	0.5±0.4	44±26^c	14±16^c
MPD	8	0.7±0.7	1.0±1.2	1.6±1.5	1.4±1.5	0.8±1.0	0.38±0.24	36±17^c	8±3^c
APR	8	0.39±0.09	0.6±0.3	0.73±0.19	0.7±0.3	0.37±0.10	0.5±0.4	33±25^c	7±5^c

are required to confirm why PLD activity kept high level for such a long time. There is no special inhibitor for PLD to date, many reports declaimed that the mechanism of some anti-inflammatory agent, such as quercetin, was involved in inhibition of PLD. The present study demonstrated that methylprednisolone 10 mg/kg or aprotinin 10^5 kIU/kg partly inhibited the increment of CD11b expression in neutrophils, IL-6, IL-8, and C-reactive protein levels in plasma. These results were coincided with other studies[11-14]. These results indicated that methylprednisolone or aprotinin relieve CPB-induced systemic inflammatory response or shorten the duration of acute inflammation. Although we found that aprotinin had hardly effect on PLD, it might be inferred that the anti-inflammation mechanism of aprotinin did not related to PLD or the target of aprotinin was the down-stream of PLD. Hill *et al*[15] reported that aprotinin reduced the CPB-induced inflammatory response by enhancing the endogenous release of IL-10 which was antagonist of IL-6 and IL-8 or by inhibiting CD11b expression of neutrophils[16].

Methylprednisolone could inhibit the increment of PLD activity of leukocytes at the time points before CPB stopped and lead to postponement of the time point that PLD activity significantly elevated. This result suggested that the mechanism by which methylprednisolone relieved post-CPB inflammation was involved in inhibition of PLD, and PLD might be the fundamental substance of leukocytes priming. To avoid the side effects of glucocorticoid, we used large pharmacological dose of methylprednisolone once during CPB. The half-life of methylprednisolone was usually passed at the end of CPB, which could explain why the PLD activity in methylprednisolone group kept a high level after that time point.

Taken together, our results demonstrated that the

PLD activation in leukocytes was an early response in CPB-induced inflammation and blunting post-CPB inflammation by methylprednisolone was related to inhibiting the activation of PLD. These results suggested that inhibiting PLD activation in leukocytes in early phase of CPB could be an approach to deal with CPB-induced systemic inflammatory response syndrome.

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甲泼尼龙与抑肽酶对心肺转流术致全身炎症反应过程中白细胞磷脂酶 D 活性的影响[1]

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关键词 心肺转流术；炎症；白细胞；磷脂酶 D；甲泼尼龙；抑肽酶

目的：探讨白细胞磷脂酶 D（PLD）在心肺转流术（CPB）致全身性炎症反应过程中的作用及甲泼尼龙和抑肽酶对 PLD 活性的影响。**方法**：42 例接受 CPB 心脏直视手术病人，分为对照组、甲泼尼龙组和抑肽酶组，患者于术前、心肺转流术中及术后 8 个不同时点采集动脉血，测定白细胞 PLD 活性，并检测髓过氧化物酶活性、CD11b 表达及血浆 IL-6、IL-8 和 C-反应蛋白的含量。**结果**：在升主动脉开放时点，对照组白细胞 PLD 活性为（18±8）nmol choline·h^{-1}·mg^{-1}，明显高于术前；甲泼尼龙组为（10±6）nmol choline·h^{-1}·mg^{-1}，明显低于对照组，但与术前相比差别无显著意义；甲泼尼龙组白细胞 PLD 活性升高时点后移至停 CPB 即刻点；抑肽酶组 PLD 活性与对照组相比差别无显著意义；但两药均不同程度降低 CPB 围术期血浆中 IL-6、IL-8 和 C-反应蛋白水平及 MPO 活性、白细胞 CD11b 表达。**结论**：CPB 致全身炎症反应过程中白细胞的 PLD 活性持续升高；甲泼尼龙部分抑制 CPB 引起的炎症反应，其抗炎机制与抑制 PLD 有关。

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