Activated protein C attenuates cardiopulmonary bypass-induced acute lung injury through the regulation of neutrophil activation

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Objective: Cardiopulmonary bypass is known to induce systemic inflammatory responses that injure multiple organs, especially the lungs. Activated protein C has been demonstrated to play an important role in the regulation of inflammation in addition to coagulation. We investigated the anti-inflammatory effects of activated protein C in a rat model of cardiopulmonary bypass.

Methods: Rats were randomized to receive an intravenous bolus of vehicle (control), 0.1 mg/kg diisopropyl fluorophosphate-activated protein C, or 0.1 mg/kg activated protein C 10 minutes before the initiation of cardiopulmonary bypass. Rats underwent cardiopulmonary bypass for 60 minutes followed by another 60-minute observation.

Results: The activated protein C group showed significantly higher mean arterial oxygen pressure and lower mean lung wet-to-dry weight ratio after cardiopulmonary bypass than the control and diisopropyl fluorophosphate-activated protein C groups. Furthermore, lung pathology revealed minimal inflammatory change in the activated protein C group. A marked increase in CD11b expression and a decrease in CD62L expression after cardiopulmonary bypass were observed in the control and diisopropyl fluorophosphate-activated protein C groups. However, administration of activated protein C significantly attenuated these changes. Lung content of tumor necrosis factor- α and interleukin-1 β in the activated protein C group tended to be lower than in the other groups. Lung content of macrophage inflammatory protein-2 in the activated protein C group was significantly lower than in the diisopropyl fluorophosphate-activated protein C group.

Conclusions: Administration of activated protein C before cardiopulmonary bypass attenuates acute lung injury induced by cardiopulmonary bypass at least in part through the inhibition of neutrophil activation and possibly via the attenuation of proinflammatory cytokine production in this rat model of cardiopulmonary bypass. (*J Thorac Cardiovasc Surg* 2011;141:1246-52)

Cardiopulmonary bypass (CPB) is a common procedure in heart surgery. Despite advances in surgical procedures and material technology, CPB induces both coagulopathy and inflammation when blood touches the bypass circuit. Because the lung is vulnerable to attack by neutrophils, CPB is often followed by pulmonary dysfunction, which varies widely, ranging from a clinically undetectable condition to the most severe form, acute respiratory distress syndrome.¹ These responses are associated with endotoxin release; accelerated thrombin generation; activation of complement, leukocytes, and platelets; and the production of cytokines and other inflammatory mediators.²⁻⁵ This complex chain of events seems to have similarities to sepsis, which

results from a generalized inflammatory and procoagulant response to an infection.⁶ These findings raise the possibility that adequate regulation of coagulation and inflammation may prevent CPB-induced lung injury, which is strongly related to an adverse outcome.¹

Activated protein C (APC) is an important natural anticoagulant that is generated from protein C by the action of the thrombin-thrombomodulin complex on the endothelial cell.⁷ APC inactivates factors Va and VIIIa, and neutralizes the activity of plasminogen activator inhibitor-1, thereby increasing fibrinolysis. Apart from the anticoagulant effects of APC, its direct anti-inflammatory properties have recently been identified.⁷ In vitro studies demonstrated that APC down-regulates proinflammatory cytokines, such as interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)- α release, from both endothelial cells and leukocytes through a specific surface molecule, endothelial protein C receptor (EPCR).^{13,16} In addition, expression of intercellular adhesion molecule (ICAM)-1 stimulated by IL-1 β and TNF- α was suppressed by APC.¹² In animal models, APC reduced ischemia-reperfusion injury in a variety of organs by inhibiting monocyte activation and cytokine-induced neutrophil chemoattractant expression.⁸⁻¹¹ In a clinical study, Lamarche and colleagues²⁴ reported successful

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Abbreviations and Acronyms

ACT	= activated clotting time
APC	= activated protein C
CPB	= cardiopulmonary bypass
DIP	= diisopropyl fluorophosphate
EPCR	= endothelial protein C receptor
ICAM	= intercellular adhesion molecule
IL	= interleukin
MIP	= macrophage inflammatory protein
TNF	= tumor necrosis factor
W/D	= wet-to-dry

treatment of severe acute respiratory distress syndrome and septic shock by the combined use of extracorporeal membrane oxygenation and APC. Furthermore, a past trial showed that APC significantly reduced 28-day mortality rate when compared with placebo control in patients with severe sepsis.¹⁴ These findings suggest that APC may have beneficial effects on acute organ damage. However, the effect of APC on CPB-induced acute lung injury remains unknown.

The purposes of this study were to (1) investigate whether the administration of APC has a protective effect on acute lung injury in a rat model of CPB and, if so, (2) verify its mechanism.

MATERIALS AND METHODS

Animals and Reagents

Adult male Sprague-Dawley rats (aged 14–17 weeks, weighing 400–580 g) from a licensed vendor (Japan SLC Inc, Shizuoka, Japan) were used in the present study. APC and diisopropyl fluorophosphate (DIP)-APC, an inactive derivative of APC, were provided by the Chemo-sero-therapeutic Research Institute, Kumamoto, Japan. Rats were randomized to receive intravenous bolus of vehicle, 0.1 mg/kg DIP-APC, or 0.1 mg/kg APC 10 minutes before the initiation of CPB. This protocol resulted in the creation of 3 groups: rats given vehicle before CPB (control group, $n = 6$), rats given DIP-APC (DIP group, $n = 6$) before CPB, and rats given APC (APC group, $n = 6$) before CPB. The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

Surgical Procedure

Animal procedure was a modification of the technique described previously.¹⁵ In brief, with the animals under anesthesia via intraperitoneally administered pentobarbital sodium (50 mg/kg) and artificial ventilation at a tidal volume of 8 mL/kg, the right jugular vein and left carotid artery were exposed for CPB. Then, an 18-gauge cannula with side holes and a 22-gauge cannula were placed for venous drainage and arterial blood return, respectively. A 22-gauge cannula was inserted into the right femoral vein to infuse saline with 5% bovine serum albumin, and a 24-gauge cannula was inserted into the femoral artery to monitor arterial blood pressure. The bypass circuit was primed with 9 mL of saline with 5% bovine serum albumin. After administration of heparin (200 U/kg), CPB was established with the perfusion rate maintained at 60 mL/kg/min for 60 minutes. Just before the establishment of CPB, clamping of the left pulmonary hilum was performed via anterolateral thoracotomy to simulate the lung condition

when total extracorporeal circulation was performed, and the tidal volume was decreased to 6 mL/kg during CPB. When CPB was terminated, the pulmonary clamp was released and the tidal volume was returned to the initial level. The rat was observed for another 60 minutes after CPB. Arterial blood samples were obtained 3 times (before CPB, at the end of CPB, and at the end of the experiment) to measure arterial oxygen pressure and blood hemoglobin level.

Wet-to-Dry Weight Ratio of Left Lung

The left lung was excised immediately after animal sacrifice and divided into 3 parts. The superior third was weighed before and after desiccation in a dry oven at 70°C, and wet-to-dry (W/D) weight ratio was calculated as a marker for lung edema.

Measurement of Lung Cytokine Content

The middle third of the left lung sample was used to measure the lung contents of cytokines. The sample was homogenized in 0.5 mL phosphate-buffered saline containing a protease inhibitor, sonicated, and then centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatants were stored at –80°C until the cytokines were measured. Lung levels of rat TNF- α , IL-1 β , and macrophage inflammatory protein (MIP)-2 were measured using corresponding enzyme-linked immunosorbent assay kits (BioSource International, Inc, Camarillo, Calif). The results were corrected by the protein content determined by the Bradford method.¹⁸

Expression of Adhesion Molecules on Circulating Neutrophils

The expression of CD11b and CD62L on the neutrophil surface was analyzed by fluorescence-activated cell sorting (FACS SCAN flow cytometer; Becton Dickinson, San Jose, Calif) before CPB, at the end of CPB, and at the end of the experiment. Whole blood was incubated with phycoerythrin-conjugated anti-rat CD11b (Serotec Ltd, Oxford, England) and fluorescein isothiocyanate-conjugated anti-rat CD62L (BD Bioscience Pharmingen, San Diego, CA). Erythrocytes were then destroyed by incubation with lysing solution (BD Bioscience Pharmingen). After washing twice with phosphate-buffered saline and centrifuge, neutrophils were fixed with CELLFIX (Becton Dickinson). Isotype-identical antibodies served as controls.

Activated Clotting Time

The measurement of activated clotting time (ACT) (Hemocron Jr Signature; International Technidyne Corporation, Edison, NJ) was performed before CPB and at the end of the experiment. In the APC and DIP groups, the first blood samples were collected 10 minutes after the administration of drugs.

Serum Fibrinogen Level

Serum level of fibrinogen was measured by the thrombin method at the end of the experiment by SRL, Inc, Tokyo, Japan.

Pathologic Study

The inferior third of the lung was fixed with 10% formalin, embedded in paraffin, cut into 4- μ m sections, and stained with hematoxylin-eosin for pathologic study.

Statistical Analysis

Numeric values are expressed as mean $\pm$ standard error of the mean unless otherwise indicated. Comparisons of parameters among the 3 groups were made using 1-way analysis of variance, followed by the Newman-Keuls test. Comparisons of changes in parameters among the 3 groups were made by 2-way analysis of variance for repeated measures, followed by the Newman-Keuls test.

FIGURE 1. Microphotograph of the left lung. The left lung section in each group was stained with hematoxylin–eosin. Lungs in the control and DIP groups show interstitial edema, alveolar hemorrhage, and severe neutrophil accumulation. Inflammatory change in the APC group lung is minimized. Original magnification: 200 $\times$. Black bar = 50 μ m. Control (A), DIP (B), and APC (C) groups.

RESULTS

Histologic and Functional Improvement of the Lung by Activated Protein C

Microscopic observation of lung tissue revealed interstitial edema, alveolar hemorrhage, and infiltration of polymor-

phonuclear leukocytes in the control and DIP groups. APC administration markedly minimized these abnormalities (Figure 1). The CPB itself and ischemia of the left lung during CPB caused the increase in peak airway pressure afterward. However, peak airway pressure in the APC group remained at a plateau and tended to be lower than that in the control and DIP groups 60 minutes after reperfusion (APC: 11.8 ± 0.6 vs control: 13.5 ± 0.8 and DIP: 13.0 ± 0.5 cmH₂O, Figure 2, A). The arterial Po₂ level in the control and DIP groups decreased significantly to 226 ± 55 mm Hg and 233 ± 22 mm Hg, respectively, at the end of the experiment, whereas it remained high in the APC group (APC: 409 ± 33 mm Hg, Figure 2, B). The APC group also showed significantly lower W/D weight ratio than the other 2 groups (APC: $6.2 \pm .2$ vs control: $7.7 \pm .4$ and DIP: 8.0 ± 0.5 , $P < .01$, Figure 1, C), although there was no significant difference in body weight among the 3 groups.

Suppressive Effect of Activated Protein C on Proinflammatory Cytokines

Lung content of MIP-2 in the APC group was significantly lower than in the DIP group and relatively lower than in the control group (APC: 0.7 ± 0.3 vs DIP: 2.2 ± 0.5 , $P < .05$, and control: 1.3 ± 0.4 pg/ μ g protein, $P =$ not significant, Figure 3, A). The lung content of TNF- α and IL-1 β in the APC group was also approximately 50% or less than in the control and DIP groups, although this was not significant among the groups (TNF- α : APC; 5.4 ± 1.3 vs control; 9.6 ± 1.1 and DIP; 11.5 ± 2.5 pg/ μ g protein, $P = .07$, Figure 3, B, and IL-1 β : APC; 4.0 ± 1.1 vs control; 8.4 ± 2.1 or DIP; 11.4 ± 2.8 pg/ μ g protein, $P = .07$, Figure 3, C).

Attenuation of Neutrophil Activation by Activated Protein C

As indexes of neutrophil activation induced by CPB, changes in the expression of surface adhesion molecules of CD11b and CD62L were analyzed. The signal intensity of CD11b in the control and DIP groups was markedly increased at 60 and 120 minutes after the initiation of CPB (Figure 4, A). However, administration of APC significantly inhibited the increase in CD11b. The signal intensity of CD62L was markedly decreased by CPB in the control and DIP groups, whereas administration of APC completely inhibited the decrease in CD62L 60 minutes after the initiation of CPB (Figure 4, B).

Activated Clotting Time, Hemoglobin, and Fibrinogen

Our preliminary ACT measurements in rats revealed that the mean ACT value of non-heparinized blood was approximately 100 seconds. ACT was prolonged by the administration of heparin, and APC, unlike DIP-APC, induced further extension of ACT (APC: 504 ± 34 vs control: 398 ± 22 and

FIGURE 2. A, Peak airway pressure before CPB and at the end of the experiment. The APC group shows a relatively lower value than the other groups at the end of the experiment, although there are no significant differences among the groups. Results are expressed as mean $\pm$ standard error of the mean (SEM). B, Arterial oxygen pressure before CPB and at the end of

DIP: 393 ± 13 seconds, $P < .05$, Figure 5, A). At the end of the experiment, however, ACT decreased to a similar value in all 3 groups (control: 317 ± 13 , DIP: 288 ± 10 , APC: 331 ± 18 seconds). Macroscopically, APC-treated rats did not show an obvious bleeding tendency during the experiments compared with other group animals. Hemoglobin levels in all groups were approximately 14 g/dL initially and then similarly decreased to approximately 10 g/dL after CPB and at the end of the experiment, mainly because of hemodilution. There was no significant difference among the groups before CPB, after CPB, and at the end of the experiment (control: 14.3 ± 0.2 , 9.0 ± 0.2 , and 9.8 ± 0.5 , DIP: 13.8 ± 0.4 , 9.8 ± 0.6 , and 10.3 ± 0.9 , APC: 14.0 ± 0.7 , 9.5 ± 0.4 , and 10.0 ± 0.8 g/dL, respectively, Figure 5, B). The serum fibrinogen level at the end of the experiment did not significantly differ among the 3 groups (APC: 99 ± 20 , control: 118 ± 7 , DIP: 129 ± 11 mg/dL, Figure 5, C).

DISCUSSION

The present study demonstrated that the administration of APC attenuated CPB-induced edematous changes and neutrophil sequestration in the lung, and maintained higher arterial P_{O_2} and lower W/D weight ratio after CPB compared with vehicle or DIP-APC. We also clarified that APC attenuated neutrophil activation induced by CPB, as indicated by normalized expression of CD11b and CD62L, and tended to decrease intrapulmonary concentrations of proinflammatory cytokines after CPB.

A previous study, using the same CPB circuit, showed that 60 minutes of CPB induces acute lung injury afterward and that the inhibition of neutrophil activation by phosphodiesterase type 4 inhibitor or anti-neutrophil monoclonal antibody clearly reduced post-CPB lung injury, indicating neutrophils activated by CPB play a pivotal role in the process of post-CPB lung injury.¹⁵ We used this rat CPB model to evaluate lung protection by APC. Lung injury that occurred in the vehicle (control) group was compatible with previous observations. APC administration before CPB markedly attenuated physiologic and pathologic pulmonary deterioration after CPB. The results clearly revealed the *in vivo* anti-inflammatory impact of APC.

We then examined underlying mechanisms of lung protection by APC. The anti-inflammatory activity of APC is known to be EPCR dependent. EPCR is primarily found on endothelial cells of arteries, veins, and capillaries.

the experiment. The APC group shows a significantly higher value than the other groups at the end of the experiment. Results are expressed as mean $\pm$ SEM. [#] $P < .01$ versus the control or DIP group. C, W/D weight ratio of the left lung. The APC group shows a significantly lower value than the other groups. Results are expressed as mean $\pm$ SEM. [#] $P < .01$ versus the control or DIP group. APC, Activated protein C; CPB, cardiopulmonary bypass; DIP, diisopropyl fluorophosphate; W/D, wet-to-dry.

FIGURE 3. A, Tissue MIP-2 concentration in the left lung. The APC group shows a relatively lower value than the control group and a significantly lower value than the DIP group. Results are expressed as mean $\pm$ SEM. $\#P < .01$ versus the DIP group. B, Tissue TNF- α concentration in

EPCR has also been confirmed to be expressed on the membrane of monocytes, CD56⁺ natural killer cells, neutrophils, and eosinophils.^{13,16} Sturm et al¹⁷ showed that APC directly inhibits chemotaxis of stimulated neutrophils by means of binding to EPCR in vitro. However, the in vivo effects of APC on neutrophils still remains unknown. This study analyzed changes in the signal intensity of adhesion molecules, CD62L and CD11b, on neutrophil surface by flow cytometry to evaluate their excitation. CD62L, a member of the selectin family, is present on the surface of circulatory non-activated leukocytes.^{19,20} The molecule CD11b is classified as one of the leukocyte integrins, which are largely responsible for increasing adhesion and transendothelial migration of neutrophils through interactions with their endothelial counter-receptors, ICAM-1 and ICAM-2.²¹ In general, CD62L is rapidly shed after neutrophil activation with concomitant up-regulation of Mac-1 (CD11b/CD18).^{4,19} During CPB, neutrophils are activated by the contact with the surface of the bypass circuit, and chemical mediators, such as TNF- α and IL-8 (MIP-2), also stimulate neutrophils. Although direct effects of APC on CD11b and CD62L remain unknown, Franscini and colleagues¹² showed that APC down-regulates expression of ICAM-1 stimulated by IL-1 β and TNF- α . Our observation that the administration of APC normalized the expression of CD11b and CD62L after CPB indicates that APC effectively down-regulates neutrophil functions. Notably, in our previous studies, the expression of CD62L increased after CPB and neutrophil inhibitors did not alter the trend. We speculated that CPB did not reduce CD62L expression in those study settings because of the influence of new neutrophils from bone marrow. The difference between this study and Franscini and colleagues' is the surgical procedure for the setup of CPB. We did not perform a sternotomy to insert a drainage line but introduced a catheter from the right jugular vein to the right atrium instead. This minor improvement, however, should significantly reduce bone marrow stimulation to release neutrophils.

Proinflammatory cytokines are important mediators in various disease processes. An increase in the lung contents of TNF- α , IL-1, and IL-8 during and after CPB was reported in several studies.^{1,15} A recent in vivo study showed that APC inhibits lipopolysaccharide-induced production of TNF- α by the inhibiting activation of both nuclear factor- κ B and activator protein-1 in monocytes.²² The ability of

the left lung. The APC group shows a relatively lower value than the other groups, although there are no significant differences among the groups. Results are expressed as mean $\pm$ SEM. C, Tissue IL-1 β concentration in the left lung. The APC group shows a relatively lower value than the other groups, although there are no significant differences among the groups. Results are expressed as mean $\pm$ SEM. *DIP*, Diisopropyl fluorophosphate; *APC*, activated protein C; *TNF*, tumor necrosis factor; *MIP*, macrophage inflammatory protein; *IL*, interleukin.

FIGURE 4. A, CD11b expression on circulating neutrophils. Percentage change in geometric mean from the value before CPB was calculated. The APC group maintains leveled CD11b expression at all time points, whereas expression in the control and DIP groups increases approximately double at 60 and 120 minutes after the initiation of CPB. Results are expressed as mean $\pm$ SEM. * $P < .05$ versus the DIP group. ** $P < .05$ versus the control or DIP group. B, CD62L expression on circulating neutrophils. Percentage change in geometric mean from the value before CPB was calculated. CD62L expression remains level in the APC throughout the experiment, whereas expression in the control and DIP groups gradually decreases to approximately 50% at 120 minutes after the initiation of CPB. Results are expressed as mean $\pm$ SEM. # $P < .01$ versus the control or DIP group. * $P < .05$ versus the DIP group. CPB, Cardiopulmonary bypass; DIP, diisopropyl fluorophosphate; APC, activated protein C.

APC to inhibit the production of IL-1 β , IL-6, and IL-8 was also reported using the immortalized human monocytic cell line.²³ In the present study, mean values of intrapulmonary TNF- α , IL-1 β , and MIP-2 in the APC group were

FIGURE 5. A, ACT before CPB and at the end of the experiment. The APC group shows a significantly higher value than the others only before CPB. The values decrease in all groups at the end of the experiment and become similar to one another. Results are expressed as mean $\pm$ SEM. * $P < .05$ versus the control or DIP group. B, Blood hemoglobin level before CPB, after CPB, and at the end of the experiment. In all groups, the values after CPB and at the end of the experiments similarly decrease to approximately 70% of initial values. There are no significant differences among the groups at any time point. Results are expressed as mean $\pm$ SEM. C, Serum fibrinogen level at the end of the experiment. There are no significant differences among the groups. Results are expressed as mean $\pm$ SEM. ACT, Activated clotting time; DIP, diisopropyl fluorophosphate; APC, activated protein C; CPB, cardiopulmonary bypass.

approximately 50% of those in the other groups, although the statistical significance was not recognized because of a relatively wider sample distribution. TNF- α and IL-1 β are secreted primarily by activated monocytes and cause subsequent activation of neutrophils, macrophages, and endothelial cells. MIP-2 is considered to be a rat homologue of IL-8, a potent neutrophil chemoattractant. These findings imply the inhibitory effect of APC on CPB-induced production of TNF- α , IL-1 β , and MIP-2, which also may contribute to neutrophil activation and sequestration.

Exogenously administered APC may affect the hemostatic function, because APC is an endogenous anticoagulant. Indeed, elongated ACT by heparin was further prolonged by the administration of APC. The effect of APC on ACT value, however, had declined by the end of the experiment, and no obvious bleeding tendency was seen in the APC group in terms of hemoglobin levels and macroscopic findings. In addition, serum fibrinogen level at the end of the experiment did not significantly differ among the 3 groups. Taken together, a bolus injection of the APC dose used in this study did not seem to significantly worsen or improve clinical coagulability during and after CPB in this model. In contrast, the inhibitory effect of APC on neutrophil activation lasted more than 120 minutes.

CONCLUSIONS

APC prevented acute lung injury induced by CPB at least in part through the inhibition of neutrophil activation, possibly via the attenuation of cytokine production in this setting. The administration of APC may be a promising therapeutic strategy for the treatment of postoperative complications induced by CPB.

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