

Endogenously released adenosine causes pulmonary vasodilation during the acute phase of pulmonary embolization in dogs☆

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ABSTRACT

Background: Endogenous adenosine levels increase under stress in various organs. Exogenously administered adenosine is a well-known pulmonary vasodilator. However, the physiology and therapeutic potential of endogenous adenosine during alteration in pulmonary hemodynamics such as pulmonary embolism is not elucidated. We hypothesized that the adenosine level increases following an acute elevation of pulmonary resistance, resulting in pulmonary vasodilation.

Methods: We induced acute pulmonary embolization by injecting plastic beads in anesthetized dogs. Plasma adenosine levels, defined as the product of plasma adenosine concentration and simultaneous cardiac output, were assessed from blood samples from the superior vena cava, main pulmonary artery (MPA), and ascending aorta 1 and 10 min following injection. Hemodynamics were assessed with ($n = 3$) and without ($n = 8$) administration of the adenosine receptor blocker, 8-(*p*-sulfophenyl)theophylline (8SPT).

Results: Mean pulmonary arterial pressure (PAP) increased from 11 ± 1 mmHg, peaking at 28 ± 4 mmHg at 52 ± 13 s after injection. During this period, total pulmonary resistance (TPR) elevated from 11 ± 1 to 33 ± 6 Wood unit. Plasma adenosine levels increased in the MPA from 14.5 ± 2 to 38.8 ± 7 nmol/min 1 min after injection. TPR showed greater elevation under 8SPT treatment, to 96 ± 12 Wood unit at PAP peak.

Conclusions: Endogenously released adenosine after acute pulmonary embolization is one of the initial pulmonary vasodilators. The immediate surge in plasma adenosine levels in the MPA could lead to a hypothesis that adenosine is released by the right heart in response to pressure overload.

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1. Introduction

Endogenous adenosine levels rise in various types of organs including the heart and lungs in response to the pathophysiological stresses such as inflammation and energetic perturbations as typified by ischemia, resulting in the activation of tissue-protective signaling [1–6]. Adenosine is also a well-known pulmonary vasodilatory agent as demonstrated by an experimental administration in intact lungs [3] and in conditions of reversible vascular precontraction such as hypoxic pulmonary vasoconstriction [3,7] and abnormal vasoconstriction in pulmonary arterial hypertension (PAH) [8,9]. Pharmacological studies suggested that when vascular tone is low or high, adenosine A₁ and A₂

receptors activation appears to independently mediate vasoconstriction and vasodilation, respectively [10]. However, the physiological mechanism of endogenous adenosine-mediated regulation of the pulmonary circulation is still unclear. As the pulmonary circulation is a high-flow, low-pressure circuit, vasodilatory action is crucial to prevent circulatory collapse particularly during critical alterations of the pulmonary hemodynamics such as an acute elevation of pulmonary vascular resistance as observed in acute pulmonary embolism (PE). In acute PE, active vasoconstriction of the remaining non-occluded vasculature contributes to increased pulmonary vascular resistance [11,12], and has been shown to be reversible by intravenously injected vasodilators such as guanylate cyclase stimulator [13]. Thus, intrinsically released adenosine would be a beneficial vasodilator for normal vascular tone or functionally constricted vasculature following PE. However, alterations in endogenous adenosine levels and its physiological role in vasoactivity or therapeutic potential for acute PE have not yet been elucidated. We hypothesized that the endogenous adenosine level increases in response to an acute elevation of pulmonary resistance, resulting in pulmonary vasodilation.

☆ All of these authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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The aim of our study was to investigate the alteration of plasma adenosine levels and its resultant effect on hemodynamics during acute elevation in pulmonary vascular resistance. We used an experimental model of acute pulmonary embolization with plastic beads to eliminate the confounding effects of clot-derived chemical factors and focused on the acute phase to minimize an influence of secondary reaction to hypoxia and inflammation. Furthermore, we tested whether a blockade of adenosine receptors using 8-(*p*-sulfophenyl)theophylline (8SPT) modulates pulmonary vascular resistance.

2. Methods

All procedures involving the handling of animals in the present study adhered to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (the 8th Edition, NRC 2010) and were conducted with the approval of the Committee for Laboratory Animal Use of the National Cerebral and Cardiovascular Center.

2.1. Animal preparation and instrumentation

Studies were performed in 11 adult female beagle dogs (Kitayama Labes, Nagano, Japan) weighing 9–11 kg. Dogs were anesthetized with intravenous pentobarbital sodium (25 mg/kg) and, after endotracheal intubation, were maintained with 0.5%–1% isoflurane delivered via a volume-controlled ventilator with a FiO_2 of 0.3, a respiratory rate of 9–10 breath/min, and a tidal volume of 15–25 ml/kg adjusted to maintain arterial PCO_2 between 35 and 45 Torr. Fluid-filled polyvinyl catheters were inserted in the ascending aorta and the superior vena cava (SVC) via the left cervical artery and vein, respectively, for monitoring of systemic blood pressure and central venous pressure (CVP) with pressure transducers (DX-200, Nihon Kohden, Tokyo, Japan) and for blood sampling. A balloon-tipped pulmonary arterial catheter (6Fr) for blood sampling and a catheter-tipped micromanometer (SPR-524, Millar Instruments, Houston, TX) for monitoring of pulmonary arterial pressure (PAP) were advanced into the main pulmonary artery (MPA) through the left and the right femoral vein, respectively. After thoracotomy in the left fifth intercostal space, an ultrasonic flow probe (12PAX, Transonic, Ithaca, NY) was adjusted around the MPA to obtain cardiac output. Standard electrocardiogram was used to monitor heart rate and rhythm. All hemodynamic data were recorded using PowerLab system (ADInstruments, Bella Vista, Australia). Total pulmonary resistance (TPR) was calculated as mean PAP divided by cardiac output, expressed as Wood unit or $\text{mm Hg} \cdot \text{s} \cdot \text{ml}^{-1}$. Pulmonary arterial compliance (PAC) was estimated by stroke volume divided by pulmonary arterial pulse pressure ($\text{ml} \cdot \text{mm Hg}^{-1}$) [14]. Stroke volume was calculated by dividing cardiac output by heart rate. Arterial blood samples were analyzed with a blood gas analyzer (IRMA Trupoint system, International Technidyne Corporation, Edison, NJ) for PO_2 and PCO_2 .

2.2. Protocol

Saline was infused at a rate of 200 ml/h throughout the experiment. After an administration of heparin (3000 U/body, IV) followed by a stabilization period, baseline hemodynamic parameters of PAP, systemic blood pressure, cardiac output, CVP and heart rate were measured. All pressures and flows were recorded during the expiratory phase of ventilation. In the 8SPT group ($n = 3$), 8SPT, an adenosine receptor antagonist, (80 mg/body (8 mg/kg for the average weight) dissolved in saline, IV) was administered instead of saline infusion in control group ($n = 8$). After a 10-min stabilization period and assessment of hemodynamic measurements (pre-injection), 0.25 g of 110–300 μm plastic beads (M-4003, SE-090T, Negami chemical industrial, Ishikawa, Japan) suspended in 10 ml of 10% dextran 40 containing 0.1% of Tween-80 were injected into the SVC via cervical vein catheter followed by 20 ml of saline flush. Hemodynamics were continuously measured after bead injection until hemodynamic stabilization was reached.

Arterial PO_2 was assessed at pre-injection and 10 min after injection. In the control group, adenosine levels, defined as the product of plasma adenosine concentration and simultaneous cardiac output, were assessed from blood samples from the SVC, MPA and ascending aorta at pre-injection, 1 min, and 10 min after injection. At the conclusion of the protocol, the dogs were euthanized with an intravenous overdose of pentobarbital and potassium chloride.

2.3. Adenosine concentrations

Blood samples (1 ml) were mixed with EDTA2-Na (4 mM final concentration), 100 μM dipyrindamole (0.01%), and 100 μM 2'-deoxycoformycin (0.5%) to block uptake of adenosine by red blood cells and degradation of adenosine. Supernatant plasma samples after centrifugation were stored frozen. Plasma concentrations of adenosine were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at Nemoto Science (Tsukuba, Japan). Plasma samples were prepared by fortifying 100 μl of plasma with 10 μl of internal standard (0.06 mmol/L $^{13}\text{C}_5$ -labeled adenosine). Proteins were removed by adding trifluoroacetic acid to the samples, followed by 5-min centrifugation (12,000 g, 4 °C). LC-20AD LC system (Shimadzu, Kyoto, Japan) was coupled to API 5000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) operating in electrospray ionization mode. Chromatographic separation took place on Hypercarb PGC column (150 $\times$ 2.1 mm i.d., 5 μm particle, ThermoFisher) maintained at 60 °C. The sample injection volume was 5 μl at the final dilution and the mobile phase was 10 mmol/L ammonium formate/acetonitrile (60/40 vol/vol). Samples were eluted within 3 min at a flow rate of 250 $\mu\text{l}/\text{min}$. For mass spectrometry detection the multiple reaction monitoring was implemented using the following mass transitions: m/z 268 to 136 for adenosine, and m/z 273 to 136 for the internal standard. Data processing and quantification were performed using Analyst version 1.5 (AB Sciex).

2.4. Statistical analyses

Data are expressed as means $\pm$ standard error of the mean. Adenosine levels and hemodynamics variation over time in the control group were assessed by one-way repeated measures analysis of variance with post-hoc Tukey's honestly significant difference method when appropriate. When Mauchly's test of sphericity was significant, Greenhouse-Geisser epsilon and paired *t*-test with Bonferroni correction was applied to correct the family-wise error rate. Within-subject disparities of adenosine levels between SVC blood and MPA blood were assessed using paired *t*-test. For hemodynamics comparisons between the two groups, Mann-Whitney *U* test was used. All *P* values were 2-sided and a $P < 0.05$ was considered statistically significant. All statistical analyses were performed using JMP version 9 (SAS Institute Inc., Cary, NC) and GraphPad Prism version 7 (GraphPad Software, San Diego, CA).

3. Results

3.1. Plasma adenosine levels after the beads injection

In one of eight dogs, the plasma adenosine concentrations and levels were not available due to a failure in blood sampling. The plasma adenosine concentration measurements are shown in Table 1. Comparisons of plasma adenosine levels are shown in Fig. 1. One min after injection, plasma adenosine levels significantly increased in the main pulmonary artery (MPA) compared to pre-injection levels ($P = 0.01$, Fig. 1B). Plasma adenosine levels in the superior vena cava (SVC) tended to increase 1 min after injection ($P = 0.06$, Fig. 1A) but returned to pre-injection levels after 10 min ($P = 0.04$ vs. 1 min, NS vs. pre-injection). In contrast, the disparity in plasma adenosine levels between SVC and MPA continued to increase, reaching a significant level 10 min after injection (95% CI, 4.5–26.1 nmol/min, $P = 0.01$, Fig. 1D), despite the

Table 1

The plasma adenosine concentration measurements.

Baseline				1 min			10 min		
				Time after injection					
SVC, nmol/L	12	±	2	24	±	5	12	±	3
MPA, nmol/L	13	±	2	32	±	6	26	±	4
Aorta, nmol/L	8	±	2	17	±	4	11	±	2

Data are shown as mean ± standard error of the mean. SVC, superior vena cava; MPA, main pulmonary artery.

insignificant disparity at pre-injection (95% CI, −6.2–8.5 nmol/min, $P = 0.71$).

3.2. Effects of the pulmonary embolization in the presence or absence of adenosine receptor blockade on pulmonary and systemic hemodynamics

3.2.1. The control group

The time courses in hemodynamics in the control group are illustrated in Fig. 2. Mean pulmonary arterial pressure (PAP) rapidly increased, peaking at 28 ± 4 mm Hg ($P = 0.01$ vs. pre-injection, Fig. 2A) 52 ± 13 s after injection and started to decline immediately thereafter. Although the overall change in cardiac output was not significant, after PAP peak, cardiac output was maintained equal to or higher than the pre-injection level (Fig. 2B). Total pulmonary resistance (TPR) changed parallel to PAP; TPR rapidly elevated, peaking at PAP peak and started to decline (Fig. 2C). At 1 min after PAP peak (~2 min after injection), mean PAP and TPR showed a recovery of about 30% to 50% of the initial elevation (PAP peak minus pre-injection). At 10 min after PAP peak, both PAP and

TPR appeared to stabilize. Pulmonary arterial compliance (PAC) decreased after injection and then also recovered, inversely to TPR (Fig. 2E). Central venous pressure (CVP) was increased transiently at PAP peak ($P = 0.006$ vs. pre-injection, Fig. 2F). Neither blood pressure nor heart rate changed significantly during the experiment (Fig. 2F). Arterial PO_2 decreased 38% (139 ± 17 to 85 ± 12 Torr, $P < 0.001$) 10 min after injection.

3.2.2. The 8SPT group

Fig. 2 illustrate the time course of hemodynamics in the 8SPT group. Patterns of time courses of PAP and TPR after injection were similar to those in the control group, rapid elevation peaking at 76 ± 38 s after injection ($P = 0.78$, vs. control) and then started to decline. However, the maximal changes in PAP, TPR, and PAC that observed in the initial phase (from pre-injection to PAP peak) were more significant in the 8SPT group than in the control group (Fig. 2A, C, and D, insets). Cardiac output values dropped after injection in all dogs in the 8SPT group, but the initial changes in cardiac output at PAP peak were not significantly different between the two groups ($P = 0.08$, Fig. 2B, inset). The inverse relationship between TPR and PAC was consistently maintained in the 8SPT group as in the control group (Fig. 2E). There was no significant difference between the two groups regard to response of blood pressure, heart rate, or CVP at PAP peak (Fig. 2F). Arterial PO_2 decreased 46% 10 min after injection; that was not significantly different from the control group either ($P = 0.63$).

4. Discussion

This study demonstrated an immediate increase in plasma adenosine levels in the MPA in response to induced elevation of pulmonary

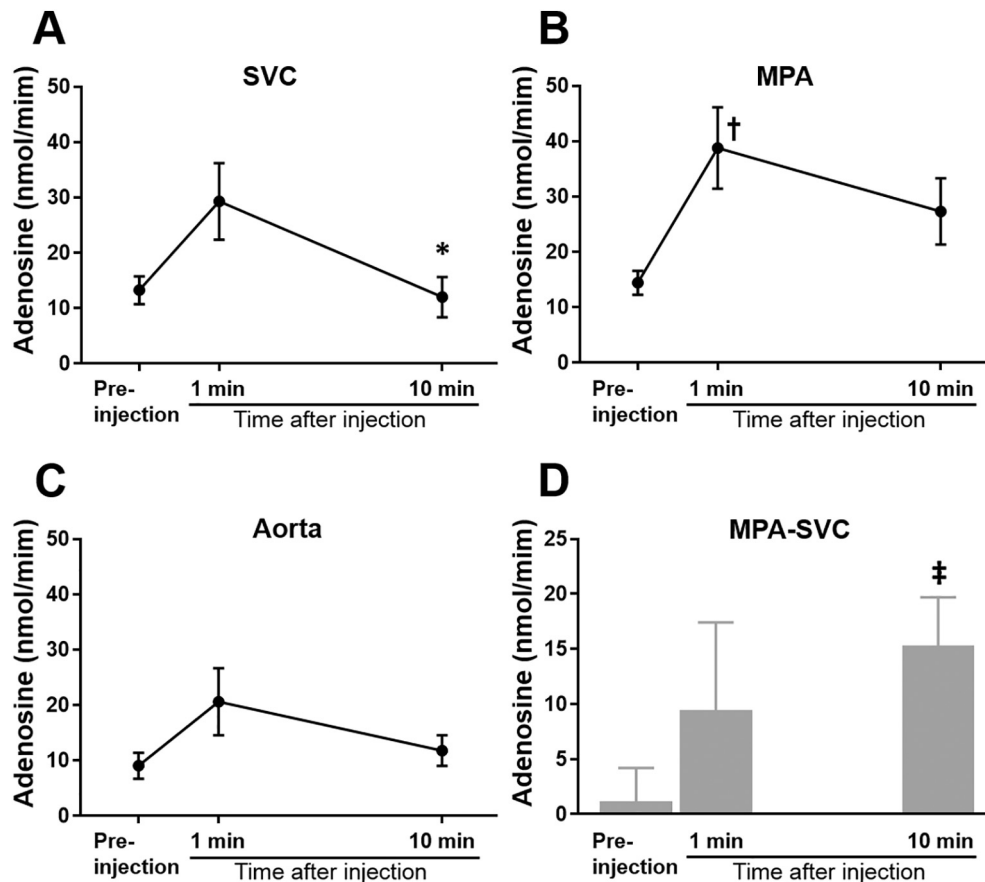


Fig. 1. Adenosine levels in the blood of the SVC (A), MPA (B), and aorta (C), defined as the product of plasma adenosine concentration and simultaneous cardiac output. Adenosine level disparities between MPA and SVC are also shown (D). Data are shown as mean ± standard error of the mean. * $P < 0.05$ vs. 1 min after injection and † $P < 0.05$ vs. pre-injection in one-way repeated measures analysis of variance with post hoc Tukey HSD method. ‡ $P < 0.05$ for the difference between MPA and SVC in a paired t-test. SVC, superior vena cava; MPA, main pulmonary artery; HSD, honestly significant difference.

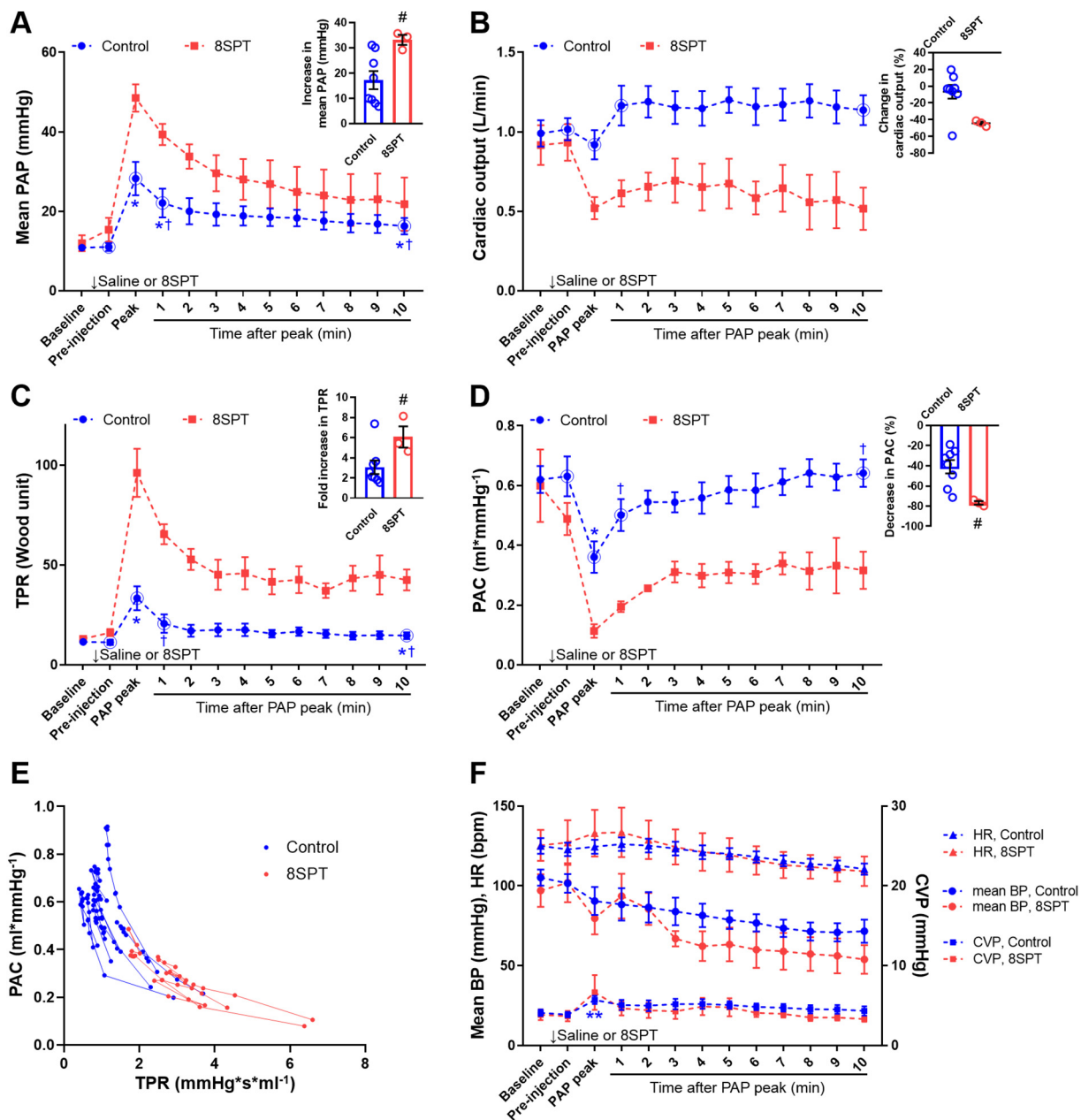


Fig. 2. Hemodynamic changes in the control group and the 8SPT group. The time courses of mean PAP (A), cardiac output (B), TPR (C), PAC (D), the relationship between TPR and PAC (E), mean BP, HR, and CVP (F). Comparisons among time points within the Control group with one-way repeated measures analysis of variance followed by paired t-test with Bonferroni correction were performed using pre-injection, PAP peak, 1 and 10 min after PAP peak data (bordered symbols): ** $P < 0.01$ and * $P < 0.05$ vs. pre-injection and † $P < 0.05$ vs. PAP peak. Plots and connecting lines in E represent the exact values and time courses of each dog from PAP peak to 10 min after. Insets represent quantification of the hemodynamic response by calculating the maximal increase from pre-injection for mean PAP (A) and increase or decrease at PAP peak from pre-injection for cardiac output, TPR, and PAC (B, C, and D). # $P < 0.05$ vs. the Control group, Mann-Whitney U tests. Data are shown as mean $\pm$ standard error of the mean. PAP, pulmonary arterial pressure; TPR, total pulmonary resistance; PAC, pulmonary arterial compliance; BP, blood pressure; HR, heart rate; CVP, central venous pressure; 8SPT, 8-(*p*-Sulphophenyl) theophylline hydrate.

vascular resistance via experimental acute pulmonary embolization. Administration of an adenosine receptor blocker (8SPT) exaggerated the pulmonary vascular resistance elevation. This suggests that endogenous adenosine is increasingly released after acute pulmonary embolization and mediates pulmonary vasodilation.

4.1. Adenosine production following pulmonary embolization

Given the extremely short half-time of adenosine in blood [15], adenosine is assumed to be released locally or from upstream of the sampling site. The significant increase in plasma adenosine levels in the MPA implies two possibilities. First, adenosine was released from the endothelium of the MPA near the sampling catheter. The pulmonary

hypertension induced by the embolization imposes a mechanical stretch on endothelial cells of the pulmonary artery. However, whether mechanical stretch of endothelial cells triggers adenosine production has not been indicated. The second (and more likely) possibility is that adenosine was produced by the right ventricular (RV) myocardium, and released to coronary venous blood and/or directly to blood in the RV cavity. Acute elevation of pulmonary vascular resistance increases RV pressure load, and concomitantly, myocardial oxygen demand rises as RV workload increases to reach optimal RV-pulmonary vascular coupling [16]. On the other hand, oxygen supply to the myocardial tissue is reduced when the elevated RV end-diastolic pressure impairs subendocardial coronary perfusion, leading to subendocardial ischemia [17,18]. Enhanced adenosine formation following significant

myocardial ischemia has been well documented [19,20]. But, some reports suggested that increased ventricular workload alone triggers adenosine formation by the myocardium. Bian et al. demonstrated that RV dialysate adenosine levels increased when RV myocardial oxygen consumption was enhanced by experimentally elevating right coronary pressure in dogs [21]. In a model of ventricular pressure overload, Foley et al. reported that adenosine levels in left ventricular tissue increased during aortic constriction in rats [22]; similarly, during pulmonary arterial constriction, Schwartz et al. observed a decline in the ratio of phosphocreatine to adenosine 5'-triphosphate (PCr/ATP) in RV free wall in pigs [23,24], which also supports an increased adenosine formation. The ratio of PCr/ATP in the working heart is an index of cytosolic ATP phosphorylation potential and thus a reduction in PCr/ATP implies an increase in free ADP [24] that is hydrolyzed into AMP and then adenosine. In these studies ([21–24]), they ruled out tissue ischemia during the observation; thus their results suggested that workload-induced myocardial adenosine formation does not require significant evidence of ischemia. In the present study, acute pulmonary embolization caused RV pressure overload and might have caused RV subendocardial ischemia, overlapping the high plasma adenosine levels in the MPA. Plasma adenosine concentrations in coronary sinus have reportedly increased to several hundreds of nanomolar concentrations even during significant ischemia or hyperemia [25–27]. Our observed adenosine concentrations in the MPA in the current study (32 ± 6 nmol/l) are reasonable after being diluted with systemic venous return which has 20 times the flow of the myocardial circulation.

Another issue is that adenosine levels in the SVC seemed to increase 1 min after injection. We interpret this as blood reflux from the right atrium involved in acute cor pulmonale. The growing disparity in adenosine levels between the MPA and SVC also supports the idea that SVC blood is not a source of adenosine in this experiment.

4.2. Changes in hemodynamics due to 8SPT following pulmonary embolization

The current study demonstrated notable hemodynamic changes in the immediate phase following the embolization. TPR quickly elevated, peaking at about 1 min after injection, and subsequently declined and stabilized, all while maintaining an inverse relationship between PAC. This pattern of events supports that the pulmonary vasculature adapts strategies to maintain low pressure and high flow (pulmonary vascular reserve) [28], such as capillary recruitment and/or active dilation; this is in addition to passive distension which alone decreases PAC, although it remains uncertain whether humoral or neural active factors are involved [29,30]. Restoration or, in some dogs, increase of cardiac output after embolization in the control group might be attributed to inotropic stimulation and the Frank-Starling mechanism associated with sympathetic nerve activity [31,32]. Blocking of the adenosine receptors using 8SPT resulted in an unaltered pattern of pulmonary vasculature adaptation following the early peaking of TPR, thereby suggesting adaptive strategies independent of adenosine, such as capillary recruitment dynamically induced by increased pressure and flow [33]. In contrast, the higher initial peak of TPR with 8SPT suggests that adenosine instantly vasodilates prior to the completion of the adaptive strategies.

As proven by infusion studies, adenosine is a potent pulmonary vasodilatory agent [3,7] and is used clinically for testing vasoreactivity in PAH [8,9]. However, the behavior of endogenous adenosine in pulmonary circulation has not been elucidated. Adenosine deaminase and adenosine receptor antagonists have failed to alter hypoxic pulmonary vasodilatation in isolated lungs or pulmonary arterial rings [34,35], and also failed to alter pulmonary resistance reduction during graded exercise in swine [36]. In this study, in vivo observation and intense focus on the immediate phase of acute embolization clearly indicated an increase in endogenous adenosine. Adenosine causes vasodilation via A_2 receptors in the pulmonary vasculature, which are classified into two subtypes: high-affinity A_{2a} and low-affinity A_{2b} [37]. It is

difficult to determine the exact source of adenosine causing the pulmonary vasodilation in this study, and an insignificant increase in adenosine in the aorta does not preclude paracrine release and consumption of adenosine in the pulmonary bed. However, it should be noted that the A_{2a} receptor potency of adenosine is reported to be 10^{-8} to 10^{-7} M [38]. This suggested that the plasma adenosine concentration observed in MPA ($3.2 \pm 0.6 \times 10^{-8}$ M) might be adequate to activate A_{2a} receptors. Hence, we propose that adenosine is released from the heart in association with RV myocardial energy perturbation in response to a sudden elevation of the pulmonary resistance; this works as the initial pulmonary vasodilator to prevent circulatory collapse and protect the right heart from overload. Future studies should verify this proposed mechanism to further explore the physiology of endogenous adenosine in a relationship between the right heart and pulmonary vasculature, as well as the therapeutic potential of augmentation of endogenous adenosine in the setting of high pulmonary vascular resistance.

4.3. Limitation of the study

There are a few inherent limitations of this study. First, the exact source and trigger for adenosine release were not confirmed. Hemolysis has a potential impact on plasma adenosine concentrations, and some published studies have reported hemolysis in pulmonary embolization with severe pulmonary hypertension [39,40]. According to the hypothesis that increased turbulence of blood via the tricuspid regurgitant jet leads to hemolysis [41], a little hemolysis could occur at the sampling time of 1 min after injection in this study. However, the effect of hemolysis on plasma adenosine level is disputable because damaged erythrocytes might release ATP which is converted to adenosine, or release adenosine deaminase [42,43]. An earlier report showed no relationship between hemolysis and endogenous adenosine concentrations during blood cell incubation [44]. Therefore, at this time, the relationship between hemolysis and plasma adenosine concentration is inconclusive. Microdialysis techniques commonly used to sample extracellular adenosine [38] could be worth considering in future studies. However, for detection in the pulmonary bed, results would strongly depend on sampling sites because embolization induces the heterogeneous distribution of lesions. Secondly, because 8SPT is a non-specific adenosine receptor antagonist, the exact type and subtype of adenosine receptor that attributed to pulmonary vasodilation in this study was not clarified. In a review article published in 2001, Tabrizchi and Bedi described that adenosine receptors that contribute to pulmonary vasodilation in the large pulmonary artery were reported to be of the A_{2b} subtype and those in small arteries appear to be of the A_{2a} subtype [37]. However, in our view, that is still not definitive owing to the limited number of studies up to this point. This issue should be investigated in future studies. Thirdly, adenosine receptor blockade prior to the beads injection might have led to a hypersensitive condition to the subsequent intervention, although we cannot comment on this conclusively with a sample of three dogs. Finally, we ignored left atrial pressure when calculating pulmonary resistance; 8SPT might have affected the coronary circulation in which adenosine is a key mediator of vasodilation under pathological conditions [20,45]. Thus, limiting coronary perfusion might have caused an increase in left atrial pressure via left ventricular dysfunction. However, we believe there was little (if any) increase in left atrial pressure, and it likely did not affect our main findings due to the transient nature of our intervention and measurements.

5. Conclusions

We observed an immediate surge in plasma adenosine levels in the MPA after acute pulmonary embolization. Also, we blocked adenosine receptors and observed an exaggerated initial pulmonary resistance elevation after acute pulmonary embolization. These results demonstrated that endogenously released adenosine is one of the initial

pulmonary vasodilators after acute pulmonary embolization, and led to a hypothesis that adenosine is released by the right heart in response to acute elevation of right heart afterload, causing a pulmonary vasodilatory effect. We suggest that this approach should be tested in future studies to better understand the physiological mechanism and therapeutic potential of endogenous adenosine-mediated pulmonary vasodilation during alteration of pulmonary hemodynamics with right ventricular overload.

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Declaration of Competing Interest

Drs. Takahama, Asanuma, Tsukamoto, Ito have no conflict of interest. Kitakaze reports grants and personal fees from Takeda, during the conduct of the study; grants from Japanese government, grants from Japan Heart Foundation, grants from Japan Cardiovascular Research Foundation, grants and personal fees from Asteras, grants and personal fees from Sanofi, personal fees from Daiichi-sankyo, grants and personal fees from Pfizer, grants and personal fees from Ono, personal fees from Bayer, grants and personal fees from Novartis, personal fees from Bheringer, grants and personal fees from Tanabe-mitubishi, personal fees from Kowa, grants and personal fees from Kyowa-hakko-kirin, personal fees from Dainihon-sumitomo, personal fees from Sawai, personal fees from MSD, grants and personal fees from Abbott, grants and personal fees from Otsuka, grants from Calpis, grants from Nihon Kohden, personal fees from Shionogi, personal fees from Astrazeneca, personal fees from Asahikasei Med., personal fees from Novo nordisk, personal fees from Fuji-film RI, and personal fees from Japan Medical Data, outside the submitted work.

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