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SHORT COMMUNICATION

Phosphoenolpyruvate administration protects ischemia–reperfusion injury in isolated rabbit lungs

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Abstract Phosphoenolpyruvate (PEP) is an intermediate metabolite of the glycolytic pathway and an in vivo high-energy phosphate compound. We have examined the protective effects of PEP on ischemia–reperfusion lung injury in isolated rabbits lungs perfused with a physiological salt solution. The lungs were divided into three treatment groups: (1) ischemia–reperfusion (IR), (2) ischemia–reperfusion with PEP treatment (PEP-IR), in which 1 mM PEP was pre-administered into the perfusate during the stable period, and (3) ventilation–perfusion continued without interruption (Cont). In the IR and PEP-IR groups, ventilation–perfusion was discontinued for about 60 min after a 30-min stable period and then restarted. The capillary filtration coefficients (K_{fc}) and pyruvate concentration in the perfusate were determined immediately before ischemia and 30 and 60 min after reperfusion. The left lungs were dried at the end of the experiment to calculate the tissue wet-to-dry weight ratio (W/D). The K_{fc} values after reperfusion were significantly higher in the IR group than in the other two groups. Pyruvate concentrations were significantly higher at three time-points in the PEP-IR group than in the other two groups. The W/D was significantly higher in the IR group than in the other two groups. Based on these results, we conclude that the administration of PEP prior to lung ischemia alleviates lung ischemia–reperfusion injury.

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The conversion of phosphoenolpyruvate (PEP), an intermediate metabolite of glycolysis, to its final product, pyruvate, generates one molecule of ATP and one molecule of pyruvate per molecule of PEP. High-energy phosphate compounds, such as ATP, do not pass through the cell membrane, but PEP does pass through the erythrocyte membrane, prolonging the lifetime of red cells [1]. It has recently been shown that the storage of isolated livers or kidneys in phosphate-buffered saline (pH 7.4) containing PEP enhances the organ-protecting effects of the solution [2]. Ischemia–reperfusion (IR) lung injury remains a serious clinical problem, particularly after lung transplantation, with the main problem being dysfunction of the pulmonary vascular endothelium, manifested by increased vascular permeability. Here we report the results of our study on the protective effects of PEP on IR injury in isolated rabbit lungs.

The experimental protocol was approved by the Tottori University Laboratory Animal Care Committee. Isolated rabbit lungs were prepared using the method described in detail by Liu et al. [3] with minor modifications. Male white Japanese rabbits weighing 1.3–2.4 kg were used. The lungs were ventilated with 93.5 % air and 6.5 % CO_2 (tidal volume 6 ml/kg; frequency 40/min; positive end-expiratory pressure 2 cm H_2O) and perfused with bicarbonate-buffered physiological salt solution [PSS (in mM): NaCl, 119; KCl, 4.7; $MgSO_4$, 1.17; $NaHCO_3$, 22.61; KH_2PO_4, 1.18; $CaCl_2$, 3.2] in a recirculating manner at a constant flow rate of 30 ml/kg/min. To each 100 ml of PSS stock solution, we added 100 mg dextrose, 20 mU insulin, and 5 g

hydroxyethyl starch (Ajinomoto Pharmaceuticals, Tokyo, Japan). The partial pressure of carbon dioxide (PCO_2) in the perfusate was adjusted to 35–40 mmHg during the experiment by continuous aeration of the reservoir liquid surface with a mixed gas having the same composition as the inspired gas. Gas analysis of the perfusate was performed during the experiment, and a small amount of sodium bicarbonate ($NaHCO_3$) was added to the perfusate to maintain the pH at 7.35–7.45. The lungs were removed en bloc and enclosed in a humidified chamber which was positioned on an electronic scale (GX4000; A and D, Tokyo, Japan) to allow for continuous monitoring of lung weight. The left atrium pressure (P_{LA}) was set at 2 mmHg (referenced at the hilum), and the whole system was equilibrated at 37 °C.

The lungs were divided into three groups. In the Cont group ($n = 6$), the lungs were continuously perfused and ventilated for 120 min after an initial equilibration period. In the IR group ($n = 6$), ventilation and perfusion were interrupted (ischemia) for 60 min after an initial equilibration period, during which time the lungs were maintained in the humidified chamber at 37 °C while airway pressure was maintained at 3.5 cm H_2O by administrating a constant flow of the mixed gas. After 60 min of ischemia, the lungs were reperfused and reventilated for a further 60 min. In the PEP-IR group ($n = 6$), phosphoenolpyruvic acid monosodium salt (Wako Pure Chemical Industries, Osaka, Japan) was added to the perfusate at the start of the equilibration period to a final concentration of 1 mM. Ischemia was then performed for 60 min, followed by 60 min reperfusion and reventilation, similar to the treatment for the IR group.

The pulmonary capillary filtration coefficient (K_{fc}) was determined after 30 min of equilibration and at 30 and 60 min after reperfusion in the IR and PEP-IR groups, and at these same measurement points in the control group. P_{ISO} was measured as described by Pearl et al. [4]: the shunt between the pulmonary and left atrial cannulae was opened and perfusion was discontinued, and changes in lung weight were observed by gradually increasing the reservoir height and pulmonary capillary pressure (P_{PC}) to determine maximum P_{PC} without any increase in the lung weight, i.e., P_{ISO}. The P_{PC} was then changed to the $P_{ISO}+7$ mmHg by rapidly elevating the reservoir to a height corresponding to +7 mmHg, and the reservoir was kept at this height for 7 min. The rate of lung weight gain every minute from 2 to 6 min was then recorded on a semilogarithmic plot and extrapolated to time 0 by linear regression. The logarithm of lung weight gain at time 0 was converted to an antilog, and the resulting value was used to calculate K_{fc}. K_{fc} was normalized using the baseline wet lung weight and expressed in milliliter/minute/millimeter Hg per 100 g lung tissue. The baseline wet lung weight was calculated from the body weight (BW) of the animals using the formula: BW(g) $\times 0.0024$ [5]. Although positive pressure ventilation was interrupted during the K_{fc} determination, a constant flow of the mixed gas was administered at 3 cm H_2O airway pressure.

Samples of perfusate were obtained immediately after 30 min of equilibration and at 30 and 60 min after reperfusion in the IR and PEP-IR groups, and at these same measurement points in the Cont group. Pyruvate concentrations were measured using a clinical chemistry analyzer (JCA-BM8000; Japan Electron Optics Laboratory, Tokyo, Japan). Lactate concentrations were measured using a blood gas analyzer (ABL800 FLEX; Radiometer, Tokyo, Japan).

The left lung was excised at the end of the experiment and its wet weight measured. The left lung was dried at 60 °C under a heating lamp for 2 weeks and its dry weight was measured to determine lung water weight compared with pulmonary tissue weight (W/D) using the formula: W/D = (wet weight − dry weight)/dry weight.

All data are presented as the mean ± standard deviation (SD). Significant differences within each group were determined by a one-way repeated-measures analysis of variance (ANOVA) followed by the post hoc Student–Newman–Keuls test. Differences between groups were tested by a one-way ANOVA followed by the post hoc Tukey's test. Significance was determined at $P < 0.05$.

In the IR group, the K_{fc} value after reperfusion significantly increased compared to the baseline value and was also significantly higher than that of the PEP-IR group (Fig. 1). The W/D was 7.7 ± 0.7 in the Cont group, 8.9 ± 0.9 in the IR group, and 7.5 ± 0.5 in the PEP-IR group, with the W/D value for the IR group being significantly higher than that for the other two groups. The pyruvate concentration had significantly increased for all groups at 60 min after reperfusion, but pyruvate concentration at all measurement points was significantly higher for the PEP-IR group than for the other two groups (Fig. 2). In all groups, the lactate concentration at 60 min after reperfusion was significantly higher than the baseline value, but there were no significant differences among the three groups at any measurement point (Fig. 2).

In our study on the protective effects of PEP on IR lung injury, we found that pretreatment with 1 mM PEP suppressed the increase in K_{fc} at 30 and 60 min after reperfusion and the increase in W/D.

Golbidi et al. conducted in vivo experiments on guinea pigs and demonstrated that pretreatment with 0.2 mM PEP prevented the enhancement in oleic acid-induced pulmonary vascular permeability and the decrease in the partial pressure of oxygen in arterial blood (PaO_2) [6]. These authors histologically examined pulmonary vascular permeability using Evans Blue dye. PEP has been demonstrated to permeate the erythrocyte membrane through a band 3-mediated anion permeation system in accordance

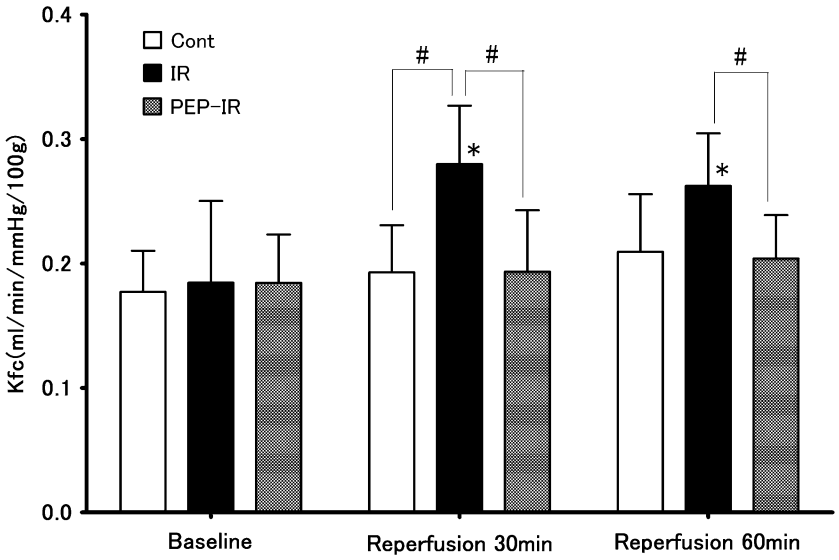

Fig. 1 Changes in the coefficient of filtration (K_{fc}). Data are presented as the mean ±standard deviation (SD) ($n = 6$). $^{*}P < 0.05$ vs. baseline value, $^{\#}P < 0.05$ vs. the ischemia–reperfusion (*IR*) group. *PEP-IR* ischemia–reperfusion with phosphoenolpyruvate (PEP) treatment, *Cont* ventilation–perfusion continued without interruption. For a more detailed description of the treatments, see text

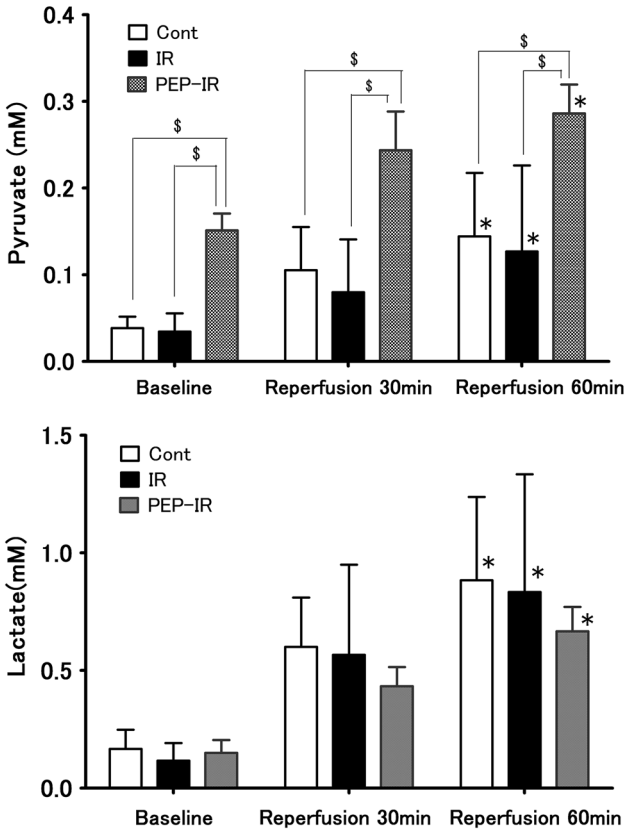

Fig. 2 Changes in pyruvate and lactate concentration in the perfusate. Data are the mean ± SD ($n = 6$). $^{*}P < 0.05$ vs. baseline value, $^{\$}P < 0.05$ vs. PEP-IR group

with the concentration gradient [1]. Band 3 is a major integral membrane protein and is found in all cells. PEP protectively acts against liver and renal ischemia [2].

Intracellular pyruvate and lactate flow into the perfusate, an extracellular area, in accordance with the concentration gradient. Briefly, increases in the perfusate concentrations of pyruvate and lactate may reflect increases in their intracellular levels. PEP is converted to pyruvate by pyruvate kinase (PK), which is present in 98 % of all cells, in an irreversible reaction [7]. In our experiments, PEP may have penetrated the cellular membrane and converted to pyruvate by PK in an intracellular area. A high perfusate concentration of pyruvate may indicate that PEP was utilized for the final step of the glycolytic pathway in an intracellular area.

K_{fc}, measured under the isogravimetric condition, can be reliably and reproducibly determined in isolated rabbit lungs [3–5]. A high K_{fc} value reflects high capillary permeability. In this experiment, K_{fc} also corresponded to W/D.

Under aerobic conditions one molecule of pyruvate enters the TCA cycle, and 19 molecules of ATP are produced. In contrast, under anaerobic conditions, pyruvate is metabolized to lactate, and 1 molecule of ATP is produced. In this study, the levels of adenine nucleotides, such as lung tissue ATP, were not measured, and it is unclear how much of the pyruvate increased by PEP was utilized in the TCA cycle.

As our aim was to investigate the direct effect of PEP on the lung, we did not add red blood cells to the perfusate to avoid an indirect influence of the effect of PEP on red blood cells. Therefore, in the Cont group, tissue hypoxia may have occurred. However, a previous study with isolated rabbit lungs similar to our study showed that there was no increase in the K_{fc} during a 150-min period of observation when neither pulmonary blood flow nor ventilation was interrupted under the conditions of a hematocrit of 0 and inhaled oxygen concentration of 21 % [8].

Oxygen supply to lung tissues can come from both the intravascular and the alveolar space. As oxygen is present in the alveolus, rapid hypoxia does not occur in the lung tissues even if the blood flow into the lung is suddenly interrupted. Fisher et al. reported that the alveolar oxygen tension must be reduced to $\leq$7 mmHg to reproduce IR injury in the isolated rabbit lungs [9]. In our study, the lactate concentrations were similar among all groups at all measurement points, and the perfusate concentrations of lactate were not altered until the alveolar oxygen tension was decreased to 0.7 mmHg [9]. The absence of blood flow into the rabbit lungs may have caused oxidant injury despite the lung energy status. The endothelium appears to be one of the predominant sources of oxidants during lung ischemia [10]. Endothelial cells are highly sensitive to physical forces resulting from blood flow variation [10]. In their study with isolated rabbit lungs, Schütte et al. reported that maintenance of a positive intravascular pressure throughout the ischemic period alleviated postischemic lung injury [8]. Other authors have reported that PEP or pyruvate is able to exert anti-oxidative activity and attenuate cell injury induced by oxidative stress [2, 11]. In our study, PEP may have alleviated IR lung injury through antioxidative mechanisms. PEP may also have reduced IR lung injury by acting not only on the endothelium but also on alveolar epithelial cells. Thus, several issues remain to be examined.

In summary, IR lung injury poses serious clinical problems in lung transplantation. PEP, supplemented to the perfusion preservation solution of donors, may extend the storage periods of organs or increase engraftment rates. Our study shows that pretreatment with PEP alleviated IR lung injury in isolated rabbit lungs.

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