Remote Reperfusion Lung Injury is Associated With AMP Deaminase 3 Activation and Attenuated by Inosine Monophosphate

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**Background** Remote reperfusion lung injury occurs in patients with vascular occlusion and surgical procedures. Inosine monophosphate (IMP) produced by adenosine monophosphate deaminase (AMPD) 3 is involved in the remote reperfusion injury. The purpose of the present study was to identify whether IMP administration attenuated the remote reperfusion lung injury in a skeletal muscle ischemia-reperfusion model.

**Methods and Results** A remote reperfusion lung injury was created using reperfusion after the bilateral ligation of the hind-limb. AMPD activity, myeloperoxidase (MPO) activity, IMP, AMPD3 mRNA and tumor necrosis factor (TNF)-α in the lungs before and after reperfusion were analyzed. Furthermore, the effects of IMP on these parameters were examined. AMPD3 mRNA, AMPD activity and IMP production in the lungs significantly increased after ischemia-reperfusion with increases in MPO activity, TNF-α level and decreased oxygen saturation (SpO2). Histological examination of the lungs demonstrated significant neutrophil infiltration and accumulation. IMP administration significantly reduced MPO activity, TNF-α and neutrophil infiltration, with ameliorated SpO2.

**Conclusions** Along with the activation of AMPD3, ischemia-reperfusion-induced lung inflammation is associated with increased MPO activity and TNF-α level. IMP significantly decreased the lung injury, MPO activity, TNF-α and increased SpO2. These findings may lead to the development of a new therapeutic strategy for remote reperfusion lung injury. (Circ J 2007; 71: 591–596)

**Key Words:** AMP deaminase; IMP; Neutrophil; Remote reperfusion injury; TNF-α

Skeletal muscle ischemia-reperfusion occurs in patients with different vascular diseases and following surgical operations, including cardiopulmonary bypass, revascularization and organ transplantation. It may result in local and multiple organ dysfunction involving the lungs, brain, kidneys and intestines.1,2 In particular, remote lung injury accounts for high morbidity and mortality. Currently, there are no effective intervention strategies for modulating remote organ injury.

The onset of reperfusion stimulates some unidentified circulating mediators that lead to neutrophil activation and recruitment in remote organs.3,4 Neutrophils interact with the vascular endothelium in target organs and then release pro-inflammatory mediators, chemokines and cytokines, causing remote organ inflammation and injury.3,4 Increases in neutrophil infiltration and tumor necrosis factor (TNF)-α production in the lungs, both of which play a central role in organ damage, have been demonstrated in a hind limb ischemia-reperfusion model.5,6 In addition, TNF-α triggers neutrophil infiltration into the lungs and stimulates the release of pro-inflammatory mediators by neutrophils, a phenomenon that was prevented by the TNF-α antibody.5 The anti-adhesion molecule antibodies and depletion of neutrophils from peripheral blood can also attenuate reperfusion-induced injury significantly.7,8 However, there have been few reports regarding treatment for remote reperfusion lung injury.

Adenosine 5’-triphosphate (ATP) is degraded to nucleotides and nucleosides during ischemia and hypoxia. It has been well recognized that adenosine and inosine can exert a powerful regulating effect on tissue inflammation in many systems.9-15 In contrast, inosine monophosphate (IMP) is a naturally occurring nucleotide, formed from adenosine monophosphate (AMP) by AMP deaminase (AMPD); however, the anti-inflammatory properties of IMP have been poorly researched. Because it has been reported that the ischemic reperfusion induced the AMPD3 transcripts and its activity in the lung, IMP can affect the ischemic reperfusion injury on the lung.16

The purpose of the present study was to identify whether pharmacological intervention with IMP attenuated the re-
stroke lung injury in a skeletal muscle ischemia-reperfusion model.

Methods

Skeletal Muscle Ischemia-Reperfusion Model

Male BALB/C mice, aged 6–8 weeks, were randomized to 5 groups (n=4–8, each). After being anesthetized using an intraperitoneal injection with 3.5 mg ketamine and 0.2 mg xylazine, bilateral hind-limb ischemia was created using a Hemorrhoidal Ligator (Miltex Inc, York, PA, USA) at hip region for 3 h. Then, the rubber bands were removed and the lungs were excised before and 0, 3, 6, 24 h after reperfusion. The tissues were immediately placed in liquid nitrogen, then stored at –80°C until assayed. All animals were handled in strict accordance with the Tottori University Guide for the Care and Use of Laboratory Animals.

Determination of AMPD Activity

The lung tissues were homogenized using a homogenate buffer (100 mmol/L potassium phosphate at pH 6.5) and then centrifuged. The supernatant was dialyzed overnight against a dialysis buffer containing 50 mmol/L imidazol HCl, 150 mmol/L KCl and 1 mmol/L dithiothreitol at pH 6.5. The dialyzed samples were incubated with 1 mol/L KCl, 0.125 mol/L imidazol HCl containing 0.1% bovine serum albumin and 25 mol/L AMP at 37°C for 1 h or 2 h, respectively. Each sample was mixed with an equal volume of methanol and then centrifuged at 12,000 rpm and 4°C for 10 min. The supernatant was vacuumed and then dissolved in a high performance liquid chromatography (HPLC) buffer containing 10.0 mmol/L NaH$_2$PO$_4$·2H$_2$O, 0.45 mmol/L tetrabutylammonium phosphate and 1.26 mol/acetonitrile. AMPD activity was measured using HPLC (D-2500 chromatography, Hitachi, Japan) and presented as the change of IMP amount h$^{-1}$ g tissue$^{-1}$.

Transcripts of AMPD2 and AMPD3 Analysis

RNA was isolated from mice lungs using an RNeasy Mini Kit (QIAGEN GmbH, Hiden, Germany). Reverse transcription-polymerase chain reaction (PCR) was performed to generate first-strand cDNA using a ReverTra Ace-_INSTANCE-$TM$ Kit (TOYOBO Ltd, Osaka, Japan). The transcripts were analyzed using real-time PCR (LightCycler Roche Diagnostic, Hiden, Germany). The forward and reverse primers for AMPD2 were 5’-ACCTGCGACCCTGTTGAGAT-3’ and 5’-TTTCCTGCT-TCTCCTGATGAGA-3’, respectively. The results were calculated using LightCycler Software, Version 3.5 (Roche Diagnostic GmbH).

Measurement of IMP

Mice lungs were homogenized with 0.4 mol/L HClO$_4$, placed on ice for 10 min and then centrifuged at 14,000 rpm at 4°C for 10 min. The supernatant was neutralized and stored at –80°C for 2 h. After centrifugation, the IMP in the supernatant was measured using HPLC as described above. The IMP in the blood was also measured using HPLC.

Myeloperoxidase (MPO)

This assay was completed using an Myeloperoxidase Assay Kit (Cytostore, Calgary, Alberta, Canada) and a micro plate reader (Bio-RAD, Tokyo, Japan). The results were expressed as change of absorbance at 450nm units/gram tissue.

TNF-$

TNF-$

in the lungs was assayed using enzyme-liked immunosorbent assay (ELISA) with a commercially available ELISA kit (R&D Systems Inc, Minneapolis, MN, USA). The experiment was performed according to the manufacturer’s instructions. All standards and samples were assayed in duplicate.

Lung Histological Study

The lungs were excised before ischemia and 3 h after reperfusion. After being fixed in 10% formalin overnight at room temperature, the tissues were embedded in OCT compound. Sections (10μm) were collected on microscope slides and then stained with hematoxylin and eosin (HE).

IMP Administration

An Alzet Mini-Osmotic 2001D pump (Alzet, Cupertino, CA, USA) was used to deliver IMP (Sigma, St. Louis, MO, USA) at a constant rate of 1.6 mg/h. The pump was implanted subcutaneously 12 h before inducing skeletal muscle ischemia. As a control, the same volume of saline was administered using the same type of pump.

Measurement of Oxygen Saturation of Hemoglobin (SpO$_2$)

SpO$_2$ was analyzed using pulse oximeter (2000, ATOM medical corporation, Tokyo, Japan) with a slender digit SpO$_2$ sensor (Masimo Corporation, Irvine, CA, USA) fixed.
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A reading that was stable for at least 5 min was recorded. The measurements were performed after 3-h reperfusion following 3-h ischemia with 12-h IMP or control saline administration, respectively.

Statistical Analysis

Data are presented as the mean value±SEM. The statistical significance was determined using one-way analysis of variance (ANOVA), followed by Fisher’s post hoc test as appropriate. A p value of <0.05 was considered statistically significant. All analyses were performed using statistical software (Stat view version 5.0.1, SAS Institute Inc Cary, NC, USA).

Results

Activation of AMPD3 Expression and Increased Endogenous IMP Production Associated With Ischemia-Reperfusion Lung Injury

AMPD activity in the lungs significantly increased after the reperfusion and peaked at 3 h after reperfusion during the first 24-h period; however, the group without reperfusion
did not show any increase in AMPD activity (Fig 1). The transcripts of AMPD3 in lungs also significantly increased after reperfusion, compared to those before ischemia, and peaked at 3 h after reperfusion (Fig 2A), while there was no significant difference between results before ischemia and 3 h after ischemia alone. In contrast, the transcripts of AMPD2, another subtype of AMPD expressed in the lungs, did not change significantly regardless of whether skeletal muscle ischemia-reperfusion was induced or not (Fig 2B). These results suggest that up-regulated AMPD3 transcripts, but not AMPD2, participate in the remote reperfusion lung injury. IMP significantly increased in mice lungs 3 h after reperfusion, and then decreased at 24 h after reperfusion. There were no significant differences in IMP concentrations before ischemia and 3 h after ischemia without reperfusion (Fig 3). MPO was measured in the lungs as an index of neutrophil sequestration and oxidative damage. MPO activity in the lungs significantly increased after reperfusion compared to that before ischemia. No significant difference in MPO activity was observed before ischemia and 3 h after ischemia without reperfusion (Fig 4). HE staining showed that the lungs had a normal architecture before ischemia and no inflammatory cells were found within the alveoli or the alveolar interstitium. Three hours after reperfusion, marked neutrophil infiltration and hemorrhage were exhibited in the alveoli (Figs 5A, B). Since TNF-α production plays a central role in remote reperfusion lung injury, we measured TNF-α in the lungs. TNF-α significantly increased in the lungs at 3 h after reperfusion compared to the control mice (Fig 6).

Effects of IMP on Ischemic Reperfusion Lung Injury
IMP administration did not alter MPO activity before ischemia; in contrast, it significantly suppressed MPO activity 3 h after reperfusion compared to the controls (saline treatment) (Fig 7). IMP administration had no effect on lung histological findings before ischemia but significantly decreased neutrophil infiltration and hemorrhage in the lungs 3 h after reperfusion (Figs 5C, D). The increase of TNF-α in the lungs was remarkably inhibited by IMP treat-
AMPD3.20,21 Qiu et al demonstrated that up-regulated AMPD3 in rodents. Mice lungs contain AMPD2 and that IMP exerted an anti-inflammatory effect in the AMPD3 were involved in the remote reperfusion injury suggested that purine metabolism and the activation of following 3 h ischemia.

In addition, we measured SpO2 before and after ischemia-reperfusion with IMP or saline vehicle administration to evaluate the clinically relevant parameters. In the control mice, skeletal muscle ischemia-reperfusion decreased SpO2: after 3 h reperfusion compared with that before ischemia (before ischemia: 95.8%, after reperfusion: 76.3%, p<0.001). IMP treatment significantly ameliorated the decreased SpO2: after 3 h reperfusion compared to saline control administration (IMP administration: 88.8%, p<0.001 vs control).

**Discussion**

The purine metabolism pathway is involved in regulation of tissue inflammation. ATP is progressively degraded to nucleotides and nucleosides under ischemia or hypoxia. The major metabolic event during the ischemic period is salvage of the nucleotides such as IMP. However, the role of the purine nucleotide pathway and the metabolites that participate in the regulation of reperfusion injuries remain unknown. In the present study, skeletal muscle ischemia-reperfusion led to remote lung inflammation, which was characterized by increased MPO activity and significant neutrophil infiltration in the lung. The injury was also associated with an elevation of TNF-α and AMPD activity. Furthermore, IMP administration significantly attenuated the lung inflammation, decreased SpO2: and suppressed MPO activity and TNF-α. These results suggested that purine metabolism and the activation of AMPD3 were involved in the remote reperfusion injury and that IMP exerted an anti-inflammatory effect in the injury.

There are 3 AMPD subtypes: AMPD1, AMPD2 and AMPD3 in rodents. Mice lungs contain AMPD2 and AMPD3. Qiu et al demonstrated that up-regulated AMPD3 gene expression was identified in bronchial and alveolar epithelium, using in situ hybridization in the lungs that underwent limb ischemia-reperfusion, and that coformycin, a nonspecific AMPD inhibitor, decreased AMPD activity and the production of IMP, resulting in an enhanced the remote lung injury. However, they did not analyze AMPD2 transcripts. In the present study, AMPD3 transcripts and AMPD activity increased in the lungs after the reperfusion. In contrast, AMPD2 transcripts did not increase after the reperfusion. These results indicate that AMPD3, but not AMPD2, is involved in remote reperfusion lung injury and that the up-regulated AMPD3 expression is likely to account for the elevation of AMPD activity. We cannot exclude the possibility that the increased AMPD activity is mediated via a post-transcriptional mechanism because the AMPD3 mRNA and AMPD activity increased at the same time. Further investigation will be needed to clarify the relationship between AMPD3 expression and AMPD activity.

Ischemia alone without reperfusion did not alter the activity of MPO or the histopathological findings in the lungs significantly, nor did it alter AMPD3 activity and IMP. These results suggest that the skeletal muscle reperfusion following the ischemia, in the case of this study, induces the specific transcripts of AMPD3 and IMP production. Thus, ischemia-reperfusion plays a pivotal role in the development of the remote lung injury, although the factors triggering the up-regulation of AMPD3 transcripts and lung injury by the reperfusion are still unknown.

The mechanism for remote reperfusion lung injury is still unclear. Mouse experiments showed that the trafficking of neutrophils was inhibited by IMP and IMP acted on neutrophils to reduce rolling in microvessels in vitro, thereby restricting the entry of neutrophils into tissue. A previous study showed that IMP also suppressed TNF-α production in mice peritoneal macrophages. In a rat skeletal muscle ischemia-reperfusion model, TNF-α was found to be strongly implicated in systemic inflammation, including lung injury, by evoking neutrophil infiltration in the lungs. Furthermore, ischemia-reperfusion induces a marked increase of TNF-α that in turn increases pulmonary endothelial permeability. In the present study, TNF-α increased after reperfusion and decreased with IMP administration, resulting in changes in MPO activity, neutrophil infiltration and SpO2. Taken together, it is possible that skeletal muscle ischemia-reperfusion induces TNF-α production, leading to lung inflammation, and that IMP administration ameliorates the lung injury via the suppression of TNF-α.

Pharmacological modulation of the remote lung injury induced by skeletal muscle ischemia-reperfusion may im-

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**Fig 6.** Tumor necrosis factor (TNF-α) in the lungs administered inosine monophosphate (IMP) or saline. n=7 or 8. *p<0.001 vs before ischemia. †p<0.01 vs before ischemia.

**Fig 7.** Myeloperoxidase (MPO) activity in lungs administered inosine monophosphate (IMP) or saline. n=4 or 5. *p<0.05 vs saline administration after 3 h reperfusion following 3 h ischemia.
prove the mortality rate after surgery and permit longer tourniquet application periods. The administration of IMP prior to ischemia maintains a high concentration of IMP during ischemia. In the present study, constant administration of IMP also resulted in a significant elevation of plasma IMP concentration (before IMP administration: 0.035 mmol/L, after IMP administration: 0.2 mmol/L, p < 0.05) and ameliorated neutrophil infiltration and reduced MPO activity and TNF-α production in the lungs, with amelioration of decreased SpO2. We acknowledge the limitations of the present study because we did not evaluate the effects of AMPD inhibition and endogenous IMP production on the remote lung injury. However, our findings are consistent with a report that the administration of an AMPD inhibitor (coformycin) significantly decreased AMPD activity and IMP production, enhancing lung injury. Further studies using AMPD knockout mice are necessary to clarify the roles of IMP and AMPD in the reperfusion injury, because coformycin is not a specific AMPD inhibitor. Taken together, IMP administration may be a potential pharmacological therapy for the remote reperfusion lung injury, while some intervention strategies, such as inhibitors like arachidonic acid and lipid peroxidation metabolites, have some limitations because of their side effects and toxicity.

In conclusion, AMPD3 and IMP play a pivotal role in the remote reperfusion injury. Our findings suggest that AMPD3 and IMP are potential candidates for a pharmacological therapeutic strategy for remote reperfusion lung injury.

References