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AMP deaminase 3 plays a critical role in remote reperfusion lung injury

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ABSTRACT

Remote reperfusion lung injury following skeletal muscle ischemia and reperfusion accounts for high morbidity and mortality. AMP deaminase (AMPD), a key enzyme for nucleotide cycle, has been implicated in the regulation of this phenomenon. However, the function of Ampd2 and Ampd3 subtype has not been elucidated in remote reperfusion rodent lung injury. We utilized AMPD3 and AMPD2-deficient mice. The two types of AMPD-deficient mice and wild-type (WT) littermates were subjected to ischemia–reperfusion injury. After 3 h bilateral hind-limb ischemia and reperfusion, AMPD3 mRNA, AMPD activity and inosine monophosphate (IMP) increased significantly in WT and AMPD2-deficient mice lungs, while they did not show significant alterations in AMPD3-deficient mice lungs. Genetic inactivation of Ampd3 resulted in markedly accelerated myeloperoxidase (MPO) activity along with exaggerated neutrophils infiltration and hemorrhage in the lungs compared to WT and AMPD2-deficient mice, furthermore, IMP treatment significantly attenuated MPO activity and neutrophils infiltration in WT and the two types of AMPD-deficient mice lungs after 3 h reperfusion. These findings demonstrate for the first time in AMP-deficient mice models that AMPD3 plays a critical role in remote reperfusion lung injury via generation of IMP and validate the potential to use IMP into the clinical arena to attenuate remote ischemia–reperfusion lung injury.

1. Introduction

Skeletal muscle ischemia–reperfusion happens to many morbid situation and surgical procedures, including aortic bypass, organ transplantation with tourniquet [1]. Skeletal muscle reperfusion provokes aberrant neutrophils accumulation within the lungs, brain, kidney and liver [2,3]. Neutrophils release a cascade of pro-inflammatory mediators, chemokines and cytokines by interacting with vascular endothelium resulting in remote organs inflammation and injury, which has been called multiple organs dysfunction syndrome [4,5]. Among these damages, remote lung inflammation plays a pivotal factor that is responsible for high mortality and influences outcome. The lung injury is characterized by severe neutrophils sequestration and increased pulmonary vascular permeability even though, what factors stimulate neutrophils activation is still unknown [5,6]. Prevention of neutrophil accumulation and sequestration within lung becomes a key step to minimize the lung injury [4,5]. Currently there are no effective intervention strategies for this setting, although conventional intensive care support has existed.

Purine metabolism pathway is linked to regulate tissue inflammation. For example, IMP, inosine and adenosine have shown anti-inflammatory and tissue protective properties in inflammatory responses to ischemia–reperfusion injury and endotoxin exposure [7–9]. Adenosine 5′-triphosphate (ATP) is degraded into adenosine monophosphate (AMP) progressively, which is converted by AMP deaminase to form IMP, and other nucleotides in the tissues during episodes of tissue ischemia and hypoxemia [10]. It was reported that transcripts of AMPD3 gene (Ampd3) and its enzymatic activity increased in lungs after skeletal muscle ischemia and reperfusion; furthermore, coformycin inhibited AMPD activity and IMP production causing enhanced remote reperfusion lung injury [11]. However, coformycin is a nonspecific AMP deaminase inhibitor which also hampers adenosine deaminase activity to change levels of several nucleosides [12]. Besides, lungs of rodent contain Ampd2 and Ampd3 which govern the activity of AMP deaminase [13]. Genetic

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efforts of Ampd3 and Ampd2 in remoter lung injury processes seen in skeletal muscle ischemia–reperfusion have not been well examined. To elucidate the role of AMPD subtypes genetically, we analyzed AMPD3 and AMPD2-deficient mice to clarify the genetic function of the two subtypes AMPD in remote reperfusion lung injury model.

In current study, we found that only Ampd3 disruption exaggerated remote reperfusion lung injury with low levels of AMPD activity and IMP within lungs after skeletal muscle reperfusion. The inflammation of lungs was significantly attenuated by IMP treatment. The results indicate that Ampd3 plays a pivotal role and IMP protects lungs from inflammation in this setting.

2. Materials and methods

2.1. Mice

AMPD3 and AMPD2-deficient mice were generated and genotyped as described [14,15]. The primers were used to track AMPD2 or AMPD3 gene disruption as shown in Table 1. All mice were on C57BL/6J background and handled in strict accordance with the Tottori University Guide for the Care and Use of Laboratory Animals.

2.2. Murine skeletal ischemia–reperfusion model

C57BL/6J mice were obtained from Japan SLC (SIZUOKA, Japan) as WT and AMPD2 and AMPD3-deficient mice were received from National Cardiovascular Center Research Institute (Osaka, Japan). Three kinds of mice, male and aged 6–8 weeks, were randomized to 3 groups ($n = 4–8$) after being anesthetized by intraperitoneal injection with 3.5 mg Ketamine and 0.2 mg Xylazine. Bilateral hind-limb ischemia was created by Hemorrhoidal Ligator (Miltex, INC, York, PA USA) at hip region for 3 h. The rubber bands were removed and lungs were excised before and 0, 3 h after reperfusion, respectively. Lungs were placed in liquid nitrogen immediately then stored at −80 °C for investigation.

2.3. RT-PCR and real-time quantitative RT-PCR

Total RNA was isolated from mice lung using RNeasy Mini Kit (QIAGEN GmbH, Hiden, Germany). Reverse transcription (RT)-PCR was performed to generate first strand cDNA by ReverTra Ace-α-TM Kit following manufacturer's instructions (TOYOBO, Ltd., Osaka, Japan). Alternatively, the transcripts levels were quantified by real-time quantitative RT-PCR (Light Cycler Roche Diagnostic, Hiden, GmbH, Germany). The primers are shown in Table 2. The results were calculated by the LightCycler Software Version 3.5 (Roche Diagnosatic GmbH).

2.4. 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 DTT at pH 6.5. Dialyzed samples were incubated with 1 mol/L KCl, 0.125 mol/L imidazol HCl containing 0.1% BSA and 25 mol/L AMP at 37 °C for 1 h or 2 h, respectively. Each sample was mixed with an equal volume of MeOH and then centrifugation at 12,000 rpm and 4 °C for 10 min. The supernatant was vacuumed and then solubilized in HPLC buffer containing 100 mmol/L $NaH_2PO_42H_2O$, 0.45 mmol/L tetrabutylammonium phosphate and 1.26 mol/lacetonitrile. AMPD activity was measured by high performance liquid chromatography (HPLC: D-2500 chromatography, Hitachi, Japan) and presented by the change of IMP amount per hour, per gram tissue.

Table 1
Sequence of the RT-PCR product for APD2 and AMPD3.

AMPD2	Forward: 5′-AGATGCTGGAGAACATCTTTCTG-3′; Reverse: 5′-GATGCTGTACTCCTCCATCAGG-3′;
AMPD3	Forward: 5′-ATCACTCACCTAGTGTCTGCCTTC-3′ Reverse: 5′-CAGGTACTCAGCTTCCACACTTG-3′

Table 2
Sequence of the real time PCR product for AMPD2, AMPD3 and β-actin.

AMPD2	Forward: 5′-GCTCAGCTCCTGCGATATGTG-3′ Reverse: 5′-GCTTTTCACCTTGTGAGAGAAG-3′
AMPD3	Forward: 5′-ACCTGCGACCTGTGTGAGAT-3′ Reverse: 5′-CTTCTGCTTCTCCTGATGTGAGA-3′
β-Actin	Forward: 5′-CAACCGTGAAAAGATGAC-3′ Reverse: 5′-CAGGATCTTCATGAGGTAGT-3′

2.5. 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, IMP in the supernatant was measured by HPLC as described above.

2.6. Myeloperoxidase

This assay was completed by 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 450 nm units per gram tissue.

2.7. Lung histological study

Lungs were excised before ischemia and 0 h, 3 h after reperfusion. After being fixed in 10% formalin overnight at room temperature, the tissues were embedded in O.C.T. compound. Sections (10 μmol/L) were collected on microscope slide and then stained with hematoxylin and eosin.

2.8. IMP administration

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

2.9. Statistical analysis

Data are presented as the mean value ± SEM. Statistical significance was determined by 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.).

3. Result

3.1. Verification of AMPD2 and AMPD3-deficient mice

A targeting construct was generated to inactivate the AMPD2 and AMPD3 gene by inserting puromycin between exon 14 and

exon 16 and neomycin within exon 13, respectively. Successful genomic integration of mutant alleles was determined by PCR. A 572-bp product for AMPD2 was detected in WT and AMPD3-deficient mice lungs but it was undetectable in AMPD2-deficient mice (Fig. 1A). WT and AMPD2-deficient mice lungs presented a 296-bp bands products of AMPD3 but it was absent in AMPD3-deficient mice (Fig. 1B).

3.2. Activation of AMPD3 and elevated endogenous IMP production associated with skeletal muscle ischemia–reperfusion

The transcripts of Ampd3 in AMPD2-deficient and WT mice lungs significantly increased about 3–4-fold after 3 h reperfusion compared to before ischemia but remained at base line in AMPD3-deficient mice (Fig. 2A). There were no significant differences of the transcripts among WT, AMPD2-deficient and AMPD3-deficient before ischemia, while AMPD3-deficient mice exhibited a significant differences compared to WT and AMPD2-deficient mice 3 h after reperfusion. On the other hand, skeletal muscle ischemia–reperfusion did not enhance the transcripts expression of Ampd2 in WT, AMPD2 and AMPD3-deficient mice lung. No significant differences were found among WT, AMPD2 and AMPD3 before ischemia and after reperfusion (Fig. 2B). These results strongly indicate only Ampd3 participates in remote reperfusion lung injury setting. AMPD activity elevated significantly in WT and AMPD2-deficient mice lungs that underwent 3 h skeletal muscle ischemia with 3 h reperfusion compared with before ischemia and 3 h ischemia alone. However, the augmented AMPD activity was not induced in AMPD3-deficient mice lung. AMPD activity in AMPD2-deficient and AMPD3-deficient lungs before ischemia was lower than WT but no significant differences were existed. (Fig. 2C) These results suggested up-regulated AMPD3 gene plays a functional role in this setting. IMP, an unique product of AMPD, increased remarkably in WT and AMPD2-deficient mice lung after 3 h reperfusion but was unable to increase until 24 h reperfusion in AMPD3-deficient mice lung (data not shown). Amount of IMP in AMPD2 and AMPD3 mice lungs represented at almost same level before ischemia, although they were lower than WT but no significant differences were detected (Fig. 2D). Ischemia alone did not alter Ampd3 transcripts, AMPD activity and IMP in WT, AMPD2 and AMPD3 deficient mice lungs (Fig. 2A, C and D). These data demonstrate that inactivated Ampd3 is responsible for the low level of IMP in lungs after reperfusion and reperfusion triggers these events.

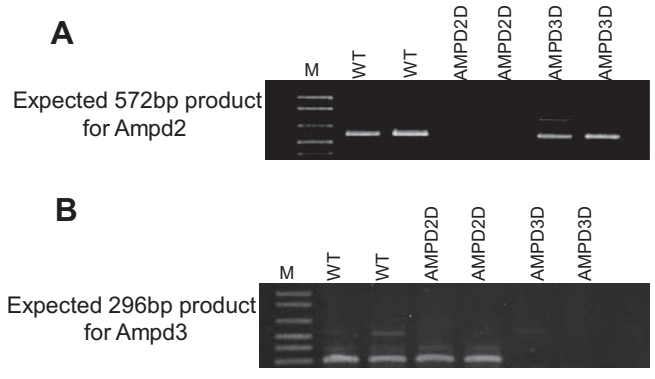

Fig. 1. Ampd2 and Ampd3 expression in WT, AMPD2 deficient (AMPD2D) and AMPD3 deficient (AMPD3D) mice lungs. Finding from 2 different pairs of littermates are shown. M, DNA size ladder. (A) The expected Ampd2 products in WT and AMPD3 deficient mice are indicated. No same products were detected in AMPD2 deficient mice lung. (B) Ampd3 products were found in WT and AMPD2 deficient mice but lack in AMPD3 deficient mice lung. $n = 2$ respectively.

3.3. Remote reperfusion lung injury

Significantly increased MPO activities were found within WT and AMPD2-deficient mice lungs after 3 h reperfusion compared to before ischemia and ischemia alone, respectively. There were no significant differences between WT and AMPD2-deficient mice before ischemia and after reperfusion. MPO activity in AMPD3-deficient mice lungs accelerated markedly after 3 h reperfusion compared to WT and AMPD2-deficient mice (Fig. 3A). MPO activities were not shown alterations in ischemia alone groups among WT, AMPD2 and AMPD3 deficient mice lung. Histological analysis of hematoxylin and eosin-stained sections of the lungs showed that before ischemia lungs of WT, AMPD2-deficient and AMPD3-deficient had normal architecture and no inflammatory cells were found within alveoli and the alveolar interstitium (Fig. 3B-a,e,i). There were no detectable alterations relative to ischemia alone (Fig. 3B-b,f,j). After 3 h reperfusion, the alveoli in WT and AMPD2-deficient mice lung showed a large number of neutrophil infiltration and light hemorrhage; moreover, AMPD3-deficient mice exhibited much more exaggerated inflammation by accumulation of huge number of neutrophils and red blood cells within lungs (Fig. 3B-c,g,k).

3.4. IMP administration significantly attenuated remote reperfusion injury

IMP treatment significantly suppressed the increased MPO activity after 3 h reperfusion in WT, AMPD2-deficient and AMPD3-deficient mice lungs compared to saline administration as a vehicle (Fig. 4). Neutrophils and red cells infiltration in WT, AMPD2-deficient and AMPD3-deficient mice lungs were significantly attenuated by IMP administration (Fig. 3B-d,h,l). However, IMP administration had no influence in lung histological findings (Supplementary Fig. 1) and MPO activity before ischemia in three types of mice.

4. Discussion

We present evidence that after skeletal muscle ischemia–reperfusion the transcripts of Ampd3, AMPD activity and IMP in WT and AMPD2-deficient mice lungs increased significantly and exhibited an increased MPO activity in lungs with neutrophil and red cell infiltration within lungs. Inactivation of AMPD3 gene (Ampd3) diminished the elevation of AMPD activity and IMP production in AMPD3-deficient mice lung after 3 h reperfusion, furthermore, accompanied a significantly enhanced MPO activity and exaggerated inflammation in lungs compared to WT and AMPD2-deficient mice. LDH in WT, AMPD2-deficient and AMPD3-deficient mice blood increased significantly in ischemia–reperfusion groups after 3 h reperfusion compared with before ischemia but there were no significant differences between the three types of mice (data not shown). These data suggests that the exaggerated remote reperfusion lung injury in AMPD3-deficient mice was caused by AMPD3 inactivation and it was not related to different level of muscle damage.

AMPD gene family has 3 subtypes in rodent animals, including Ampd1 (skeletal muscle type), Ampd2 (liver type), and Ampd3 (heart type) [16]. Mice lung tissues dominantly express Ampd2 and Ampd3. Ampd2 contributes the majority of AMPD activity [17] and Ampd3 is expressed low levels in healthy lung [11]. In order to determinate whether the increase of Ampd3 transcript is specific, we examined the transcripts of Ampd2 in mice lungs in this setting. Skeletal muscle ischemia–reperfusion did not generate the significant increase of AMPD2 transcripts in WT and AMPD3-deficient mice lung compared to before ischemia. On the other

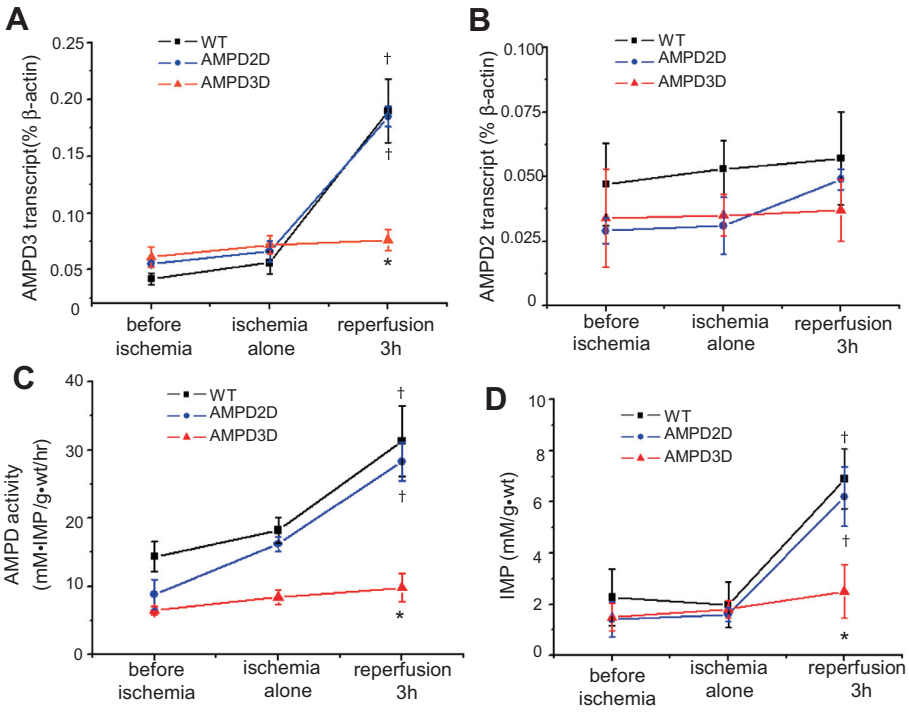

Fig. 2. Ampd transcripts and enzymatic activity in mice lungs. (A) The level of Ampd3 in WT, AMPD2 and AMPD3 deficient mice lungs. $n = 4–6$. $^{\dagger}p < 0.01$ vs before ischemia and ischemia alone, $^{*}p < 0.01$ vs WT and AMPD2 deficient mice after 3 h reperfusion. There was no significant differences between WT and AMPD2 deficient mice after 3 h reperfusion. (B) Transcripts level of Ampd2 in WT, AMPD2 and AMPD3 deficient mice lungs. There were no significant differences among the 3 types of mice lungs before ischemia, ischemia alone and after reperfusion. $n = 4$. (C) AMPD activity in WT, AMPD2 and AMPD3 deficient mice lungs. $n = 4–5$. $^{*}p < 0.01$ vs WT and AMPD2 deficient mice after 3 h reperfusion, respectively; $^{\dagger}p < 0.01$, <0.05 vs WT and AMPD2 deficient mice before ischemia and ischemia lone, respectively. There were no significant differences in WT, AMPD2 deficient and AMPD3 deficient mice before ischemia and no significant difference was found between WT and AMPD2 deficient after 3 h reperfusion. (D) IMP in WT, AMPD2 and AMPD3 deficient mice lungs. $n = 4–6$. $^{*}p < 0.05$ vs WT or AMPD2 deficient mice after 3 h reperfusion; $^{\dagger}p < 0.01$ vs WT and AMPD2 deficient mice before ischemia. There were no significant differences among the two kinds of AMPD deficient mice and WT before ischemia and ischemia alone.

hand, skeletal muscle ischemia–reperfusion triggered Ampd3 transcripts increased significantly in AMPD2-deficient mice lungs as WT mice. Ischemia alone without reperfusion was unable to alter Ampd3 expression. AMPD3 transcripts were identified with located in bronchial and alveolar epithelium by in situ hybridization [11]. These results indicate that increased transcripts of Ampd3, not Ampd2, is involved in remote reperfusion lung injury and reperfusion switches on these events.

AMPD activity and IMP production increased significantly in WT and AMPD2-deficient mice lungs after reperfusion but remained lower level in AMPD3-deficient with a significantly exaggerated lung injury, although, these were no significant differences among three types of mice before ischemia. IMP in blood and hind-limb muscle did not alter after ischemia–reperfusion compared with before ischemia [11]. These evidences demonstrate that increased Ampd3 transcripts play a functional role and IMP may protect lung from injury. What factors stimulate Ampd3 transcripts up-regulated is remained to be elucidated.

The purine metabolism pathway is involved in regulation of tissue inflammation. Under ischemic condition, ATP is mainly degraded by AMPD to form IMP which is called IMP pathway [17]. The level of IMP in lungs is consistent with AMPD activity in lungs and the degree of lung inflammation. Disruption of Ampd3 gene induced a significantly decreased AMPD activity and IMP production in lungs accompanied a remarkable MPO elevation and accelerated neutrophil infiltration within lungs. These evidences revealed a clear correlation between IMP level and lung inflammation. Constant administration of IMP increased the level of IMP in blood (data not shown) resulting in a significant amelioration of MPO in WT, AMPD2 and AMPD3-deficient mice lungs and an attenuated lung inflammation that were identified by histological study. The major metabolic event during the ischemic period is salvage of the nucleotides pool such as IMP. Administration of IMP prior to ischemia maintains the high concentration of IMP during ischemia. Therefore, the depletion of ATP in tissue is slowed down thus supplying a pool of ATP precursors at the reperfusion [18].

Mice experiment showed that IMP acts on neutrophils to inhibit their infiltration and accumulation *in vivo* and reduces rolling in microvessels in a dose-dependent fashion [11]. It is likely that IMP modulates the function of epithelial to spare the neutrophil penetration into alveoli [11]. Previous study showed that TNF-α production in mice peritoneal macrophages was decreased by IMP [19,20]. TNF-α has been strongly implicated in remote lung injury associated with ischemia–reperfusion by evoking neutrophils infiltration to increase pulmonary endothelial permeability [18,22]. In mice skeletal muscle ischemia–reperfusion model, IMP administration significantly suppressed TNF-α production in lungs resulting in an attenuated lung inflammation [21]. Taken together, we clearly demonstrate that skeletal muscle reperfusion stimulates the transcripts Ampd3 up-regulate and its enzymatic product IMP increases thus protecting lung from remote reperfusion injury. The pathway about anti-inflammation of IMP is remained to be further investigated.

In conclusion, current study has demonstrated that Ampd3, not Ampd2 increased in lungs after skeletal muscle ischemia–reperfusion specifically and elevated AMPD activity and IMP play an anti-inflammatory function in this setting. Manipulation these pathway may lead a new approach to modulate remote reperfusion lung injury.

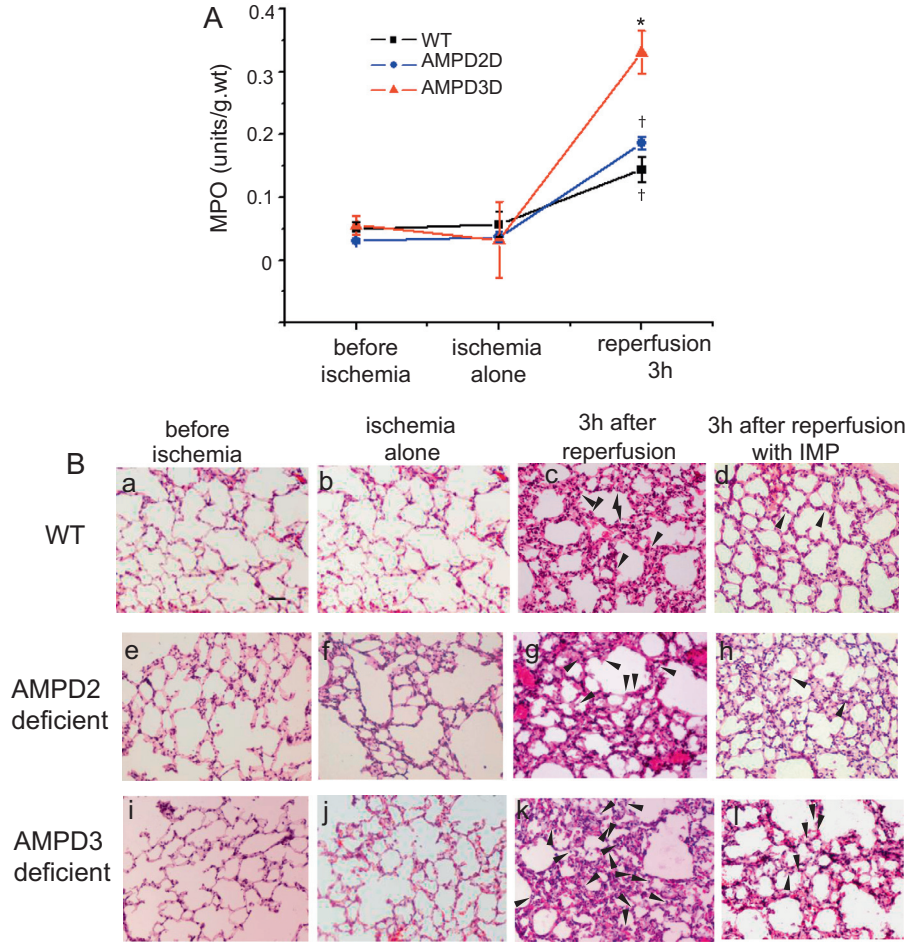

Fig. 3. MPO activity and histological study in WT, AMPD2 and AMPD3 deficient mice lungs. (A) MPO activity in WT, AMPD2 and AMPD3 deficient mice lung. $n = 4–6$. $^{\dagger}p < 0.01$ vs WT or AMPD2 deficient mice before ischemia and ischemia alone, respectively. $^{*}p < 0.01$ vs AMP3 deficient before ischemia and WT and AMPD2 deficient mice after 3 h reperfusion. There were no significant differences between WT and AMPD2 deficient mice before ischemia and after reperfusion. (B) Hematoxylin and eosin staining of representative lung section. (a) (e) (i) before ischemia of WT, AMPD2 and AMPD3 deficient mice, respectively. (b) (f) (j) ischemia alone of WT, AMPD2 deficient and AMPD3 deficient mice, respectively. (c) (g) (k) after 3 h reperfusion and (d) (h) (l) after 3 h reperfusion with IMP administration in WT, AMPD2 deficient and AMPD3 deficient mice lung, respectively. Scale bar: 100 μm. Arrows denote neutrophils.

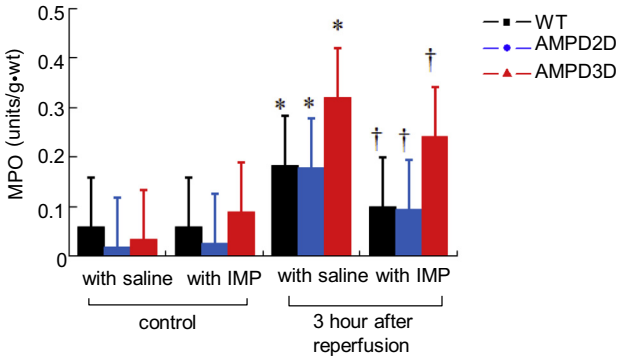

Fig. 4. MPO activity in WT, AMPD2 deficient and AMPD3 deficient mice lungs with vehicle (saline) or IMP treatment. $n = 4–6$. $^{*}p < 0.01$ vs before ischemia with vehicle treatment, respectively; $^{\dagger}p < 0.05$ vs after 3 h reperfusion with vehicle administration. No significant differences existed between vehicle and IMP administration before ischemia.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.056.

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