Targeting oxygen sensing prolyl hydroxylase (PHD) for metformin-associated lactic acidosis treatment

Running head: PHD inhibitor for lactic acidosis treatment

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Abstract: Metformin is one of the most widely used therapeutics for type 2 diabetes mellitus and also has anti-cancer and anti-aging properties. However, it is known to induce metformin-associated lactic acidosis (MALA), a severe medical condition with poor prognosis, especially in individuals with renal dysfunction. Inhibition of prolyl hydroxylase (PHD) is known to activate transcription factor HIF (hypoxia-inducible factor) that increases lactate efflux as a result of enhanced glycolysis, but it also enhances gluconeogenesis from lactate in the liver that contributes to reducing circulating lactate levels. Here we investigated the outcome of pharmaceutical inhibition of PHD in mice with MALA induced through the administration of metformin *per os* and an intraperitoneal injection of lactic acid. We found that the PHD inhibitors significantly increased the expression levels of genes involved in gluconeogenesis in the liver and the kidney, and significantly improved the survival of mice with MALA. Furthermore, the PHD inhibitor also improved the survival rate of MALA aroused on chronic kidney disease (CKD) mice. Thus, PHD represents a new therapeutic target for MALA, which is a critical complication of metformin therapy.

Introduction

Hypoxic response is mainly regulated by heterodimeric transcription factor HIF (hypoxia-inducible factor) composed of stable β-subunit (HIFβ/ARNT) and labile α-subunit (HIFα). Protein expression level of HIFα is negatively regulated by prolyl hydroxylase PHD1–3 (prolyl hydroxylase domain-containing protein 1–3). In normoxic conditions where oxygen is available, PHDs hydroxylate proline residues on HIFα targeted for von Hippel–Lindau (VHL) E3 ubiquitin ligase-dependent proteasomal degradation. On the other hand, enzymatic activities of PHDs are inhibited under the hypoxic conditions where available oxygen becomes limited, as PHDs are 2-oxoglutarate-dependent dioxygenases which require molecular oxygen for their enzymatic activities. Thus, HIFα escapes from prolyl hydroxylation-dependent protein degradation, accumulates, binds to HIFβ/ARNT and activates transcription of hypoxic mRNA including *Epo* (erythropoietin) or *Vegf* (vascular endothelial growth factor) under the hypoxia. (1). We previously reported that liver-specific inactivation of *Phd2*, the dominant prolyl hydroxylase for HIFα, improved the survival rate of mice with lactic acidosis by activating hepatic gluconeogenesis from circulating lactate, which contributed to reducing the blood lactate level (2).

Metformin is a biguanide drug that reduces blood glucose levels by suppressing hepatic gluconeogenesis. However, it is also known to induce metformin-associated lactic acidosis (MALA) (3), which is particularly prevalent in patients with chronic kidney disease (CKD) due to a decrease in urinary excretion of lactate. MALA has a high mortality rate of up to 50%, but only early diagnosis and renal replacement therapy are currently the treatment of choice (4). Therefore, an established treatment is required for overcoming such circumstances and we hypothesized that PHD inhibitors could be used to improve the survival rate of mice with MALA.

69 In the present study, we investigated the effects of PHD inhibitors on mice with MALA. We
70 found that treatment with PHD inhibitors *per os* improved the survival rate of mice with
71 MALA and also rescued MALA in CKD mice, which represents a more clinical model.
72 These findings indicate that PHD represents a new therapeutic target for MALA.

Materials and Methods

Mice

All experiments were approved by the Animal Care and Utilization Committee of the Keio University School of Medicine, Tokyo, Japan (#11050-2). Wild-type mice used in this study were C57BL/6J male mice aged 7–13 weeks, which were obtained from CLEA Japan, Inc. (Tokyo, Japan). All *Phd2* flox/flox ($Phd2^{F/F}$) mice in this study were backcrossed to C57BL/6J strain at least five times, as previously reported (5). The transgenic mice expressing albumin promoter-driven Cre-recombinase (*Alb-Cre*) were also described previously (1, 6, 7). The mice were maintained on a 12:12 hour light–dark cycle in a specific pathogen-free facility and were fed *ad libitum*.

Development of a mouse model of MALA

Nine-week-old wild-type C57BL/6J male mice were administered metformin (0.25 mg/g body weight) dissolved in distilled water in the morning and evening of day 1, and in the morning of day 2, *per os*. After 4 hours, the mice were then administered an intraperitoneal (i.p.) injection of lactic acid (0.4 mg/g body weight; 252476; Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% sodium chloride.

RNA isolation and real-time reverse transcription (RT)-PCR analysis

Total RNA was isolated from the livers, kidneys, muscles and hearts of each mouse using TRIzol reagent (15596-026; Invitrogen, Carlsbad, CA) and an RNeasy column (74104; Qiagen, Hilden, Germany). Real-time RT-PCR was performed with an AffinityScript QPCR cDNA Synthesis Kit (600559; Agilent Technologies, Santa Clara, CA) and THUNDERBIRD SYBR qPCR Mix (QPS-201; TOYOBO, Osaka, Japan) using a real-time PCR system (Model

7300; Applied Biosystems, Foster City, CA). The mRNA levels were normalized to β-actin (*Actb*). The primer sequences are shown in **Table 1.**

Venous blood gas analysis

Eight-week-old wild-type C57BL/6J male mice administered either the vehicle or REC2923 were anesthetized with isoflurane (AbbVie Inc., Tokyo, Japan), and after 4 hours, whole-blood samples were taken from the retro-orbital sinus using Micro-Hematocrit Capillary Tubes (22-362-566; Thermo Fisher Scientific Inc., MA, USA). Hematocrit and blood urea nitrogen (BUN) levels were measured using i-STAT 1 analyzer (Abbott, IL, USA) and EC8+ cartridge (AB-3P7925; Abbott).

Treatment of mice with MALA using a PHD inhibitor per os

Mice diagnosed MALA were treated with either the vehicle (n = 18. 0.5% methyl cellulose; 64625; Sigma-Aldrich), REC2923 (n = 21. 30 mg/kg body weight; Daiichi Sankyo, Tokyo, Japan), or FG-4592 (n = 21. 50 mg/kg body weight; 15294; Cayman Chemical, Michigan, USA) *per os* immediately after metformin administration on day 2. After 4 hours, the mice were then administered an i.p. injection of lactic acid (0.4 mg/g body weight) dissolved in 0.9% sodium chloride. Kaplan–Meier survival analysis was performed.

Development of a mouse model of CKD

Seven-week-old wild-type C57BL/6J male mice were fed either a normal CE-2 diet (CLEA Japan, Tokyo, Japan) or a 0.2% adenine-containing CE-2 diet (CLEA Japan) for 6 weeks *ad libitum* (8, 9). Blood lactate and serum creatinine levels were then measured 0, 3, and 6

weeks after starting each diet. To measure blood lactate levels, whole-blood samples (5 µl) were obtained from the tail veins, which were tested using a Lactate Pro Test Meter (Cycle Classic Imports, New South Wales, Australia) and Lactate Pro Test Strip (Cycle Classic Imports). To measure serum creatinine levels, the mice were anesthetized with isoflurane (AbbVie Inc.) and whole-blood samples were obtained from the retro-orbital sinus using Micro-Hematocrit Capillary Tubes. Each sample was then collected in a 1.5 ml tube and left at room temperature for 30 min to separate the hemocytes from the serum. The serum was then transferred into a new tube and centrifuged at 20,000 × *g* for 5 min. This procedure was repeated twice per sample. The isolated serum (10 µl) was placed on a FUJI DRI-CHEM slide CRE-PIII (14A2X10004000004; FUJIFILM Inc., Tokyo, Japan) and serum creatinine levels were measured with a DRI-CHEM 7000i (FUJIFILM Inc.).

Histological analysis

The normal diet mice and adenine-induced CKD mice were anesthetized with isoflurane (AbbVie Inc.). Their kidneys were then removed and separated into upper, middle, and lower parts. The middle part of each kidney, which contained the renal pelvis, was fixed in Bouin's solution and embedded in paraffin. Thin-sliced sections were then made, which were stained with hematoxylin (Hematoxylin3G 8656; Sakura Finetek Japan, Tokyo, Japan) and eosin (Eosin 8659; Sakura Finetek Japan). Photomicrographs were obtained using a BZ-9000 Fluorescence Microscope (Keyence, Osaka, Japan) viewed at × 20 magnification. Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) analysis of 2,8-dihydroxyadenine (*m/z* 168.05) in the kidneys of normal diet or adenine-containing diet mice was performed in the positive ion mode using an atmospheric pressure MALDI-QIT-TOF-MS (Shimadzu, Kyoto, Japan) as described previously (10-12).

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145 *Treatment of MALA in CKD mice using a PHD inhibitor per os*

146 The CKD mice, as described under *"Development of a mouse model of CKD"*, were
147 administered metformin (0.5 mg/g body weight) and blood lactate levels were then measured
148 after 4 hours. Mice with blood lactate levels > 8 mmol/L were treated with either the vehicle
149 (n = 8. 0.5% methyl cellulose) or REC2923 (n = 11. 10 mg/kg body weight) *per os*. For mice
150 whose lactate levels did not meet this criterion, the experiment was repeated the following
151 day. Measurement of blood lactate levels, hematocrit, serum creatinine, BUN and isolation of
152 kidneys for real-time RT-PCR analysis of genes related to inflammation were proceeded after
153 6 hours of treatment with either the vehicle or REC2923. Kaplan–Meier survival analysis was
154 performed.

155

156 *Lactate tolerance test*

157 Eight to nine-week-old *Phd2*-liver-specific knockout (*Phd2-LKO*) male mice were
158 administered metformin (0.25 mg/g body weight) dissolved in distilled water in the morning
159 and evening of day 1, and in the morning of day 2, *per os*. After 4 hours, the mice were then
160 administered an i.p. injection of lactic acid (0.4 mg/g body weight) dissolved in 0.9% sodium
161 chloride. Whole blood samples (5 µl) were obtained from the tail veins at 0, 5, 10, 15, and 20
162 minutes after the injection. The blood lactate levels were measured using Lactate Pro Test
163 Meter and Lactate Pro Test Strip.

164

165 *Statistics*

The two treatment groups were compared using unpaired *t*-tests. The survival rate of the mice in each treatment group was determined using a Kaplan–Meier survival analysis, and differences in survival rates were analyzed using a Log-rank (Mantel–Cox) test performed with the GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA). Statistical significance was defined as a p-value of < 0.05.

Results

Development of a mouse model of MALA.

To test the effect of metformin, 8-week-old C57BL/6J wild-type male mice were administered metformin *per os* followed by an i.p. injection of lactic acid. Metformin administration alone did not increase blood lactate levels, but mice administered metformin followed by an i.p. injection of lactic acid exhibited life-threatening MALA, indicating that metformin exacerbates hyperlactatemia when blood lactate levels exceed the baseline clearance levels of lactate **(Figure 1)**.

Effects of PHD inhibitors on the survival of mice with MALA

To investigate whether pharmacological inhibition of PHDs ameliorates MALA, first we tested the effects of the PHD inhibitors REC2923 and FG-4592, 2-oxoglutarate analogues, that are supposed to activate HIF. We detected the up-regulation of HIF-target genes in the livers of C57BL/6J wild-type male mice treated with REC2923 and FG-4592 *per os*, indicating that a hypoxic response was induced by the treatment with those PHD inhibitors in the liver **(Figure 2A)**. We also detected the up-regulation of HIF-target genes in the kidneys following the treatment with REC2923, but not in the muscles or the hearts **(Figure 2B)**, indicating that REC2923 preferentially inhibits PHDs in the liver and kidney. To see the effect of PHD inhibitor on hematopoiesis, we treated mice with either the vehicle or REC2923 and measured hematocrit levels before and after the treatment. There was no difference in hematocrit levels between the two groups **(Figure 2C)** indicating that a single dose treatment of PHD inhibitor up-regulates erythropoietin in both the liver and the kidney **(Figures 2A and 2B),** but does not affect hematocrit levels yet within 10 days after the treatment.

We then pretreated mice, which had been administered metformin, with the vehicle alone, REC2923 or FG-4592, 4 hours prior to an i.p. injection of lactic acid **(Figure 3, left)**. We found that mice that had been pretreated with the PHD inhibitors exhibited significantly higher survival rates than the vehicle-treated mice **(Figure 3, right)**, indicating that PHD inhibition can rescue mice with MALA.

Development of a mouse model of CKD

Healthy individuals rarely develop MALA, but individuals with renal dysfunction such as CKD patients are at a high risk (3), as lactate in the blood stream is excreted mainly in the urine. Therefore, to establish a mouse model that mimics CKD patients, 7-week-old C57BL/6J wild-type male mice were fed a 0.2% adenine-containing diet for 6 weeks (9). Mice that were fed this diet had significantly higher serum creatinine levels than mice that were fed a normal diet **(Figure 4A)**. Imaging mass spectrometry (MALDI-IMS) analysis revealed the crystals of 2,8-dihydroxyadenine deposited in the kidneys of adenine-containing diet mice **(Figure 4B)**. Tubular dilation, dilated Bowman's space, and interstitial inflammation were also evident in kidney sections from adenine-induced CKD mice **(Figure 4C)**, indicating that they had successfully developed CKD. The blood lactate levels in these CKD mice significantly increased to a lethal level following the daily administration of metformin but exhibited no change following the administration of the vehicle, indicating that metformin administration in CKD mice successfully phenocopies MALA **(Figure 4D)**.

Effects of a PHD inhibitor on MALA in CKD mice

To assess whether treatment with the PHD inhibitor could improve the survival of MALA in CKD mice, CKD mice were administered metformin in the morning and their lactate levels

were checked after 4 hours. Those with blood lactate levels exceeding 8 mmol/L were then treated with either the vehicle or the PHD inhibitor, REC2923 **(Figure 5A, left)**. For mice whose lactate levels did not meet this criterion, the experiment was repeated the following day. There was no significant difference in the amount of administered metformin between the two groups (vehicle 1.313 ± 0.458, REC2923 1.364 ± 0.393 mg/g body weight, p=0.797). Mice that were treated with REC2923 exhibited significantly higher survival rates than the vehicle-treated mice, indicating that the PHD inhibitor may act as a "rescue agent" in the setting of lactic acidosis in metformin-prescribed patients **(Figure 5A, right)**. To see whether the improved survival was a consequence of lower lactate levels, blood lactate levels were measured after 6 hours of treatment with the vehicle or REC2923. Although the difference did not reach significance, there was a tendency of lower blood lactate levels in REC2923-treated mice **(Figure 5B)**. We detected the up-regulation of erythropoietin (*Epo*) in the kidneys of CKD mice after 6 hours of treatment with REC2923 **(Figure 5C, left),** but this did not lead to hematocrit change **(Figure 5C, right)**, showing that blood lactate levels were not influenced by the red blood cell production at this time point. To check the influence of REC2923 on renal function, serum creatinine and BUN were measured after 6 hours of treatment with the vehicle or REC2923, but there was no difference between the two groups **(Figure 5D),** showing that REC2923 does not have the ability to improve renal function that increase urinary lactate excretion. We have also checked whether PHD inhibitor had any effects on the tissue inflammation as reported previously, expression of genes related to inflammation were analyzed in the kidneys of CKD mice. IL-6 was significantly down-regulated in the kidneys of PHD inhibitor-treated mice **(Figure 5E)**, suggesting that PHD inhibitor contributed to improved survival rate of MALA in CKD mice by ameliorating inflammation, at least in part.

Up-regulation of the mRNAs involved in gluconeogenesis in both the livers and kidneys of PHD inhibitor-treated mice.

To elucidate the molecular mechanism of how the PHD inhibitors rescued mice with MALA in this study, expression of genes related to gluconeogenesis were analyzed in livers of REC2923-treated mice. Lactate dehydrogenase (LDH) A (*Ldha*) and monocarboxylate transporter (MCT) 1 (*Slc16a1*), that are involved in gluconeogenesis from lactate, were up-regulated in the livers of REC2923-treated mice **(Figure 6A)**. To see whether the rescue effect of PHD inhibitors in MALA depends on the suppression of PHD2, a dominant isoform of PHDs *in vivo*, in the liver as in our previous report (12), *Phd2*-liver-specific knockout *(Phd2-LKO)* mice were administered metformin followed by an i.p. injection of lactic acid, and a lactate tolerance test was performed. Interestingly, *Phd2-LKO* mice did not show lactate tolerance in MALA **(Figure 6B)**, suggesting that the activation of the hepatic arm of Cori cycle does not occur by inhibiting *Phd2* alone in the liver of MALA mice. We then analyzed expression of genes related to gluconeogenesis in the kidneys of REC2923-treated mice **(Figure 6C)**. LDHA (*Ldha*), MCT1 (*Slc16a1*), and phosphoenolpyruvate carboxykinase (PEPCK) (*Pck1*) were up-regulated in the kidneys of REC2923-treated mice suggesting a possibility that the PHD inhibitors rescued mice with MALA by up-regulating gluconeogenic mRNAs in part in both the liver and the kidney in this model.

Discussion

Metformin is currently prescribed to over 120 million people worldwide (13), and the number of people with impaired glucose tolerance is expected to increase to 420 million by 2025 (14). However, despite being one of the most widely used drugs for the treatment of type 2 diabetes mellitus, its mechanism of action is still being debated (15) because it helps to lower blood glucose levels via multiple mechanisms. Metformin inhibits mitochondrial complex I (16), reducing ATP generation and increasing the AMP/ATP ratio, which leads to the activation of AMP-activated protein kinase (AMPK); this then suppresses gluconeogenesis by down-regulating gluconeogenic genes such as PEPCK or glucose-6-phosphatase (17-20). It has also been reported that metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerol-3-phosphate dehydrogenase (21), and improves the uptake of glucose into the skeletal muscles (22) and reduces glucose absorption in the small intestine (23).

Metformin has also been shown to suppress cancer cell growth through the inhibition of mitochondrial complex I, or the activation of AMPK (19, 24), and its use has been associated with a decreased incidence of several types of cancer (25-29). In addition, a clinical trial named “Targeting Aging with Metformin” is currently scheduled (30) which, if successful, will see metformin being approved as an anti-aging drug by the US Food and Drug Administration (FDA). This would substantially increase the number of metformin-prescribed individuals across the world, even in the developing countries as there will be no financial barriers to mass-producing this drug, which is more than 60 years old.

However, metformin has also been associated with the development of critical lactic acidosis (3, 31, 32). The inhibition of mitochondrial complex I, which converts NADH to its oxidized form (NAD^+), leads to decreased NAD^+ production (19, 24); and the inhibition of

mitochondrial glycerol-3-phosphate dehydrogenase, which converts glycerol 3-phosphate (G3P) to dihydroxyacetone phosphate (DHAP) using flavin adenine dinucleotide (FAD) as a cofactor, leads to decreased DHAP production and the accumulation of G3P in the mitochondria (21). The latter results in the accumulation of cytosolic G3P, which halts the glycerophosphate shuttle and leads to the accumulation of cytosolic NADH, inhibiting the conversion of lactate to pyruvate by lactate dehydrogenase (21). Thus, lactate accumulates in the hepatocytes, suppressing its absorption from the bloodstream into the liver.

The overall reported incidence of MALA is approximately 3–10 per 100,000 person-years (3), however, the FDA has recently announced that metformin can be used safely in patients with mild impairment in kidney function, and in some patients with moderate impairment in kidney function, and required to expand metformin's use in patients with reduced kidney function (https://www.fda.gov/Drugs/DrugSafety/ucm493244.htm). Therefore, metformin will also be used not only to treat diabetes and cancer, but also for cancer prevention and anti-aging purposes in the future, thus we may see a concurrent increase in the number of patients with MALA. MALA has a high mortality rate of up to 50% (33, 34), so an established treatment is required.

Although our *in vivo* studies previously detected the up-regulation of gluconeogenic mRNAs MCT2 (*Slc16a7*), PEPCK (*Pck1*) and glucose transporter (GLUT) 2 (*Slc2a2*) in the livers of *Phd2-LKO* mice (2), the up-regulation of these mRNAs were not identified through either *in vitro* experiments using primary hepatocytes (data not shown) or *in vivo* experiments following the treatment with PHD inhibitors in the present study. This was probably due to the previous study disrupting the *Phd2* gene alone, while the present study led to the pharmacological inhibition of not only PHD2 but also other PHD isoforms, or other 2-oxoglutarete-dependent dioxygenases as off-target effects.

310 To evaluate whether the rescue effect of PHD inhibitors in mice with MALA depends on the
311 suppression of PHD2 in the liver as in our previous report (2), *Phd2-LKO* mice were
312 administered metformin followed by an i.p. injection of lactic acid, and a lactate tolerance
313 test was performed. Interestingly, *Phd2-LKO* mice did not show lactate tolerance in MALA
314 (**Figure 6B**), suggesting that the activation of the hepatic arm of Cori cycle does not occur by
315 inhibiting *Phd2* alone in the liver of mice with MALA. Metformin activates AMPK indirectly
316 by inhibiting the mitochondrial complex I, thus inhibiting mammalian target of rapamycin
317 complex 1 (mTORC1) (35), which positively regulates HIF1α, so metformin administration
318 reduces hypoxia-induced HIF1α stabilization and diminishes expression of HIF-target genes
319 (13). HIF activation in the liver due to *Phd2* disruption in *Phd2-LKO* mice may have been
320 diminished by the anti-HIF1α effect of metformin, so the activation of the hepatic arm of
321 Cori cycle in *Phd2-LKO* mice, as previously reported, did not occur in *Phd2-LKO* mice with
322 MALA and hyperlactatemia was not ameliorated.

323 PHD inhibitors *per os* preferentially target the liver and the kidney (**Figures 2A and 2B**),
324 suggesting that the kidney, which contributes to approximately 20% of gluconeogenesis (36),
325 could be another target of PHD inhibitor *per os* in the setting of MALA treatment. We found
326 that *Pck1*, which codes PEPCK, one of the rate limiting enzymes of gluconeogenesis, was up-
327 regulated in the kidneys of REC2923-treated mice, suggesting that gluconeogenesis was
328 enhanced in the kidney, in addition to the liver, of the mice with MALA.

329 Previous study reported that adenine-induced CKD mice displayed severe anemia due to
330 decreased renal EPO production in the kidney, which activated hepatic EPO production (37).
331 It is possible that locally produced EPO in the liver affected the function of the hepatocytes
332 including gluconeogenesis, as EPO has organ protective effects in certain medical conditions
333 including ischemia in hearts, brains, and kidneys (38). In our mouse model of MALA in
334 CKD, *Epo* was induced in the kidney several hours after the PHD inhibitor treatment.

However, there was no change in kidney function by the treatment with PHD inhibitor **(Figure 5D)**, which indicates that the improved survival was not a consequence of ameliorated renal function due to increased EPO production. EPO enhances red blood cell (RBC) production, which may lead to increase in lactate excretion from RBC, but the hematocrit levels after the treatment with PHD inhibitor did not increase in our short period experiments **(Figures 2C and 5C)**, indicating that the EPO–RBC pathway also did not affect the survival of our mouse model of MALA.

Another possibility is that mice with MALA were rescued by PHD inhibitor via altered immune response (39). In our mouse model of MALA in CKD, pro-inflammatory cytokine IL-6 was significantly down-regulated in the kidneys of PHD inhibitor-treated mice, which might have had an organ protective effect and contributed to better survival **(Figure 5E)**.

Hemodynamic studies using pressure-volume conductance catheter (40) revealed that the PHD inhibitor did not ameliorate the cardiac dysfunction in mice with MALA **(Figure 3 and data not shown)**, indicating that the rescue effect of PHD inhibitor on MALA is independent of cardiac function.

It is also possible that PHD inhibitor alters the cellular uptake or excretion of metformin by organic cation transporters (OCTs) or multidrug and toxin extrusion (MATE) transporters as the off-target effects (41). Another study recently reported that systemic or skeletal muscle-specific *Phd2* inactivation protects mice against myocardial ischemia–reperfusion injury independent of the HIF pathway (42), and so this unknown HIF-independent pathway may also have been involved here. Further investigation is required to fully understand the mechanism of how PHD inhibition rescues MALA, including HIF-dependency. For the findings to be applied in the clinic in the future, we need to be aware of the complications of the high doses of oral PHD inhibitors required to ameliorate lactic acidosis.

PHD inhibitors may also be used to treat lactic acidosis induced by other clinical conditions that involve shock, severe respiratory disease, or mitochondrial disease. In septic shock patients, increased blood lactate levels have been associated with a 10-fold increase in the mortality rate compared with normal lactate levels, and the increased lactate levels have been shown to be a better predictor of morbidity and mortality than physiological triage criteria (43). Therefore, a lactate level-guided treatment with PHD inhibitors may contribute to the improvement of sepsis patients with lactic acidosis.

In summary, we have found that PHD inhibitors significantly improved the survival of mice with MALA, and furthermore, a PHD inhibitor also improved the survival rate of MALA in CKD mice. Oral PHD inhibitors have been developed and are now under clinical trial for renal anemia (1). Our findings indicate that PHD could be a new therapeutic target for MALA treatment and may also be used in a variety of pathophysiological conditions.

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517

518 **Figure Legends**

519 **Figure 1. Development of a mouse model of metformin-associated lactic acidosis**
520 **(MALA).**

521 Blood lactate levels in mice with or without the administration of metformin *per os* and an
522 intraperitoneal (i.p.) injection of lactic acid (right, $n = 3$ per treatment group). Detailed time
523 table of the experiment is also shown (left). Note that metformin exacerbated the
524 hyperlactatemia that was induced by an i.p. injection of lactic acid. Error bars indicate 1
525 standard error of the mean (SEM).

526

527 **Figure 2. Tissue dependent effects of prolyl hydroxylase (PHD) inhibitors *per os* in wild-**
528 **type mice.**

529 **(A)** Real-time RT-PCR analysis of the direct hypoxia-inducible factor (HIF)-target genes,
530 *Pdk1*, *Pgk1*, *Slc7a5* (L-type amino acid transporter [LAT] 1), and *Epo*, in the livers of mice
531 after 4 hours of administration *per os* of the vehicle ($n = 3$. 0.5% methyl cellulose), REC2923
532 ($n = 3$. 30 mg/kg body weight), or FG-4592 ($n = 3$. 50 mg/kg body weight). Error bars
533 indicate 1 SEM. **(B)** Real-time RT-PCR analysis of the direct HIF-target genes (*Pdk1*, *Pgk1*
534 and *Slc7a5*) in the kidneys, muscles and hearts of C57BL/6J male mice after 4 hours of
535 administration *per os* of the vehicle ($n = 3$. 0.5% methyl cellulose) or REC2923 ($n = 3$. 30
536 mg/kg body weight). *Epo* was also analyzed in the kidneys. Error bars indicate 1 SEM. **(C)**
537 Hematocrit levels of mice treated with either the vehicle ($n = 5$. 0.5% methyl cellulose) or
538 REC2923 ($n = 5$. 30 mg/kg body weight) on day 0 (control), 1 (after 4 hours of treatment), 5,
539 and 10 **(left)**. The area under the curve (AUC) values for each group were compared using an
540 unpaired Student's *t*-test. Error bars indicate 1 SEM.

541

542 **Figure 3. Treatment model of MALA with PHD inhibitors**

543 Schematic of the treatment model of MALA with PHD inhibitors (**left**) and the survival
544 analysis (**right**). Mice were administered metformin (0.25 mg/g body weight) with the
545 vehicle (*n* = 18. 0.5% methyl cellulose), REC2923 (*n* = 21. 30 mg/kg body weight), or FG-
546 4592 (*n* = 21. 50 mg/kg body weight) *per os* 4 hours prior to an i.p. injection of lactic acid
547 (0.4 mg/g body weight).

548

549 **Figure 4. Generation of a mouse model of MALA in adenine-induced chronic kidney**
550 **disease (CKD)**

551 **(A)** Serum creatinine levels in mice that were fed a normal diet (*n* = 3) or a 0.2% adenine-
552 containing diet (*n* = 11) for the indicated time periods. The AUC values for each group were
553 compared using an unpaired Student's *t*-test. Error bars indicate 1 SEM. **(B)** Imaging mass
554 spectrometry (MALDI-IMS) analysis of 2,8-dihydroxy adenine (2,8-DHA. *m/z 168.05*) in the
555 kidneys of the mice that were fed normal or 0.2% adenine-containing diet for 6 weeks. Note
556 that crystals of 2,8-DHA were detected in adenine-containing diet mice. Scale bar: 500 μm.
557 **(C)** Histological analysis of the kidneys in the mice that were fed a normal diet or a 0.2%
558 adenine-containing diet for 6 weeks. Representative data from three mice per treatment group
559 are shown. Tubular dilation (arrows) and dilated Bowman's space (arrowhead) are observed
560 in the adenine-induced CKD mice. Scale bar: 100 μm. **(D)** The CKD mice were administered
561 the vehicle (*n* = 5. 0.5% methyl cellulose) or metformin (*n* = 29. 0.5 mg/g body weight) *per*
562 *os* and lactate levels were measured after 4 hours. The experiment was repeated the following
563 day. The AUC values of each group were compared using an unpaired Student's *t*-test. Error
564 bars indicate 1 SEM.

565

566 **Figure 5. Treatment model of MALA in CKD mice with PHD inhibitor.**

567 **(A)** Schematic of the treatment of MALA in CKD mice (**left**) and survival analysis of MALA
568 in CKD mice that were treated with the vehicle or PHD inhibitor, REC2923 (**right**). Mice
569 were administered metformin (0.5 mg/g body weight) *per os* in the morning, and those with
570 blood lactate levels > 8 mmol/L after 4 hours of metformin administration were then treated
571 with either the vehicle (n = 8. 0.5% methyl cellulose) or PHD inhibitor, REC2923 (n = 11. 10
572 mg/kg body weight) *per os*. **(B)** Blood lactate levels of CKD mice after 6 hours of treatment
573 with the vehicle (n = 10. 0.5% methyl cellulose) or REC2923 (n = 9. 10 mg/kg body weight).
574 Error bars indicate 1 SEM. **(C)** Real-time RT-PCR analysis of the direct HIF-target gene *Epo*
575 in the kidneys of CKD mice **(left)** and hematocrit levels of CKD mice **(right)** after 6 hours of
576 treatment with the vehicle or REC2923. Error bars indicate 1 SEM. **(D)** Serum creatinine
577 **(left)** and BUN **(right)** levels in the CKD mice after 6 hours of treatment with the vehicle or
578 REC2923. Error bars indicate 1 SEM. **(E)** Real-time RT-PCR analysis of inflammatory
579 mRNAs *Tnf*, *Il1b*, *Il4*, *Il6*, and *Il10* in CKD mice treated with the vehicle (n=10) or REC2923
580 (n=9). Error bars indicate 1 SEM.

581

582 **Figure 6. Up-regulation of the mRNAs involved in gluconeogenesis in both the livers**
583 **and kidneys of PHD inhibitor-treated mice.**

584 **(A)** Real-time RT-PCR analysis of the genes involved in gluconeogenesis, *Ldha*, *Slc16a1*
585 (monocarboxylate transporter [MCT] 1), *Slc16a7* (MCT2), *Pck1*, and *Slc2a2* (glucose
586 transporter [GLUT] 2), in the livers of C57BL/6J male mice after 4 hours of administration
587 *per os* of the vehicle (0.5% methyl cellulose. n = 3) or REC2923 (30 mg/kg body weight. n =
588 3). Error bars indicate 1 SEM. **(B)** Lactate tolerance test. *Control* (n = 10) and *Phd2*-liver-
589 specific knockout (*Phd2-LKO*. n = 12) male mice were administered 0.25 mg/g body weight

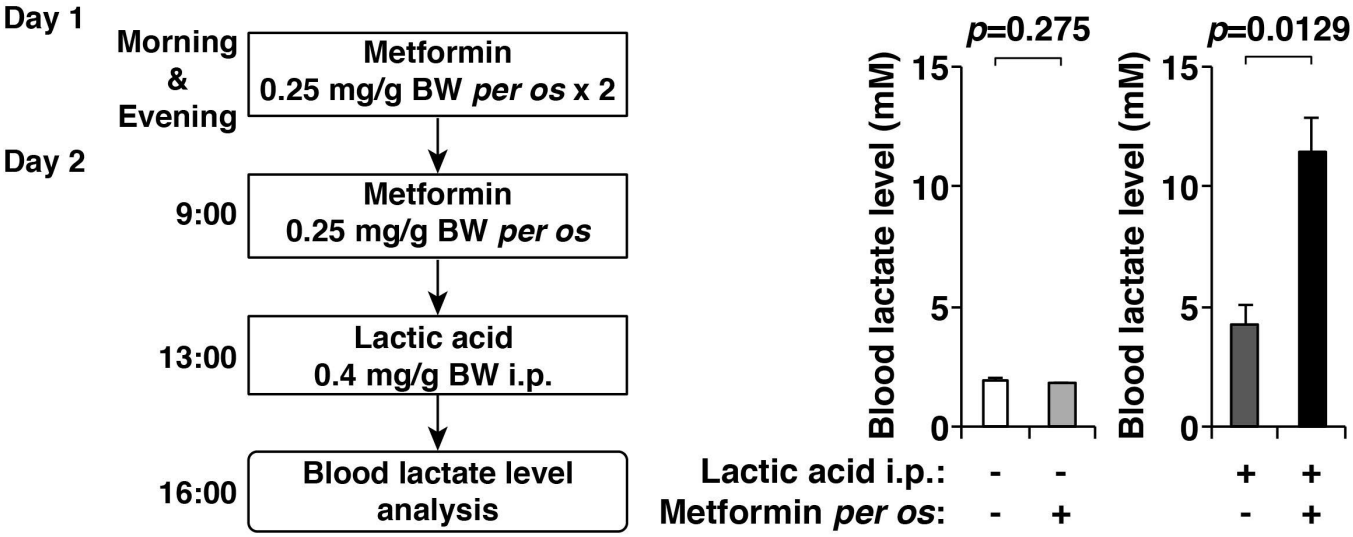
metformin dissolved in distilled water in the morning and evening of day 1, and in the morning of day 2, *per os*. After 4 hours, the mice then received an i.p. injection of 0.4 mg/g body weight lactic acid and blood lactate levels were measured at the indicated time. The AUC values of each group were compared using an unpaired Student's *t*-test. Error bars indicate 1 SEM. **(C)** Real-time RT-PCR analysis of the genes involved in gluconeogenesis, *Ldha*, *Slc16a1* (monocarboxylate transporter [MCT] 1), *Slc16a7* (MCT2), *Pck1*, and *Slc2a2* (glucose transporter [GLUT] 2), in the livers of C57BL/6J male mice after 4 hours of administration *per os* of the vehicle (n = 3. 0.5% methyl cellulose) or REC2923 (n = 3. 30 mg/kg body weight). Error bars indicate 1 SEM.

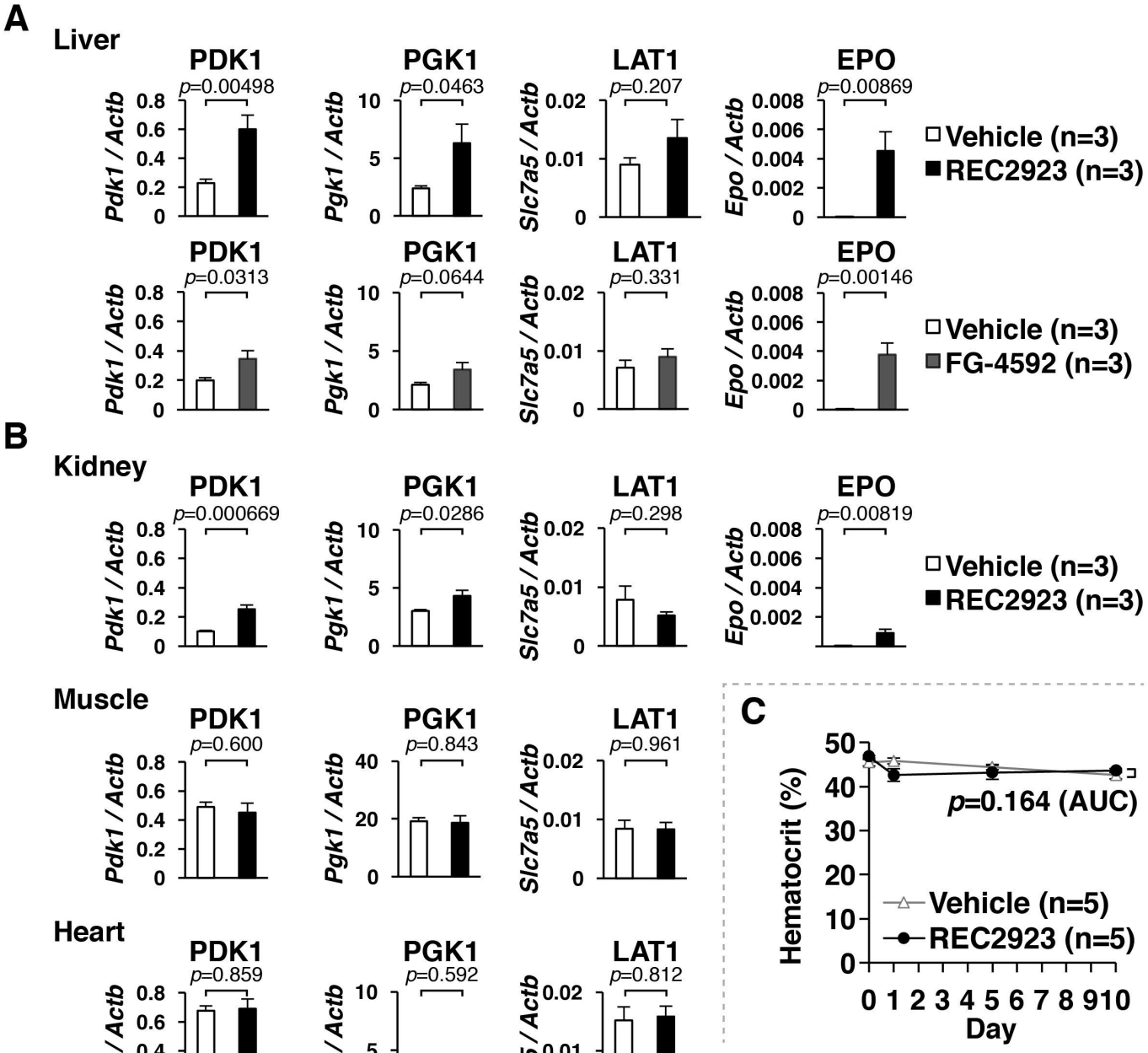
Table 1. Primer sequences for real-time reverse transcription-PCR analysis.

Gene	*Oligonucleotide sequence*	
Ldha	Forward	5′-ACAGTTGTTGGGGTTGGTGC-3′
	Reverse	5′-CGCAGTTACACAGTAGTCTTTG-3′
Pdk1	Forward	5′-ACCTCGTTTATGTTTCTGCG-3′
	Reverse	5′-CAACTCCTGAAGGCTTTGG-3′
Pgk1	Forward	5′-GATGAGGGTGGACTTCAAC-3′
	Reverse	5′-TAAGGACAACGGACTTGGC-3′
Epo	Forward	5′-CATCTGCGACAGTCGAGTTCTG-3′
	Reverse	5′-CACAACCCATCGTGACATTTTC-3′
Slc7a5	Forward	5′-GCCCTCATCATTTTGCTCG-3′
	Reverse	5′-TCAGATAGTTCCATCCTCCG-3′
Slc16a1	Forward	5′-TGGTTGTCTGTCTGGTTGC-3′
	Reverse	5′-CAGTGGTCGCTTCTTGTAG-3′
Slc16a7	Forward	5′-TTCAACACCACCTCCAGTC-3′
	Reverse	5′-CAGCATAATAGTCCTCCCAC-3′
Pck1	Forward	5′-GGAAGGACAAAGATGGCAAG-3′
	Reverse	5′-TCAGGTTCAAGGCGTTTTC-3′
Slc2a2	Forward	5′-GTCGCCTCATTCTTTGGTG-3′
	Reverse	5′-CTGATACACTTCGTCCAGC-3′
Tnf	Forward	5′-TCCCTCTCATCAGTTCTATGG-3′
	Reverse	5′-AAGAGAACCTGGGAGTAGAC-3′
Il1b	Forward	5′-GACCTGTTCTTTGAAGTTGACG-3′
	Reverse	5′-TGTTGATGTGCTGCTGCGAG-3′
Il4	Forward	5′-AGACTCTTTCGGGCTTTTC-3′

	Reverse	5′-TGATGCTCTTTAGGCTTTCC-3′
Il6	Forward	5′-CCAGAGTCCTTCAGAGAGATAC-3′
	Reverse	5′-ATGGTCTTGGTCCTTAGCC-3′
Il10	Forward	5′-GCTGGACAACATACTGCTAAC-3′
	Reverse	5′-TGCTCCTTGATTTCTGGGC-3′

Toramaru-Oyaizu et al. Figure 1





Toramaru-Oyaizu et al. Figure 3