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Metformin exerts beneficial effects in hemorrhagic shock in an AMPK α 1-independent manner

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Abstract

Despite therapeutic advances in hemorrhagic shock, mortality from multiple organ failure remains high. AMP-activated protein kinase (AMPK) is involved in cellular energy homeostasis. Two catalytic subunits, α 1 and α 2, have been identified, with α 1 subunit largely expressed in major organs. Here, we hypothesized that genetic deficiency of AMPK α 1 worsens hemorrhage-induced multiple organ failure. We also investigated whether treatment with metformin, a clinically used drug for metabolic homeostasis, affords beneficial effects. AMPK α 1 wild-type (WT) and knock-out mice (KO) were subjected to hemorrhagic shock by blood withdrawing followed by resuscitation with shed blood and Lactated Ringer's solution and treatment with vehicle or metformin. Mice were sacrificed at 3 hours after resuscitation. Compared to vehicle-treated WT animals, KO animals exhibited a more severe hypotension, higher lung and liver injury and neutrophil infiltration, and higher levels of plasma inflammatory cytokines. Metformin treatment ameliorated organ injury and mean arterial blood pressure in both WT and KO mice, without affecting systemic cytokine levels. Furthermore, metformin treatment reduced liver lipid peroxidation and increased levels of complex II co-substrate FAD and levels of ATP in WT and KO mice. Beneficial effects of metformin were associated with organ-specific nuclear-cytoplasmic shuttling and activation of liver kinase B1 and AMPK α 2. Thus, our data suggest that AMPK α 1 is an important regulator of hemodynamic stability and organ metabolic recovery during hemorrhagic shock. Our data also suggest that metformin affords beneficial effects, at least in part, independently of AMPK α 1 and secondary to AMPK α 2 activation, increase of Complex II function and reduction of oxidative stress.

Keywords

hemorrhagic shock; AMPK; metformin; myeloperoxidase; ATP; FAD; malondialdehyde

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INTRODUCTION

Trauma is the leading cause of mortality in the 1 to 46-year-old age group with significant morbidity associated with chronicity of disease post-trauma (1). Hemorrhagic shock is the second most common cause of death after central nervous system injuries but is the number one preventable cause of early death in trauma patients (2). In addition, hemorrhagic shock can lead to multiple organ dysfunction with significant morbidity and even delayed mortality (1, 2). The exact mechanisms of multiple organ dysfunction remain unknown. However, studies have suggested that organ failure may involve an impairment of mitochondria preventing utilization of oxygen at a cellular level despite early goal-oriented maximization of oxygen delivery (3).

The adenosine monophosphate-activated protein kinase (AMPK) is a crucial sensor and regulator of energy balance and contributes to many important metabolic processes. AMPK activation results in downregulation of anabolic activities and upregulation of catabolic activities (4, 5). In this context, AMPK regulates mitochondrial biogenesis through activation of the transcription co-activator peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) (6). AMPK is a heterotrimer comprised of catalytic α subunit and regulatory β and γ subunits. Each subunit has different isoforms that are expressed in varying degrees in different organs. The catalytic α subunit of AMPK is comprised of two isoforms, $\alpha 1$ and $\alpha 2$ and is activated by phosphorylation in conditions of high energy expenditure with consequent low levels of ATP and increased levels of AMP. The AMPK $\alpha 1$ isoform is present in high levels in lung, liver, and kidney and to a lesser degree in the heart (4–7).

Multiple studies have demonstrated that activation of AMPK can be beneficial in animal models of hemorrhagic shock. In our previous studies, we showed that treatment with the AMP analog, 5-amino-4-imidazole carboxamide riboside (AICAR) ameliorated lung and cardiac injury during hemorrhagic shock in young and mature adult mice (8, 9). Similarly, pharmacological activation of AMPK by AICAR has been reported to ameliorate hemodynamic parameters and survival time, and reduce metabolic acidosis in a rat hemorrhagic shock model (10).

Among drugs targeting the AMPK pathway, metformin, a widely used anti-hyperglycemic drug, has been shown to indirectly activate AMPK through activation of an upstream kinase liver kinase B1 (LKB1) and/or through alteration of the AMP/ATP ratio to maintain metabolic homeostasis (11–13).

In this study, given the previous findings showing beneficial effects of AMPK activation in the setting of shock, we sought to investigate the role of AMPK $\alpha 1$ in the systemic inflammatory response and multiple organ dysfunction following hemorrhagic shock using knockout mice genetically deficient of the AMPK $\alpha 1$ subunit (14). Furthermore, we tested whether pharmacological activation of AMPK by an indirect AMPK activator such as metformin could ameliorate the hemorrhage-induced multiple organ failure.

MATERIALS AND METHODS

Murine model of hemorrhagic shock

The experiments conformed to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (8th edition, 2011) and had the approval of the Institutional Animal Care and Use Committee. Homozygous AMPK α 1 wildtype (WT) and AMPK α 1 knockout (KO) mice were established on a C57BL/6 genetic background by the crossbreeding (>10 generations) of a breeding pair kindly provided by Dr. Benoît Viollet of the University of Paris Descartes, Paris, France (14, 15). For these experiments, male AMPK α 1 WT and KO mice were generated by a breeding scheme utilizing heterozygous mutant mice. Mice were used at the age of 3–5 months and assigned to the different experimental groups after routine genotyping by quantitative PCR. All mice were allowed free access to water and a maintenance diet in a 12-hour light/dark cycle, with room temperature at 21 $\pm$ 2 °C. Mice were anesthetized with pentobarbital (80 mg/kg) intraperitoneally (IP) and prepared for hemorrhagic shock as previously described (8, 9, 16, 17). Briefly, either the left or right femoral artery was cannulated (PE-10 tube) and connected to a blood pressure transducer (PowerLab, ADInstruments, Colorado Springs, CO) for measurement of mean arterial blood pressure (MABP) and heart rate. Blood was removed from the femoral artery over a 15-minute period until MAP reached 30 $\pm$ 5 mmHg. The mice were kept in this MABP range for 90 minutes by additional blood removal or small volume transfusion. At the end of the shock period, AMPK α 1 WT and KO mice were randomly assigned to two treatment groups: a vehicle group (distilled water) and a metformin group (metformin, 100 mg/kg, IP). Drug treatment was given as single bolus right before resuscitation. Dose of metformin was chosen according to reported pharmacokinetics and pharmacodynamics of the drug in the mice (18). The mice were then resuscitated by infusing their shed blood and twice that amount in Lactated Ringer's solution over a 10-minute period. The mice were further monitored for 3 hours for cardiovascular parameters. Control mice were anesthetized and underwent surgical preparation, but did not have any blood removed. At the end of the experimental period, mice were sacrificed. Blood, heart, lungs, liver, and kidneys were collected for biochemical assays.

Lactate assay

Plasma lactate levels were measured using commercially available lactate assay kit (Sigma-Aldrich, St Louis, MO) and following the protocol recommended by the manufacturer.

Cytokine analysis

Plasma levels of interleukin (IL) 1 β , IL-10, IL-6, IL-17, tumor necrosis factor- α (TNF- α) and keratinocyte-derived chemokine (KC) were evaluated by a commercially available multiplex array system (Linco-Research, St. Charles, MO) using the protocols recommended by the manufacturer.

Histopathologic analysis

Lungs and livers were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin, and evaluated by an independent observer blinded

to the treatment groups. Lung injury was based on the following histologic features: alveolar capillary congestion, infiltration of red blood and inflammatory cells into the airspace, alveolar wall thickness and hyaline membrane formation (8). Liver injury was based on the following histologic features: sinusoid congestion and edema, infiltration of red blood and inflammatory cells, and presence of lipid droplets.

Myeloperoxidase assay

Myeloperoxidase (MPO) activity was determined as an index of neutrophil accumulation (19). Lung and liver samples were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 minutes at 4,000 g at 4°C. An aliquot of the supernatant was allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H₂O₂. The rate of change in absorbance was measured by spectrophotometry at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μ mol of hydrogen peroxide/minute at 37°C and expressed in units per 100 mg tissue.

ATP assay

ATP levels were measured in lung, liver, kidney, and heart tissue homogenates using commercially available ATP assay kit (BioVision, Milpitas, CA) and following the protocol recommended by the manufacturer.

Cytosol and nuclear extracts

Lung and liver tissues were homogenized using a Polytron homogenizer (Brinkman Instruments, West Orange, NY) in a buffer containing 0.32 M sucrose, 10 mM TrisHCl (pH 7.4), 1 mM EGTA, 2 mM EDTA, 5 mM Na₃N, 10 mM β -mercaptoethanol, 20 μ M leupeptin, 0.15 μ M pepstatin A, 0.2 mM phenylmethanesulfonyl fluoride, 50 mM NaF, 1 mM sodium orthovanadate, and 0.4 nM microcystin. Samples were centrifuged at 1,000 g for 10 minutes at 4°C and the supernatants collected as cytosol extracts. The pellets were then solubilized in Triton buffer (1% Triton X-100, 250 mM NaCl, 50 mM Tris HCl at pH 7.5, 3 mM EGTA, 3 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM sodium orthovanadate, 10% glycerol, 2 mM p-nitrophenyl phosphate, 0.5% NP-40, and 46 μ M aprotinin). The lysates were centrifuged at 15,000 g for 30 minutes at 4°C and the supernatant collected as nuclear extracts.

Western blot analysis

Cytosol and nuclear content of AMPK α 1/ α 2 and its phosphorylated active form pAMPK α 1/ α 2, LKB1 and its phosphorylated active form pLKB1, and PGC1- α were determined by immunoblot analyses. For AMPK α 1/ α 2, pAMPK α 1/ α 2, and PGC1- α , extracts were heated at 70°C in equal volumes of 4 $\times$ Protein Sample Loading Buffer. Twenty-five μ g of protein were loaded per lane on a 10% Bis-Tris gel. Proteins were separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with Odyssey blocking buffer and incubated with specific primary antibodies; β -actin was concomitantly probed as loading control. Membranes were washed in PBS with 0.1% Tween 20 and incubated with LI-COR secondary antibodies. The Odyssey LI-COR scanner and

soft-ware (LI-COR Biotechnology, Lincoln, NE) were used for detection and quantitative analysis. For LKB1/pLKB1 immunoblotting, extracts were boiled in equal volumes of NuPAGE® LDS Sample Buffer (4×) and 40 µg of protein loaded per lane on a 16% Tris-glycine gradient gel. Proteins were separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 5% nonfat dried milk in Tris-buffered saline (TBS) and incubated with primary antibodies. Membranes were washed in TBS with 0.1% Tween 20 and incubated with secondary peroxidase-conjugated antibody; the immunoreaction was visualized by chemiluminescence. Membranes were also reprobed with primary antibody against β-actin to ensure equal loading. Densitometric analysis of blots was performed using Quantity One (Bio-Rad Laboratories, Des Plaines, IL).

Malondialdehyde assay

Malondialdehyde (MDA) activity was determined as an index of lipid peroxidation. Liver samples were homogenized in 1.15% potassium chloride solution. Then 8.1% sodium dodecyl sulfate, 20% acetic acid, 0.8% thiobarbituric acid, and distilled water were added to the homogenates. The mixed solution was boiled for 1 hour at 95°C. The samples were centrifuged at 3,000 rpm for 10 minutes. The absorbance of thiobarbituric acid reactive species was measured by spectrophotometry at 532 nm and expressed as MDA in µmol per 100 mg tissue.

Measurement of flavin adenine dinucleotide (FAD)

Liver levels of the redox co-factor FAD were determined in homogenized tissues using a Flavin Adenine Dinucleotide (FAD) Assay Kit (Abcam Cambridge, MA). The rate of change in absorbance of an OxiRed probe was measured by spectrophotometry at 570 nm. FAD levels were expressed in pmol/mg tissue.

Statistical analysis

Statistical analysis was performed using SigmaPlot for Windows Version 12.5 (Systat Software, San Jose CA). Data are represented as means ± SEM of n = 4–10 animals for each group. For multiple group analysis at a single time point, one-way analysis of variance (ANOVA) with Student-Newman-Keuls correction was used. For multiple group analysis at different time points, a two-way ANOVA with Student-Newman-Keuls correction was performed. If data failed to follow a normal distribution, a Mann-Whitney Rank Sum test or an ANOVA on ranks test was performed. *P* values less than 0.05 were considered significant.

Materials

Metformin was obtained from Enzo Life Sciences (Farmingdale, NY). Primary antibodies for AMPKα1/α2 and pAMPKα1/α2 were obtained from Cell Signaling Technology (Danvers, MA). Primary antibodies for LKB1, pLKB1 and β-actin were obtained from Santa Cruz Biotechnology (Dallas, TX). Primary antibody for PGC1-α was obtained from Abcam (Cambridge, MA). The Odyssey blocking buffer, LI-COR goat anti-rabbit IR-800 and goat anti-mouse IR-680 antibodies, and the 4× Protein Sample Loading Buffer were obtained from LI-COR Biotechnology (Lincoln, NE). The Multiplex Luminex Array kit, the

NuPAGE® LDS Sample Buffer (4×), and Western blot gels were purchased from Life Technologies (Grand Island, NY). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

RESULTS

Effect of genetic deficiency of AMPK α 1 and metformin treatment on hemodynamic instability after hemorrhagic shock

Since the degree of hypoperfusion affects the severity of organ injury, we adopted a model of pressure-controlled hemorrhage to a target MABP of 30 mmHg in both WT and KO mice (16, 17). After 90 minutes of hypoperfusion, mice were resuscitated with the shed blood and Lactated Ringer's solution. After an early increase soon after transfusion, MABP progressively declined in both vehicle-treated WT and KO animals. However, vehicle-treated KO animals showed significantly lower MABP throughout the 3-hour monitoring period compared to the vehicle-treated WT group. Treatment with metformin significantly ameliorated MABP in both WT and KO mice throughout the resuscitation phase when compared to vehicle treatment. However, metformin-treated KO mice still exhibited significantly lower MABP than metformin-treated WT mice (Fig. 1A). There were no significant differences in heart rate among the different groups (data not shown).

Effect of genetic deficiency of AMPK α 1 and metformin treatment on plasma lactate levels

To further test the efficacy of the resuscitation protocol on improvement of tissue perfusion and to rule out metformin-associated lactic acidosis (20), we measured plasma lactate levels. Interestingly, the KO control group had significantly higher levels of plasma lactate than WT control mice at basal control conditions. At 3 hours after resuscitation, plasma lactate levels in both vehicle-treated WT and KO mice were similar to baseline levels of controls, thus suggesting successful resuscitation. Treatment with metformin did not modify lactate levels in WT or KO, thus proving that the drug does not increase the risk of metabolic acidosis (Fig. 1B).

Effect of genetic deficiency of AMPK α 1 and metformin treatment on organ injury

At 3 hours after resuscitation, histological analysis revealed that both vehicle-treated WT and KO mice experienced lung and liver injury. However, vehicle-treated KO animals had more significant organ injuries when compared to WT mice. In the lung of vehicle-treated KO mice injuries were characterized by a marked neutrophil margination and infiltration, atelectasis, alveolar disruption, proteinaceous debris, and hemorrhage (Fig. 2). In the liver of vehicle-injuries were characterized by neutrophil infiltration, edema, and areas of necrosis (Fig. 3). Treatment with metformin ameliorated lung and liver damage in both WT and KO mice (Figs. 2 and 3).

Effect of genetic deficiency of AMPK α 1 and metformin treatment on tissue neutrophil infiltration

To confirm the degree of neutrophil infiltration observed at the histological analysis, we measured activity of MPO, a lysosomal enzyme specific to neutrophils. Interestingly, in the lung, the KO control group had significantly higher MPO activity than WT control mice at

basal control conditions. Following hemorrhagic shock, MPO activity significantly increased in the lung, liver, and kidney of both vehicle-treated WT and KO mice when compared to control mice of the same genotype (Fig. 4). However, in the vehicle-treated KO group, the degree of neutrophil infiltration in these organs was higher than vehicle-treated WT mice. Treatment with metformin significantly decreased MPO activity in lung and liver in both WT and KO animals, but not in the kidney. However, metformin-treated KO mice still exhibited significantly higher MPO levels than metformin-treated WT mice (Fig. 4).

Effect of genetic deficiency of AMPK α 1 and metformin treatment on plasma cytokine levels

To further measure the degree of systemic inflammation, a panel of plasma Th1/Th2/Th17 cytokines was measured. At 3 hours after resuscitation, plasma levels of IL-1 β , IL-6, IL-10, TNF- α , and KC, but not IL-17, were all significantly elevated following hemorrhagic shock in both vehicle-treated WT and KO animals when compared with levels of control mice. However, vehicle-treated KO mice had much higher levels IL-1 β , IL-6, IL-10 and TNF- α when compared to WT mice. Treatment with metformin did not affect cytokine levels (Fig. 5).

Effect of genetic deficiency of AMPK α 1 and metformin treatment on metabolic homeostasis

To determine whether genetic deficiency of AMPK α 1 might affect energy homeostasis, we measured ATP levels in major organs. In the liver, there was a significant decrease in the ATP levels after hemorrhagic shock, but the degree of reduction was most significant in the vehicle-treated KO mice when compared to WT animals. Treatment with metformin restored ATP levels in both WT and KO animals to levels like baseline conditions. In the heart, ATP levels significantly increased in vehicle-treated WT mice after hemorrhagic shock, but not in the KO group, likely suggesting a compensatory mechanism. Treatment with metformin increased cardiac ATP levels in KO animals (Fig. 6). In the kidney and lung, KO mice exhibited similar levels of ATP as WT mice at baseline conditions. There were no changes in ATP content in these organs after hemorrhagic shock in either vehicle-treated WT or KO mice. Because ATP levels in kidney and lung were normal before and after hemorrhagic shock, we did not measure ATP levels in these organs in metformin-treated animals (Fig. 6).

Molecular mechanisms of metformin in the lung

Since metformin afforded beneficial effects also in the absence of a functional AMPK α 1 gene in KO mice, next we sought to investigate the molecular mechanisms of metformin on the catalytic subunits α 1 and α 2 and the upstream kinase LKB1 in the lung. Basal ratio of the nuclear pLKB1/LKB1 levels was lower in KO mice than WT control mice and was unchanged after hemorrhagic shock. Treatment with metformin favored the expression of the active form pLKB1 in the nucleus of both WT and KO mice, while decreasing the ratio of cytosol pLKB1/LKB1 levels in WT mice only. After hemorrhagic shock, there was an increase in the ratio of pAMPK α 1/AMPK α 1 in the cytosol and nucleus in WT mice. Treatment with metformin did not affect levels of AMPK α 1 or pAMPK α 1 in the cytosol; however, it reduced the ratio of nuclear pAMPK α 1/AMPK α 1 because of increased translocation of total AMPK α 1. Expression of both cytosol and nuclear AMPK α 2 was

significantly higher in KO control mice than WT control mice, thus suggesting a compensatory increase in KO mice. Despite this increase in baseline, nuclear baseline ratio of pAMPK α 2/AMPK α 2 was higher in WT mice than KO mice. Interestingly, after hemorrhagic shock, there was a significant increase of the ratio of pAMPK α 2/AMPK α 2 in KO mice, but not WT mice, in the nucleus. Treatment with metformin increased the cytosol and nuclear expression of the pAMPK α 2 in KO mice, while it had the opposite effect in WT mice causing a downregulation of pAMPK α 2. We then evaluated the lung expression of PGC-1 α , which is the main downstream effector of AMPK pathway (5, 6). Expression of both cytosol and nuclear PGC-1 α was significantly higher in KO control mice than WT control mice. After hemorrhagic shock cytosol levels of PGC-1 α were downregulated in KO mice only. Treatment with metformin increased the cytosol expression of PGC-1 α in WT mice, while it had the opposite effect in KO mice causing a further downregulation of the transcription factor. Treatment with metformin increased PGC-1 α nuclear expression in the nucleus of both WT and KO mice (Fig. 7).

Molecular mechanisms of metformin in the liver

Because of the metabolic function of the liver, we also evaluated the molecular mechanisms of metformin on the catalytic subunits α 1 and α 2, the upstream kinase LKB1 and downstream effector PGC-1 α in this organ. Basal levels of the cytosolic LKB1 were lower in KO mice than WT control mice. After hemorrhagic shock, expression of LKB1 increased whereas, the active pLKB1 was downregulated in the cytosol and nucleus in both KO and WT mice. Treatment with metformin increased nuclear expression of the pLKB1 in both groups and in the cytosol of KO mice. After hemorrhagic shock, the ratio of pAMPK α 1/AMPK α 1 increased in the cytosol and in the nucleus in WT mice. Treatment with metformin did not modify the cytosol or nuclear ratio of pAMPK α 1/AMPK α 1 because of a concomitant increase of the content of both the total AMPK α 1 and its active form pAMPK α 1. There were no baseline differences in cytosol and nuclear AMPK α 2 between WT and KO control mice; however, the cytosol baseline ratio of pAMPK α 2/AMPK α 2 was higher in WT mice than KO mice. After hemorrhagic shock, there was no change in the ratio of pAMPK α 2/AMPK α 2 in either WT or KO mice when compared to baseline values of control mice. Treatment with metformin increased the cytosol and nuclear expression of the pAMPK α 2 of both WT and KO mice. However, the degree of cytosolic activation of pAMPK α 2 in KO mice was lower than WT mice. There were no differences in cytosol and nuclear levels of PGC-1 α at baseline. After hemorrhagic shock, PGC-1 α appeared to increase in the cytosol of KO mice. Treatment with metformin did not affect PGC-1 α nuclear translocation in the liver (Fig. 8).

Effect of genetic deficiency of AMPK α 1 and metformin treatment on liver FAD content and lipid peroxidation

Since metformin has been shown to interfere directly with the mitochondrial electron transport chain, thus causing a reduction of reactive oxygen species (21, 22), we next determined the effect of metformin on the liver levels of the complex II redox co-factor FAD and lipid peroxidation. There were no differences in liver levels of FAD at baseline conditions or after hemorrhagic shock. Treatment with metformin significantly increased FAD content in both WT and KO groups when compared to vehicle treatment. In both WT

and KO animals, MDA activity increased significantly following hemorrhagic shock. Treatment with metformin significantly decreased MDA activity in both WT and KO groups when compared to vehicle treatment (Fig. 9).

DISCUSSION

Despite advancements in management, hemorrhagic shock-associated multiple organ failure remains a significant cause of morbidity and mortality. While the exact mechanism of the organ failure is yet to be understood, loss of metabolic capacity is likely a major pathophysiological mechanism (1–3).

We have previously shown that pharmacological activation of AMPK with a synthetic AMP monolog AICAR can exert lung and cardiac protective effects and improve the hemodynamic compensatory mechanisms in a murine hemorrhagic shock model (8, 9). Similarly, we have previously described that treatment with metformin ameliorates myocardial damage in middle-aged (9–12 months old) mice subjected to severe hemorrhage (23). Conversely, in this study, we demonstrated that absence of AMPK α 1 in genetically deficient mice worsened the hemodynamic instability, the systemic inflammatory response, and the liver and lung injury after hemorrhagic shock when compared to mice with a functional AMPK α 1, thus suggesting that AMPK α 1 is an important requisite for regulating the metabolic response as well as the hemodynamic performance during hemorrhagic shock. Further investigating the prospect of targeting AMPK for the treatment of hemorrhagic shock, we also assessed the therapeutic effects of metformin. We demonstrated that metformin reduced organ injury and improved the hemodynamic compensatory response in WT mice. Interestingly, knockdown of the α 1 subunit did not abolish the protective effects of metformin since metformin-treated KO mice also experienced beneficial effects on systemic hypotension and lung and liver injury, although at lower extent when compared to WT mice. Collectively, our data suggest that metformin may exert therapeutic effects also through AMPK α 1-independent mechanisms.

To investigate the mechanisms responsible for the beneficial effects of metformin, we focused on the contribution of AMPK signaling pathway. AMPK is a serine/threonine protein kinase, which serves as a cellular energy sensor, regulating ATP levels through activation of catabolic and inhibition of anabolic processes, including mitochondrial biogenesis (4–7). Although AMPK is ubiquitously expressed, different catalytic isoforms (α 1 and α 2) display tissue-specific distribution and their phosphorylation by upstream kinases is essential for AMPK activation (24). While AMPK α 1 is predominant in the lung and AMPK α 2 is predominant in the heart, AMPK α 1 and α 2-containing complexes account each for about half of total AMPK activity in liver (15, 24). The upstream LKB1 is a critical kinase for phosphorylating and activating AMPK under energy stress conditions (25) and it is required for metformin-mediated AMPK activation (11, 12). Consistent with our previous studies that subcellular localization of AMPK could also have important functional consequences during hemorrhagic shock (8, 9), in the present study we found that there was an increase of nucleo-cytoplasmic shuttling of the active AMPK α 1 in the liver in response to cellular stress of hemorrhagic shock in vehicle-treated WT mice. As expected, treatment with metformin increased expression and activation of AMPK α 1 in both nucleus and cytosol

in WT mice. Although content of LKB1 protein was lower in the cytosol compartment of KO mice, we observed that nucleo-cytoplasmic shuttling and kinetics of activation of LKB1 and AMPK α 2 subunit were similar in WT and KO mice. Treatment with metformin increased the activation of LKB1 and AMPK α 2 in both WT and KO mice, favoring mostly a nuclear compartmentalization. Thus, our data suggest that in the liver, molecular mechanisms of metformin may also involve AMPK α 2 activation. Interestingly, in the lung both the cytosol and nuclear AMPK α 2 were significantly higher in KO control mice than WT control mice, most probably indicating a compensatory increase to overcome the lack of the AMPK α 1 subunit. These data are consistent with other studies showing that AMPK α 2 subunit is able to form various functional active combinations of heterotrimeric complexes with β 1/2 and γ 1/2 subunits in the absence of α 1 subunit (15). Strikingly, the nucleo-cytoplasmic shuttling and kinetics of activation of AMPK α 2 subunit were different between the two groups of mice, since AMPK α 2 was only activated in WT mice but not KO mice after hemorrhagic shock in the lung. Although further activating LKB1 in the lung, treatment with metformin caused only a nuclear translocation of the total AMPK α 1 but, contrary to the effect in KO mice, it failed to activate AMPK α 2 in WT mice. This event paralleled with nuclear translocation of PGC-1 α , which is the master regulator of mitochondrial biogenesis and downstream effector of the AMPK pathway (5, 6). Thus, our data suggest that in the lung, molecular mechanisms of metformin may be predominantly AMPK α 1-dependent. Furthermore, our findings paint a complex and organ-specific dynamic nucleo-cytoplasmic regulation of LKB1/AMPK pathway, which may depend on the metabolic need of the tissue.

In our study, genetic deficiency of AMPK α 1 was also associated with an exaggerated inflammatory response as demonstrated by high levels of pro-inflammatory cytokines in KO when compared to WT mice, thus suggesting that AMPK α 1 may also exert a regulatory role in the innate immune response. Surprisingly, treatment with metformin did not affect cytokine production in either WT or KO mice. These findings are in discrepancy with our previous study demonstrating that treatment with AICAR, another AMPK activator, significantly reduced elevation of several inflammatory cytokines in the plasma and in the bronchial alveolar lavage fluid in a model of hemorrhage-induced lung injury (8). Other *in vitro* studies have also suggested that metformin may have anti-inflammatory effects in hepatocytes and macrophages by specifically blunting secretion of pro-inflammatory cytokines (26). Although we could not find any effect of metformin on cytokines, we cannot rule out other potential anti-inflammatory effects of the drug. For example, it has been recently reported that metformin may directly bind the pro-inflammatory alarmin high mobility group box 1, thus neutralizing the deleterious effects of this alarmin (27). Therefore, further investigation is warranted to understand the potential anti-inflammatory mechanisms of the drug.

To further evaluate the biological function of the metformin-induced nucleo-cytoplasmic regulation of LKB1/AMPK pathway we evaluated the effect on ATP content. In support to the metabolic role of AMPK α 1 in energy metabolism during stress (4), we observed that without a functionally active AMPK α 1 subunit KO mice had higher plasma lactate levels than WT mice, although maintaining normal ATP levels in liver, heart, kidney and lung as WT mice under basal conditions. Taken together with the findings of normal tissue ATP concentrations, the elevated lactate accumulation seen in these KO mice may be

compensatory events attempting to normalize the energy homeostasis. However, we observed that AMPK α 1 genetic deficiency considerably compromised oxidative metabolism in the liver after stress, since KO mice exhibited worse energy failure than WT mice after hemorrhagic shock. Most importantly, KO mice lost the capability to manage a compensatory increase in ATP levels in the heart, but not lung and kidney, which was consistent with a more severe hypotension in KO mice than WT mice after hemorrhagic shock. Metformin treatment increased both liver and cardiac energy production. Notably, although lactic acidosis is a severe adverse effect of biguanides (20), metformin treatment did not increase lactate levels, thus indicating that the drug may be safely used in hemorrhagic shock. Whether this effect is secondary to efficient metabolic switching from mitochondrial-derived glucose oxidation to cytoplasmic-derived anaerobic glycolysis deserves further investigation.

The energy producing processes depend on electrons supplied to the mitochondrial electron transport chain in the form of reducing equivalents of nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FADH₂). The capacity of the cell to use these substrates efficiently is thus critical for its ability to adapt to changing environmental conditions and stress (28). In this context, several studies have reported that metformin selectively blocks the reverse electron flow through the electron respiratory chain Complex I, without affecting the downstream oxidative phosphorylation machinery (21, 22). This inhibitor effect on Complex I has been associated with potential redirection of substrate flux feeding into Complex II and increased mitochondrial function in conditions of stress (29). In our study, treatment with metformin increased levels of Complex II substrate FAD in WT and KO mice, thus supporting the hypothesis that metformin can shift oxidative metabolism in the electron respiratory chain from Complex I to Complex II for energy homeostasis independently of AMPK α 1. Since inhibition of Complex I by metformin has also been associated with reduced production of mitochondrial reactive oxygen species, which are potent mediators of cell injury (21), we also sought to determine the potential effects of metformin on liver lipid peroxidation. In our study, treatment with metformin significantly reduced the hemorrhage-induced lipid peroxidation in the liver of both WT and KO animals, thus suggesting potential anti-oxidant effects of metformin independently of AMPK α 1. These findings are consistent with studies reporting that metformin may hamper ROS production in hepatocytes *in vitro* (22) and may increase *in vivo* the antioxidant system in kidneys in a rat model of gentamicin toxicity (30).

Our *in vivo* model of hemorrhagic shock has limitations, potentially influencing the interpretation of our data. Firstly, it should be noted that our hemorrhagic shock model does not have any associated traumatic injury, therefore it does not replicate the clinical scenario of trauma victims. However, we chose a fixed-pressure controlled model because of the advantage of experimental standardization and reproducibility without confounding variables of severity of trauma and coagulation process (16, 17, 31). Furthermore, it should be considered that there are also medical conditions of severe acute bleeding in absence of traumatic events, such excessive bleeding from the gastrointestinal tract, from the uterus in women, and other excessive spontaneous bleeding in patients with hematological disorders. Therefore, our data, although not generalizable to clinical conditions involving trauma, may provide mechanistic information on the pathophysiology of the single hemorrhage injury. To

this goal, this controlled model is widely used to study the pathophysiology mechanisms of organ damage secondary to maladaptive decompensatory hypotension (16, 17, 31). Secondly, our resuscitation strategy may not reflect the current practice of treatment after severe trauma and blood loss in humans. Although there are numerous resuscitation methods in association with hemorrhagic shock models, a fixed volume resuscitation method of Lactated Ringer solution, equal to two-four times the shed blood volume, has been extensively used in experimental models to study mechanisms of organ injury and systemic inflammation (16, 31). In our model, we adopted a resuscitation strategy combining both administration of blood and crystalloid transfusion, a regimen that allows to reach a target blood pressure of 70–80 mmHg (32), but does not replicate clinical practices of restrictive resuscitation or permissive hypotension (33). Therefore, we recognize that uncontrolled hemorrhage models, especially combined with other traumas and different resuscitation fluids, are more clinically relevant and should be adopted for further preclinical testing of treatment strategies with metformin. Thirdly, we did not investigate long-term effects of metformin treatment. Nevertheless, a recent study from our laboratory has demonstrated that a single intra-arterial bolus of metformin at the time of fluid resuscitation significantly improved survival up to seven days in C57BL/6 middle-aged, but not young mice, subjected to hemorrhagic shock (30). Additional long-term studies are necessary to confirm the potential therapeutic effects of metformin.

In conclusion, despite the limitations of our model, our data suggests that AMPK α 1 plays a crucial role in maintaining hemodynamic stability and allowing organ metabolic recovery during hemorrhagic shock. Our results raise also the possibility that treatment with metformin, a widely-used agent used in clinical practice for diabetes type II, may provide organ protection effects in hemorrhagic shock. The molecular mechanism of actions of the drug may involve distinct AMPK α 1-dependent and independent targets, which may be also organ specific. Whether metformin has the potential to be useful in the clinical treatment of trauma and shock needs further investigation.

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FIG. 1. Effect of AMPK α 1 deficiency and metformin treatment on mean arterial blood pressure (A) and plasma levels of lactate (B)

Each data represents the mean $\pm$ SEM of 5–10 animals for each group. Vehicle (normal saline) or metformin (100 mg/kg) was administered intraperitoneally at the time of resuscitation. Arrows in panel A indicate time of induction of hemorrhage and initiation of resuscitation and metformin or vehicle administration. * P <0.05 vs baseline values at time 0 of the same group; ‡ P <0.05 vs WT group; # P <0.05 vs vehicle-treated group of the same genotype.

FIG. 2. Representative histology photomicrographs of lung sections

Normal lung architecture in control WT (A) and KO (D) presenting patent alveoli, and vessels with a few or no adhering neutrophils. Lung damage in vehicle-treated WT (B) and KO mice (E) after hemorrhagic shock with mild injury in WT mice but severe reduction of alveolar space and neutrophil adhesion along vascular wall and infiltration of inflammatory cells in KO mice. Amelioration of lung architecture in metformin-treated WT (C) and KO mice (F) after hemorrhagic shock. Magnification x400. A similar pattern was seen in n=4–6 different tissue sections in each experimental group.

FIG. 3. Representative histology photomicrographs of liver sections

Normal lung architecture in control WT (A and D) and KO (G and J). Liver damage in vehicle-treated WT (B and E) and KO mice (H and K) after hemorrhagic shock with light edema in WT mice but moderate congestion and infiltration of inflammatory cells in KO mice. Amelioration of liver architecture in metformin-treated WT (C and F) and KO mice (I and L) after hemorrhagic shock. Magnification $\times 100$ for A, B, C, G, H and I, magnification $\times 400$ for D, E, F, J, K and L. A similar pattern was seen in $n=4-6$ different tissue sections in each experimental group.

FIG. 4. Effect of AMPK α 1 deficiency and metformin treatment on myeloperoxidase activity in the lung, (A) liver (B), and kidney (C)

Each data represents the mean $\pm$ SEM of 5–8 animals for each group. * $P < 0.05$ vs control group; $\ddagger P < 0.05$ vs WT group; # $P < 0.05$ vs vehicle-treated group of the same genotype.

FIG. 5. Effect of AMPK α 1 deficiency and metformin treatment on plasma levels of IL-1 β (A), IL-6 (B), IL-10 (C), TNF- α (D), KC (E), IL-17 (F)
 Each data represents the mean $\pm$ SEM of 6–8 animals for each group. * P <0.05 vs control group; ‡ P <0.05 vs WT group.

FIG. 6. Effect of AMPK α 1 deficiency and metformin treatment on ATP levels in the liver (A), heart (B), kidney (C) and lung (D)

Each data represents the mean $\pm$ SEM of 5–7 animals for each group. * $P < 0.05$ vs control group; ‡ $P < 0.05$ vs WT group; # $P < 0.05$ vs vehicle-treated group of the same genotype. ND = Not determined.

FIG. 7. Western blot analysis of LKB1, pLKB1, AMPK α 1, pAMPK α 1, AMPK α 2, pAMPK α 2, PGC-1 α and β -actin (used as loading control protein) in lung cytosol and nuclear extracts (A). Image analyses of cytosol and nuclear ratio of relative intensity pLKB1/LKB1 (B and C), AMPK α 1/pAMPK α 1 (C and D), AMPK α 2/pAMPK α 2 (E and F), and expression of cytosol PGC-1 α (G) and nuclear PGC-1 α (H) as determined by densitometry. Each data represents the mean $\pm$ SEM of 5–7 animals for each group. * P <0.05 vs control group; ‡ P <0.05 vs WT group; # P <0.05 vs vehicle-treated group of the same genotype.

FIG. 8. Western blot analysis of LKB1, pLKB1, AMPK α 1, pAMPK α 1, AMPK α 2, pAMPK α 2, PGC-1 α and β -actin (used as loading control protein) in liver cytosol and nuclear extracts (A). Image analyses of cytosol and nuclear ratio of relative intensity pLKB1/LKB1 (B and C), AMPK α 1/pAMPK α 1 (C and D), AMPK α 2/pAMPK α 2 (E and F), and expression of cytosol PGC-1 α (G) and nuclear PGC-1 α (H) as determined by densitometry. Each data represents the mean $\pm$ SEM of 5–7 animals for each group. * $P < 0.05$ vs control group; ‡ $P < 0.05$ vs WT group; # $P < 0.05$ vs vehicle-treated group of the same genotype.

FIG. 9. Effect of AMPK α 1 deficiency and metformin treatment on FAD content (A) and MDA activity (B) in the liver

Each data represents the mean $\pm$ SEM of 5–7 animals for each group. * $P < 0.05$ vs control group; # $P < 0.05$ vs vehicle-treated group; ‡ $P < 0.05$ vs metformin-treated group.