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Original Article

Formoterol promotes mitochondrial biogenesis, improves liver regeneration, and suppresses liver injury and inflammation after liver resection in mice with endotoxemia

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Abstract: Objectives: Clinically, liver regeneration is often impaired by infections causing endotoxemia, although mechanisms are unclear. Since energy supply is essential for liver regeneration, we assessed whether formoterol (FMT), a β_2-adrenergic agonist that increases peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), the master regulator of mitochondrial biogenesis (MB), restores liver regeneration after partial hepatectomy (PHX) in endotoxin (LPS)-treated mice. Methods: Mice underwent sham-operation, two-thirds PHX, PHX with LPS injection (PHX+LPS, 5 mg/kg, *i.p.*), or PHX+LPS followed by FMT (0.1 mg/kg, *i.p.*) after 2 h. Results: At 48 h after PHX, 5'-bromo-2'-deoxyuridine incorporation, mitotic cells, proliferating cell nuclear antigen, and cyclin-D1 markedly increased, signifying liver regeneration. By contrast, after PHX+LPS, liver regeneration was almost completely suppressed. FMT restored liver regeneration after PHX+LPS. PGC1α, mitochondrial transcription factor-A (controlling mitochondrial DNA replication/transcription), and mitochondrial oxidative phosphorylation proteins ATP synthase-β and NADH dehydrogenase-3 decreased after PHX+LPS, signifying suppressed MB. FMT largely reversed these effects. Mitochondrial oxidative stress stimulates inflammation by activating inflammasomes. In addition to promoting MB, PGC1α reportedly inhibits oxidative stress and inflammation. 8-Hydroxy-deoxyguanosine, NLRP3, and inflammatory cytokines increased after PHX+LPS, demonstrating increased oxidative stress and inflammasome activation. Many necro-inflammatory foci occurred in liver sections after PHX+LPS. FMT increased expression of antioxidant protein thioredoxin-2, decreased oxidative stress, and blunted inflammatory responses. Additionally, FMT decreased alanine aminotransferase release and necrosis caused by PHX+LPS. Conclusions: FMT restores liver regeneration during endotoxemia and decreases liver injury and inflammation, most likely by increasing PGC1α. Therefore, FMT is a promising therapy for liver failure caused by loss of liver mass complicated with sepsis.

Keywords: Endotoxin, formoterol, liver regeneration, liver resection, mitochondrial biogenesis, oxidative stress

Introduction

In clinical settings, loss of liver mass occurs in many surgical procedures. Severe liver trauma, especially those ballistic in nature, typically necessitate immediate surgical intervention to stop bleeding, remove severely injured or nonviable tissue, and repair injury to vessels and the biliary system [1, 2]. Major liver resection is often performed in treatment for malignant and benign liver tumors and other liver lesions (*e.g.*, parasitic cysts, intrahepatic gallstones, etc.) [3-5]. Major liver resection also occurs in living liver donation, and partial liver transplantation continues to increase as a means to alleviate the severe shortage of donor livers [6]. In addition, massive liver damage occurring in non-surgical situations (*e.g.*, toxicant/drug-induced liver injury) also leads to loss of liver mass. Under all these conditions, liver regeneration is required to restore liver mass and function.

Although the liver typically regenerates to a functional organ after resection, liver dysfunction and failure occur under many circumstances, including a small remnant liver, pre-existing

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liver disease, and significant intra- or perioperative factors, such as infection, post-operative hemorrhage, and bile leakage. These conditions can all lead to delayed recovery or even mortality [7-9]. Small-for-size syndrome occurs after major liver resections and partial liver transplantation [10]. Clinically, the danger of liver failure increases when greater than 50% of the liver is removed [8]. After transplantation of small-for-size liver grafts that cannot meet the recipients' metabolic demands (often the consequence of using a left lobe from a living donor or in split liver transplantation), the risk of graft failure markedly increases [9]. Histopathologically, liver failure following hepatectomy shows failure of cell proliferation, cholestatic changes, and death of hepatocytes [11]. Clinically, liver failure patients exhibit progressive hyperbilirubinemia, coagulopathy, encephalopathy, and eventually death [11].

Other factors can also affect clinical outcomes, such as infection, age, pre-existing liver disease, post-operative hemorrhage, bile leakage, kidney failure, as well as others [12, 13]. After liver resection, infection may occur in the incision wound, intraperitoneal space (*e.g.*, subphrenic abscess), urinary tract, and the pulmonary system (*e.g.*, pneumonia) [12, 14]. Septicopyemia or septicemia may also develop if infection is severe [12]. Infection is more prevalent in cases of liver trauma. In addition, increased gut permeability, slow gut movement, and small intestinal bacterial overgrowth are common phenomena following major abdominal surgical procedures [15, 16]. Such changes enhance the movement of pathogens and pathogen-associated molecules (*e.g.*, endotoxin, bacterial DNA) from the intestinal lumen into blood stream. Indeed, major abdominal surgery is associated with transient endotoxemia and reduced endotoxin inactivation capacity of the plasma [17].

When patients with decreased liver mass have an infection or translocation of pathogen-associated molecules from the gut into blood, their livers experience a second "hit" promoting inflammatory responses. Endotoxin, a toxin from gram-negative bacteria, inhibits liver regeneration [6, 18, 19]. However, another study shows that pretreatment with endotoxin 24 hour (h) before partial hepatectomy (PHX) increases production of hepatotrophic factors that favor liver regeneration [20]. Therefore, more research is needed to elucidate whether endotoxin increases or hinders hepatic regeneration after injury or resection.

Sufficient energy supply is essential for liver regeneration. ATP not only supports synthetic processes during cell proliferation but also controls regenerative signaling [21, 22]. However, mitochondrial dysfunction reportedly occurs after massive liver resection and transplantation of small-for-size liver grafts [23, 24]. Therefore, we hypothesize that endotoxemia exacerbates mitochondrial dysfunction after liver resection or partial liver transplantation to inhibit liver regeneration, leading to acute liver failure (ALF).

Currently, approaches to treat ALF are mainly supportive with the aim of bridging patients to transplantation (*e.g.,* bioartificial liver assistance devices) [25, 26]. However, donor livers for transplantation are in severe shortage [27]. In the U.S., spontaneous survival after ALF is only about 45% [28]. Therefore, effective strategies to prevent/treat ALF in situations of loss of liver mass are urgently needed. We hypothesize that stimulation of mitochondrial biogenesis (MB), a process that increases mitochondrial ATP-producing capacity [29], improves mitochondrial function, thereby enhancing liver regeneration and survival after liver resection complicated with endotoxemia. Here, we examined whether formoterol (FMT), a β_2-adrenergic agonist that stimulates MB [30, 31], improves outcomes of PHX in mice with endotoxemia and explored the underlying mechanism of action.

Materials and methods

Materials

The sources of all chemicals, antibodies, and other reagents are listed in Table S1 in "Supplemental Information".

Animal model

Male C57BL/6 mice (7-8 weeks old, Jackson Laboratory, Bar Harbor, ME) were anesthetized by inhalation of 2-3% isoflurane and subjected to either sham-operation or PHX removing two-thirds of the liver mass, a model that induces robust liver regeneration in rodents [23, 32]. For PHX after a midline laparotomy, ligaments

around the liver were freed, and the left lateral, right, and caudate lobes were ligated with 5-0 silk suture at their bases and then resected [33]. For sham-operation, ligaments around the liver were freed, and the abdomen was closed 20 min later without PHX. Immediately after surgery, mice were injected intraperitoneally with endotoxin (LPS, Table S1) dissolved in normal saline (NS, 0.5 mg/mL) at a dosage of 5 mg/kg of body weight or an equal volume of NS. At 2 h after surgery, mice were injected with FMT intraperitoneally at a dosage of 0.1 mg/kg of body weight or with an equal volume of NS. The control group received sham-operation and NS treatments immediately and at 2 h after surgery. At 48 h after surgery, mice were anesthetized with ketamine/xylazine (90 mg/kg and 10 mg/kg, *i.p.*), blood was collected from the inferior vena cava, and livers were infused with 2 mL of NS via the portal vein to remove blood in the liver and then harvested. Mice were euthanized by cervical dislocation immediately after liver harvest. All animals were given humane care in compliance with institutional guidelines using protocols pre-approved by the Institutional Animal Care and Use Committee.

Serum alanine aminotransferase and histology

Serum from vena caval blood was isolated by centrifugation and stored at -80°C until use. Serum alanine aminotransferase (ALT) activity was determined using a commercial kit (Table S1) according to the manufacturer's instructions.

After harvest, portions of liver samples were fixed in neutralized 10% formaldehyde in NS for 24-48 h. Paraffin-imbedded liver samples were cut at a thickness of 5 μm. Liver sections were stained with hematoxylin and eosin (H&E) for histological analysis. Liver images were acquired using a Zeiss AX10 microscope (White Plains, NY) with 20× and 40× objective lenses.

5-Bromo-2'-deoxyuridine incorporation test and 8-hydroxy-deoxyguanosine immunostaining

Cell proliferation in liver tissue, a widely used indicator of liver regeneration, was assessed by 5-bromo-2'-deoxyuridine (5-BrdU) incorporation. At 2 h prior to liver harvest, 5-BrdU solution (2 mg/mL in NS) was injected into mice at a dose of 20 mg/kg, and livers were harvested and processed for 5-BrdU immunohistochemical staining (IHC). Tissue was also immunostained for 8-hydroxy-deoxyguanosine (8-OHdG), an indicator of DNA oxidation [34]. IHC procedures are described in "Supplemental Information".

Quantitative real-time PCR

To assess the effects of FMT on MB, mRNAs of peroxisome proliferator-activated receptor-γ coactivator-1 alpha (PGC1α), mitochondrial transcription factor-A (TFAM), ATP synthase-β (ASβ), and NADH dehydrogenase-3 (ND3) were detected by quantitative real-time PCR. Total RNA was isolated from the liver tissue using Trizol reagent, and cDNA was synthesized from the isolated mRNA (2 μg) using a Bio-Rad iScript cDNA Synthesis kit (Table S1), as described previously [35]. Primers for the mRNAs of PGC1α, TFAM, ASβ, and ND3 were designed and synthesized by Integrated DNA technologies (IDT, Coralville, IA). The primer sequences are listed in Table S2. Real-time PCR was performed using iQ™ SYBR Green Supermix and a Bio-Rad CFX 96 Real Time PCR System as described [35]. The results were normalized to the expression of hypoxanthine phospho-ribosyl-transferase (HPRT) housekeeping gene using the $\Delta\Delta Ct$ method [35].

Immunoblotting

For immunoblotting, liver tissues (~50 mg) were homogenized in lysis buffer (500 μL, Table S1) with 1% protease and phosphatase inhibitors. Immunoblotting procedures together with original blot images are provided in the "Supplemental Information".

Detection of interleukin-1β, tumor necrosis factor-α, and 8-hydroxy-2'-deoxyguanosine by ELISA

Liver tissues were homogenized in lysis buffer (Table S1), and protein was detected using a Pierce BCA protein assay kit (Table S1) according to the manufacturer's instructions. Interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), and 8-OHdG were detected using ELISA kits according to the manufacturers' instructions (Table S1). Concentrations of IL-1β, TNF-α, and 8-OHdG were normalized to protein concentrations in liver lysates.

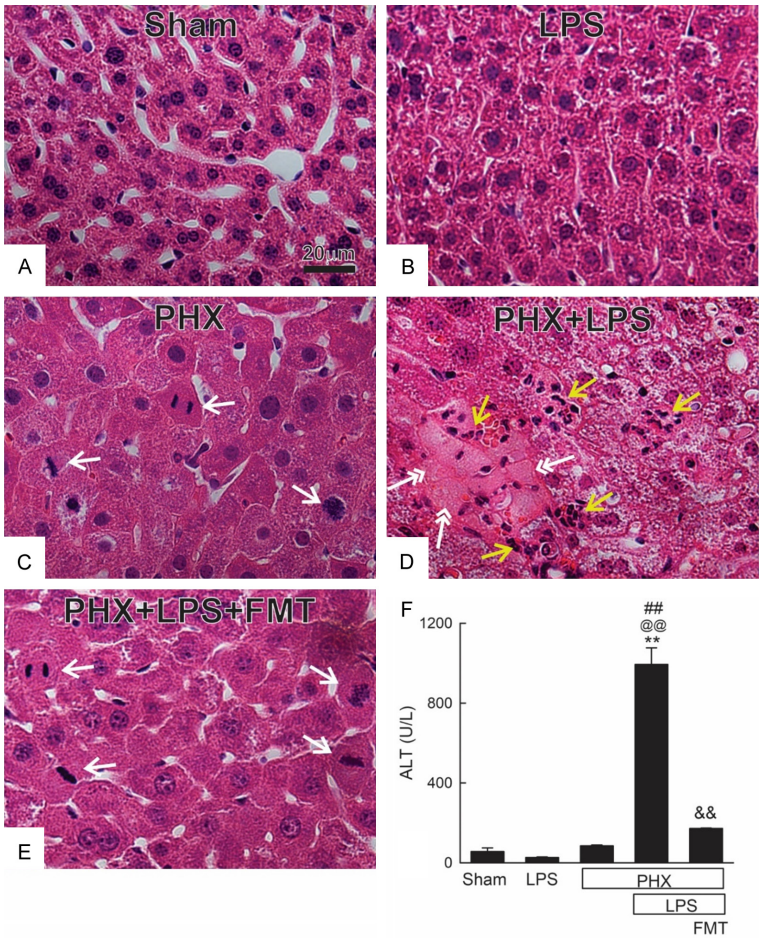

Figure 1. Endotoxemia increases liver injury and inflammation but inhibits liver regeneration after partial hepatectomy: blunting by formoterol. Mice underwent sham-operation or PHX surgeries and were treated with LPS, FMT or vehicle, as described in MATERIALS AND METHODS. Livers and blood were harvested at 48 h after surgery. A-E. Representative images of liver sections with H&E staining (200× magnification; n = 3-4 per group). White single arrowheads, mitotic figures; white double arrowheads, necrotic cells; yellow arrows, leukocytes. F. Serum ALT. Values are means ± S.E.M. **, P < 0.01 vs sham; @@, P < 0.01 vs LPS; ##, P < 0.01 vs PHX; &&, P < 0.01 vs PHX+LPS by ANOVA with Student-Newman-Keuls post-hoc test, n = 3-4 per group.

Detection of endotoxin in sera

In some mice, blood was collected at 6 h after LPS injection. LPS in sera was detected using an ELISA kit (Table S1) according to manufacturer's instructions.

Statistical analysis

Data shown are means ± S.E.M. (3-4 mice per group). ANOVA plus Student-Newman-Keuls post-hoc test were used for comparison of three or more groups, and student's t-test was used for comparison of two groups. Statistical analyses were conducted using Sigmastat software. Differences were considered significant at $P < 0.05$.

Results

Endotoxemia increases liver injury and inflammation after partial hepatectomy: blunting by formoterol

After sham-operation, no histological alterations of livers were observed (**Figure 1A**). After injection of a low dose of LPS alone, serum LPS increased significantly, as measured after 6 h, which demonstrated the occurrence of endotoxemia (Figure S1, Supplemental Information). At 48 h after this low dose of LPS, hepatic leukocyte infiltration and cell necrosis were not apparent histologically (**Figure 1B**). After PHX, cell necrosis occurred rarely, and infiltration of leukocytes was minimal (**Figure 1C**). After PHX+LPS, widespread cellular swelling, small necrotic foci shown by pyknotic nuclei or loss of nuclei (**Figure 1D**, white double arrowheads), and leukocyte infiltration (**Figure 1D**, yellow arrows) all occurred. FMT treatment decreased cell swelling, necrosis and inflammation after PHX+LPS (**Figure 1E**).

Serum ALT, an indicator of liver injury, did not change at 48 h after LPS treatment or PHX (**Figure 1F**). By contrast, after PHX+LPS, ALT increased markedly from 56 U/L to 994 U/L, indicating liver injury (**Figure 1F**). With FMT treatment, serum ALT only increased to 172/U/L after PHX+LPS.

Endotoxemia suppresses liver regeneration after partial hepatectomy: reversal by formoterol

Liver regeneration involves cell proliferation and mitosis in liver tissue. After sham-operation and LPS treatment alone, mitotic nuclear

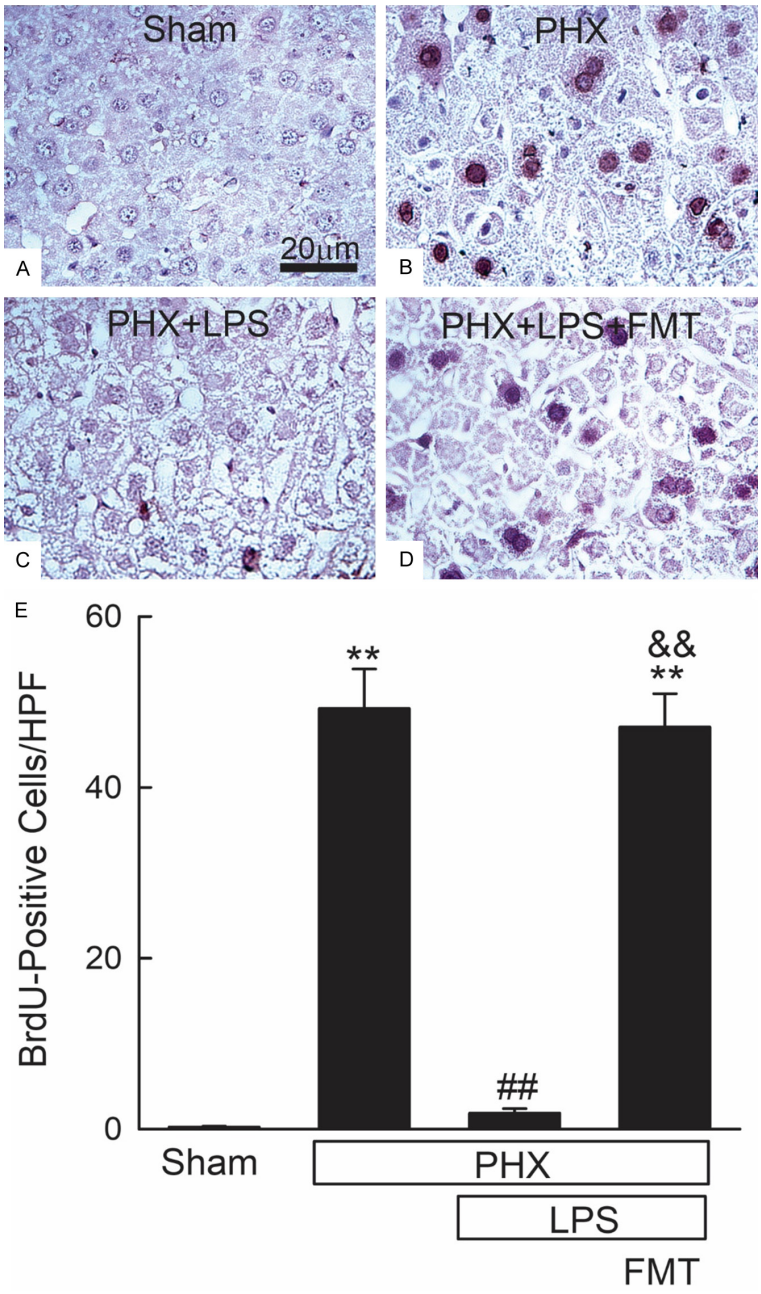

Figure 2. Endotoxemia inhibits 5-bromo-2'-deoxyuridine incorporation after partial hepatectomy: blunting by formoterol. Mice underwent sham-operation or PHX surgeries and were treated as described in **Figure 1**. Livers were harvested at 48 h after surgery. A-D. Representative images of liver sections after 5-BrdU IHC staining (200× magnification). E. quantification of 5-BrdU positive cells/HPF (under a 20× objective lens). Values are means ± S.E.M. **, $P < 0.01$ vs sham; ##, $P < 0.01$ vs PHX; &&, $P < 0.01$ vs PHX+LPS by ANOVA with Student-Newman-Keuls post-hoc test, n = 3-4 per group.

figures signifying proliferating cells undergoing mitosis were very rarely observed (**Figure 1A** and **1B**). By contrast, at 48 h after PHX, mitotic figures at various stages of mitosis were frequently observed (**Figure 1C**, white single arrowheads). By contrast, mitosis was much less frequent after PHX+LPS (**Figure 1D**), which FMT restored (**Figure 1E**).

Incorporation of 5-BrdU into DNA during the S-phase of the cell cycle is an indicator of cell proliferation. Nuclear 5-BrdU-positive cells in the liver were rarely observable (0.2 cells/high power field [HPF]) in sham-operated mice (**Figure 2A** and **2E**). After PHX, nuclear 5-BrdU-positive cells increased markedly to 49 cells/HPF (**Figure 2B** and **2E**). After PHX+LPS, nuclear 5-BrdU-positive cells were only 1.9 cells/HPF (**Figure 2C**, **2E**). FMT restored nuclear 5-BrdU-positive cells after PHX+LPS to 47 cells/HPF (**Figure 2D** and **2E**).

Proliferating cell nuclear antigen (PCNA) acts as a sliding clamp to increase the processivity of DNA polymerases in DNA replication and is also used as an index of cell proliferative activity [36]. By immunoblotting, PCNA protein expression was barely detectable in sham-operated livers, but after PHX, PCNA increased 3550% (**Figure 3A** and **3B**). However, after PHX+LPS treatment, PCNA failed to increase (**Figure 3A** and **3B**). FMT recovered the increase of PCNA after PHX+LPS to a level close to PHX alone (2720%, **Figure 3A** and **3B**).

Cyclins increase during cell proliferation to drive cell cycle progression by binding to and activating specific cyclin-dependent kinases [37]. Cyclin D1 protein expression increased ~820% after PHX but did not increase after PHX+LPS (**Figure 3A** and **3C**). With FMT treatment, the increase of cyclin D1

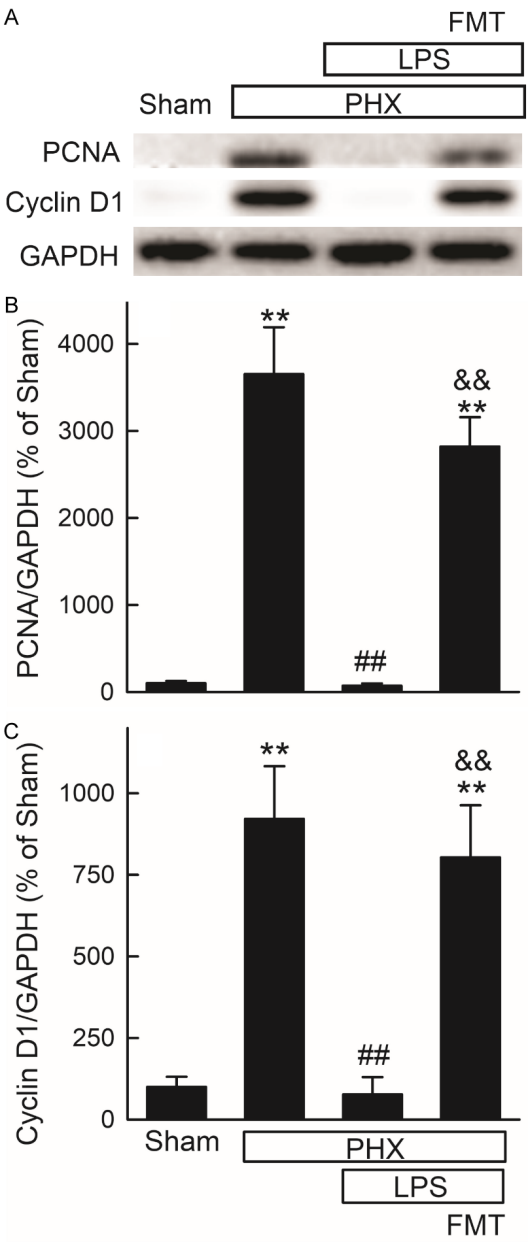

Figure 3. Endotoxemia inhibits cell cycle progression after partial hepatectomy: prevention by formoterol. Mice underwent sham-operation or PHX surgeries and were treated as described in **Figure 1**. Livers were harvested at 48 h after surgery. Proteins were detected by immunoblotting. A. Representative immunoblot images of PCNA, cyclin D1, and housekeeping protein GAPDH. B and C. Quantification of PCNA and cyclin D1 immunoblot images by densitometry. Values are means ± S.E.M. **, $P < 0.01$ vs sham; ##, $P < 0.01$ vs PHX; &&, $P < 0.01$ vs PHX+LPS by ANOVA with Student-Newman-Keuls post-hoc test, n = 3-4 per group.

expression was restored after PHX+LPS to 703% (**Figure 3A** and **3C**).

Endotoxemia suppresses mitochondrial biogenesis after partial hepatectomy: recovery by formoterol

Energy supply is essential to support liver regeneration. Therefore, we assessed MB after PHX. As assessed by qPCR, mRNA for PGC1α, the master regulator of MB [38], was not significantly different after PHX compared to sham-operation but decreased by 53% after PHX+LPS ($P < 0.01$) (**Figure 4A**). TFAM, a transcription factor that controls mitochondrial DNA replication and transcription [38], also did not change significantly after PHX but decreased by 58% after PHX+LPS (**Figure 4B**). FMT treatment during PHX+LPS restored PGC1α and TFAM mRNA to 90% and 87%, respectively, of that in sham-operated mice (**Figure 4A** and **4B**).

mRNAs for ASβ, a nuclear DNA (nDNA)-encoded mitochondrial oxidative phosphorylation (OXPHOS) enzyme, and ND3, a mitochondrial DNA (mtDNA)-encoded OXPHOS enzyme [38], did not increase significantly after PHX but decreased by 61% and 54%, respectively, after PHX+LPS (**Figure 4C** and **4D**). FMT increased ASβ and ND3 mRNAs after PHX+LPS back to 87% and 100%, respectively, of the control levels in sham-operated mice (**Figure 4C** and **4D**). Protein expression of ASβ and ND3 paralleled changes of mRNA levels and slightly increased after PHX but markedly and statistically significantly decreased after PHX+LPS (**Figure 5A-C**). FMT completely restored expression of ASβ and ND3 after PHX+LPS to control levels (**Figure 5A-C**).

Formoterol decreases oxidative stress caused by PHX and LPS treatment

Endotoxin reportedly causes mitochondrial oxidative stress, thus leading to mitochondrial dysfunction and cellular damage in conditions like sepsis [39]. 8-OHdG is a widely used indicator of DNA oxidation and mitochondrial oxidative stress [34]. As assessed by IHC, PHX slightly increased 8-OHdG staining both in cytoplasm and to a lesser extent in nuclei, as compared to sham-operation (**Figure 6A** and **6B**). By contrast, after PHX+LPS, 8-OHdG markedly increased in both cytoplasm and nuclei. 8-OHdG staining in the cytoplasm is most likely mtDNA since the only DNA normally in the cytoplasm is mtDNA. These data support DNA oxi-

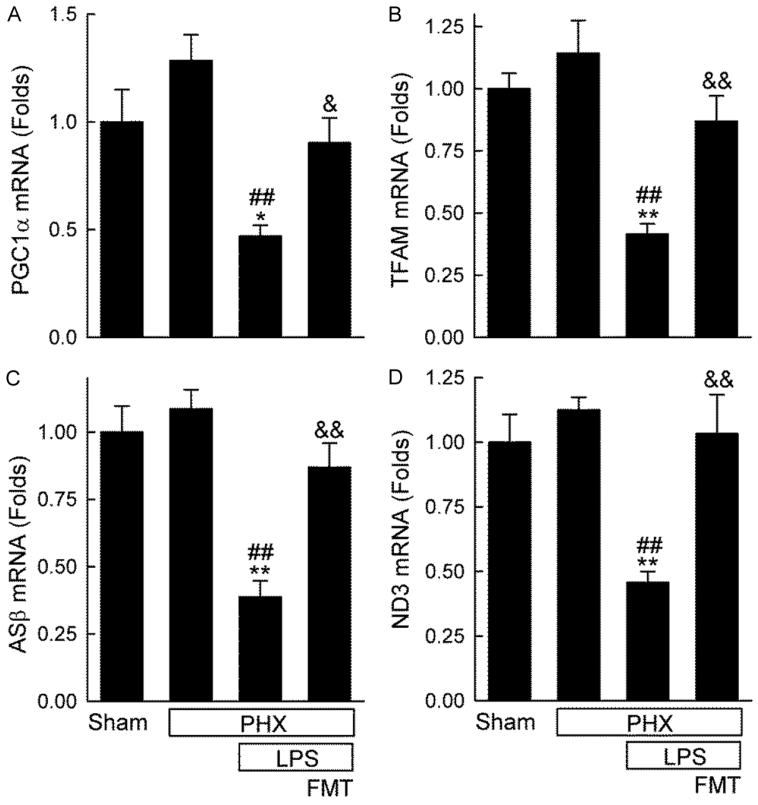

Figure 4. Endotoxemia decreases mRNAs of mitochondrial biogenesis regulators and oxidative phosphorylation proteins after partial hepatectomy: prevention by formoterol. Mice underwent sham-operation or PHX surgeries and were treated as described in **Figure 1**. Livers were harvested at 48 h after surgery. mRNAs were detected by qPCR. A. mRNA of peroxisome proliferator-activated receptor-γ coactivator-1 alpha (PGC1α). B. mRNA of mitochondrial transcription factor-A (TFAM). C. mRNA of ATP synthase-β (ASβ). D. mRNA of NADH dehydrogenase-3 (ND3). Values are means ± S.E.M. **, P < 0.01 vs sham; ##, P < 0.01 vs PHX; &, P < 0.05 and &&, P < 0.01 vs PHX+LPS by ANOVA with Student-Newman-Keuls post-hoc test, n = 3-4 per group).

dation occurring in both mitochondria and nuclei (**Figure 6C**). With FMT treatment, 8-OHdG in both mitochondria and nuclei substantially decreased (**Figure 6D**). 8-OHdG was also detected by ELISA. Consistent with the results of IHC staining, 8-OHdG slightly increased after PHX but increased markedly (> 3.5-fold) after PHX+LPS (**Figure 6E**). FMT after PHX+LPS blunted the increase of 8-OHdG to the same levels as after PHX without LPS (**Figure 6E**), indicating an inhibition of oxidative stress.

Thioredoxin-2 (TRX2) is an antioxidant mitochondrial protein that suppresses mitochondrial reactive oxygen species (ROS) generation [40]. TRX2 protein expression by immunoblotting did not change significantly after PHX but decreased 53% after PHX+LPS (**Figure 7**). FMT prevented the decrease of TRX2 after PHX+LPS (**Figure 7**).

Formoterol suppresses inflammasome activation and proinflammatory cytokine formation after PHX plus LPS treatment

Pathogen-derived signals, ROS, mitochondrial stress/damage, and metabolic disorders all stimulate activation of inflammasomes, which are multiprotein complexes within cells that play a crucial role in the innate immune system, specifically in initiating inflammatory responses and cell death. Inflammasome activation leads to maturation and secretion of proinflammatory cytokines, such as interleukin-1 and interleukin -18 (IL-1 and IL-18) [41]. In addition, pathogen-derived signals, such as endotoxin, stimulate production of proinflammatory cytokines by binding to toll-like receptor-4 [42]. In sham-operated mice, NOD-like receptor protein 3 (NLRP3), a key component of inflammasomes, was barely detectable in the liver by immunoblotting (**Figure 8A**). PHX alone did not change NLRP3 (**Figure 8A** and **8B**). By contrast after PHX+LPS, NLRP3 increased dramatically by 6520% (**Figure 8A** and **8B**). FMT totally blocked the increase of NLRP3 after PHX+LPS.

Proinflammatory cytokine IL-1β increased from 53 pg/mg protein after sham-operation to 216 pg/mg after PHX and 426 pg/mg after PXH+LPS (**Figure 8C**). With FMT treatment, IL-1β only increased to 146 pg/mg after PXH+LPS (**Figure 8C**). TNFα in the liver similarly increased from a basal level of 140 pg/mg after sham-operation to 267 pg/mg after PHX and further increased to 628 pg/mg after PHX+LPS (**Figure 8D**). FMT again decreased TNFα levels after

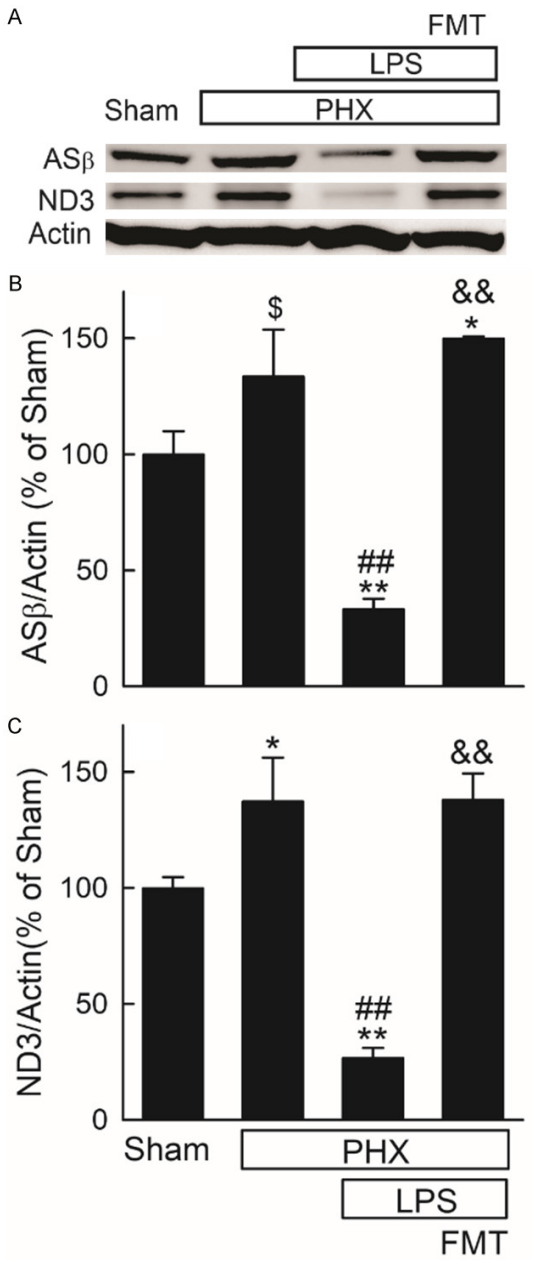

Figure 5. Endotoxemia decreases oxidative phosphorylation proteins after partial hepatectomy: prevention by formoterol. Mice underwent sham-operation or PHX surgeries and were treated as described in **Figure 1**. Livers were harvested at 48 h after surgery. Proteins were detected by immunoblotting. A. Representative immunoblot images of ASβ, ND3, and housekeeping protein β-actin. B and C. Quantification of ASβ and ND3 immunoblot images by densitometry. Values are means ± S.E.M. *, $P < 0.05$ and **, $P < 0.01$ vs Sham; $, $P = 0.06$ vs Sham; ##, $P < 0.01$ vs PHX; &&, $P < 0.01$ vs PHX+LPS by ANOVA with Student-Newman-Keuls post-hoc test, n = 3-4 per group).

PXH+LPS back to levels close to PHX alone (**Figure 8D**).

Discussion

Endotoxemia suppresses liver regeneration and increases liver injury and inflammation after partial hepatectomy: role of mitochondrial oxidative stress and dysfunction

In this study, our first important finding was that endotoxemia compromises outcomes of liver resection by causing excessive mitochondrial oxidative stress, which leads to suppression of MB and liver regeneration, as well as increases of injury and inflammatory responses after PHX. Two-thirds PHX is a widely used model for studying liver regeneration [23, 32]. After two-thirds PHX to mice, liver injury was minimal and robust liver regeneration occurred, as indicated by markedly increased 5-BrdU incorporation, expression of PCNA and cyclin D1, and many mitotic figures appearing in liver sections (**Figures 1**-**3**). However, injection of a low dose of LPS immediately after PHX suppressed almost all liver regenerative events (**Figures 1**-**3**). Moreover, liver injury (ALT, necrotic cell death) and inflammation (leukocyte infiltration in liver sections, NLRP3, IL-1β and TNFα) also increased (**Figures 1** and **8**). These results demonstrate that after loss of liver mass, endotoxemia acts as a second hit that negatively affects the outcome of liver resection (**Figure 9**).

Mechanisms underlying ALF after major liver resection and following small-for-size liver transplantation are poorly understood. Insufficient functional mass is a critical factor. Small-for-size syndrome usually occurs when the relative graft volume is < 30% of the standard liver volume, leading to slow/no recovery of liver function and ultimately liver failure [43, 44]. Suppression of liver regeneration, even when liver injury is mild, leads to liver failure after partial liver transplantation, whereas stimulation of liver regeneration markedly improves graft survival and functional recovery [45]. In mice, livers regenerate after two-thirds PHX with full recovery but fail to regenerate after 90% PHX with consequent ALF [23]. Therefore, liver regeneration is crucial for recovery after loss of liver mass. In our mouse

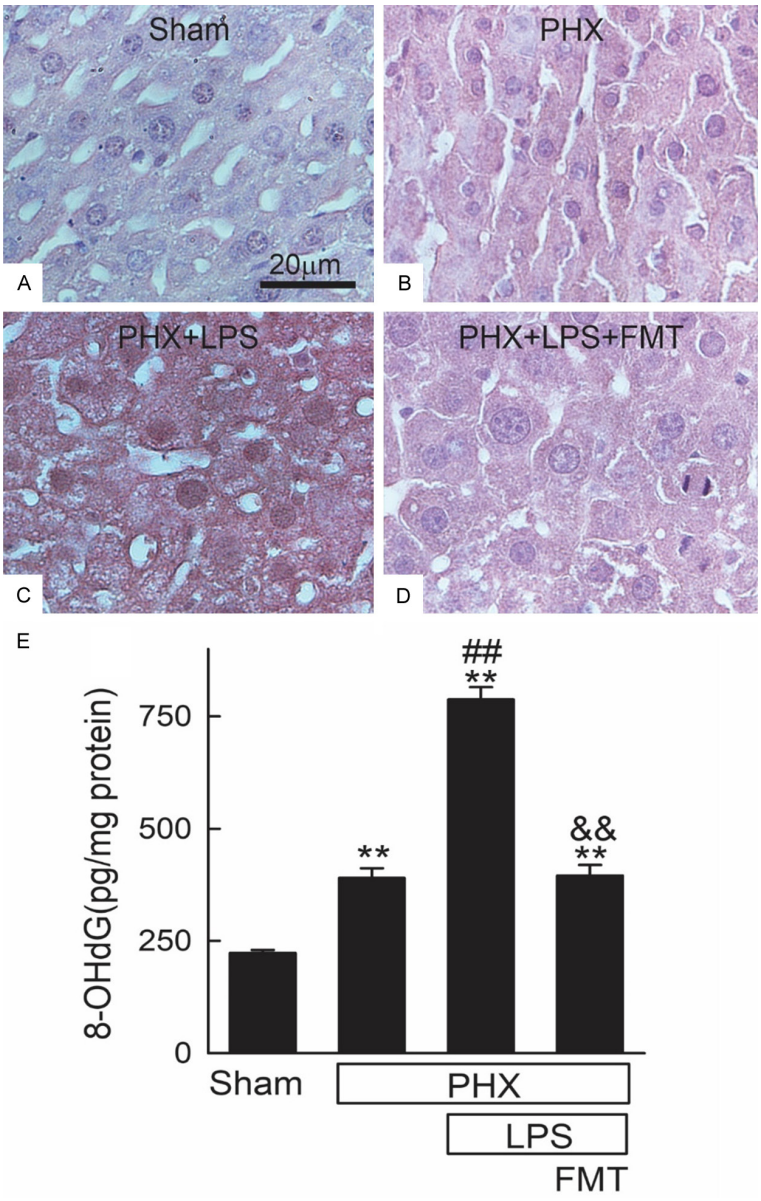

Figure 6. 8-Hydroxy-deoxyguanosine increases after partial hepatectomy and endotoxin treatment: blunting by formoterol. Mice underwent sham-operation or PHX surgeries and were treated as described in **Figure 1**. Livers were harvested at 48 h after surgery. A-D. Representative images of liver sections after 8-OHdG IHC staining (400× magnification; n = 3-4 per group). E. Detection of 8-OHdG in liver homogenates by ELISA. Values are means ± S.E.M. **, P < 0.01 vs Sham; ##, P < 0.01 vs PHX; &&, P < 0.01 vs PHX+LPS by ANOVA with Student-Newman-Keuls post-hoc test, n = 3-4 per group.

model, robust liver regeneration occurred after two-thirds PHX (**Figures 1**-**3**), consistent with previous reports [23, 32]. Endotoxemia, even at a low dose that does not cause overt liver injury and inflammation when given alone (**Figure 1**), almost completely suppressed liver regeneration after PHX to disrupt the recovery process after loss of liver mass (**Figures 1**-**3**).

A possible mechanism by which endotoxemia suppresses liver regeneration is compromised mitochondrial function. Here, we showed that mRNAs and proteins of OXPHOS components ASβ and ND3 were normal or slightly increased after PHX but substantially decreased after PHX+LPS (**Figures 4** and **5**), suggesting that liver resection complicated by endotoxemia leads to impaired mitochondrial function. After PHX, the remnant liver faces a markedly increased metabolic burden that would otherwise be met by the whole liver. Moreover, additional energy is needed to support the synthetic processes of cell proliferation. Lastly, ATP is an important signaling molecule regulating cell proliferation. ATP increases cell proliferation through activation of purinergic receptors (P2 receptors), c-Jun-*N*-terminal kinase (an important signaling molecule regulating liver regeneration) and its upstream kinases such as MAP kinase kinase (MKK) 4 and 7, and mammalian target of rapamycin (mTOR) that stimulates protein synthesis and cell growth [46-48]. Molecules involved in the G1-S transition of the cell cycle, such as ornithine decarboxylase, thymidine kinase and chromatin remodeling enzymes, also require ATP for their function [49]. Essentially, high ATP levels signal to the cell that energy is sufficient to engage in anabolic processes like cell division.

Deficiencies of ATP in remnant livers suppress liver regeneration [6, 50, 51]. Mitochondria convert metabolic fuels like glucose and fatty acids into a usable form of energy (ATP) throu-

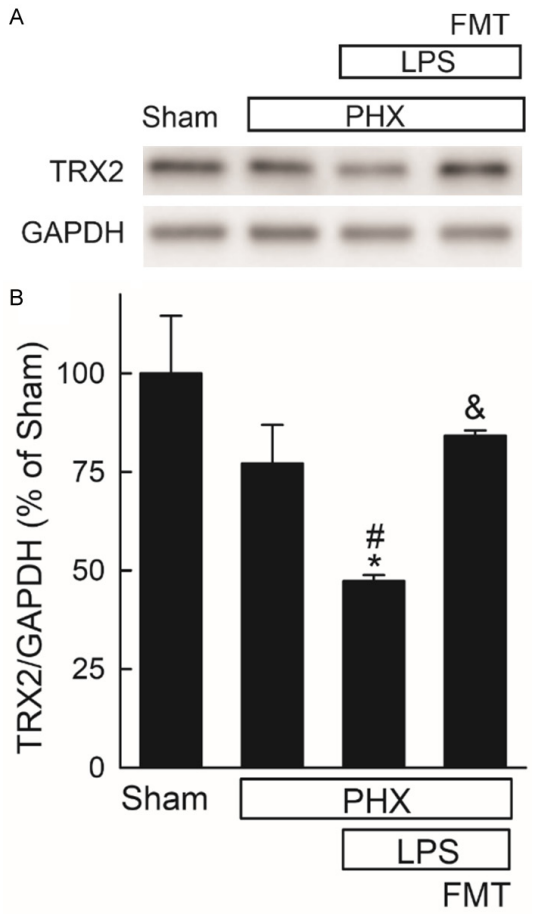

Figure 7. Endotoxemia decreases thioredoxin-2 expression after partial hepatectomy: prevention by formoterol. Mice underwent sham-operation or PHX surgeries and were treated as described in **Figure 1**. Livers were harvested at 48 h after surgery. Proteins were detected by immunoblotting. A. Representative immunoblot images of TRX2 and housekeeping protein GAPDH. B. Quantification of TRX2 immunoblot images by densitometry. Values are means ± S.E.M. *, $P < 0.05$ vs sham; #, $P < 0.05$ vs PHX; &, $P < 0.05$ vs PHX+LPS by ANOVA with Student-Newman-Keuls post-hoc test, n = 3-4 per group.

gh the process of OXPHOS. Therefore, decreases in OXPHOS proteins by endotoxemia after PHX (**Figures 4**, **5**) likely contribute to the suppression of liver regeneration and inability to meet increased metabolic demand, leading to ALF.

When tissues experience increased energy demand that exceeds mitochondrial ATP-producing capacity (*e.g.*, exercise, thermogenesis), MB is stimulated [29]. MB depends on coordinated expression of genes in both the nucleus and mitochondria. Proper function of OXPHOS in mitochondria requires an adequate copy number of mtDNA per cell [29]. Although mitochondria synthesize 13 of their own proteins, the majority of mitochondrial proteins are encoded by nDNA, translated in ribosomes, and then imported into mitochondria [52]. MB requires a coordinated and tightly regulated signaling system for eliciting mitochondrial responses to internal and external stresses [29]. The PGC1α-NRF1/2-TFAM pathway plays a key role in control of MB and mtDNA maintenance [53]. Activated PGC1α interacts with nuclear respiratory factors-1 and 2 (NRF1/2), as coactivators of their transcriptional activity [54]. NRF1/2 binds to consensus sequences in promoter regions of nDNA, causing expression of nDNA-encoded mitochondrial proteins (*e.g.*, ASβ and COX-4), TFAM (a transcription factor that translocates to mitochondria to regulate mtDNA replication and transcription), and PGC1α itself [53]. PGC1α overexpression accelerates MB in cultured renal tubular cells after damage [55]. LPS lowers PGC1α levels in different tissues, including the heart, kidney, muscle, and liver [56, 57]. We showed previously that OXPHOS proteins after two-thirds PHX markedly decrease at 24 h but recover to or slightly above the control levels at 48 h, signifying occurrence of MB [58]. In the present study, OXPHOS proteins, PGC1α, and TFAM remained at or slightly above the control levels at 48 h after PHX (**Figures 4**, **5**). By contrast, after PHX+LPS, OXPHOS proteins substantially decreased in association with decreases of the MB signaling molecules PGC1a and TFAM. These data clearly demonstrate that endotoxemia suppresses MB after PHX. Mitochondrial dysfunction not only inhibits liver regeneration but can also cause cell death. Indeed, serum ALT signifying liver injury increased markedly after PHX+LPS but not after PHX alone (**Figure 1**).

Endotoxin is reported to increase ROS production, leading to mitochondrial dysfunction, cellular damage and multiple organ damage during sepsis [59, 60]. Mitochondria are a major source of ROS, since some electrons escape the electron transport chain prematurely to form superoxide especially at Complexes I and III [61, 62]. Moderately increased ROS act as a signal from mitochondria to the nucleus, thus stimulating MB [63]. However, excessive ROS damages DNA, proteins, and lipids in both

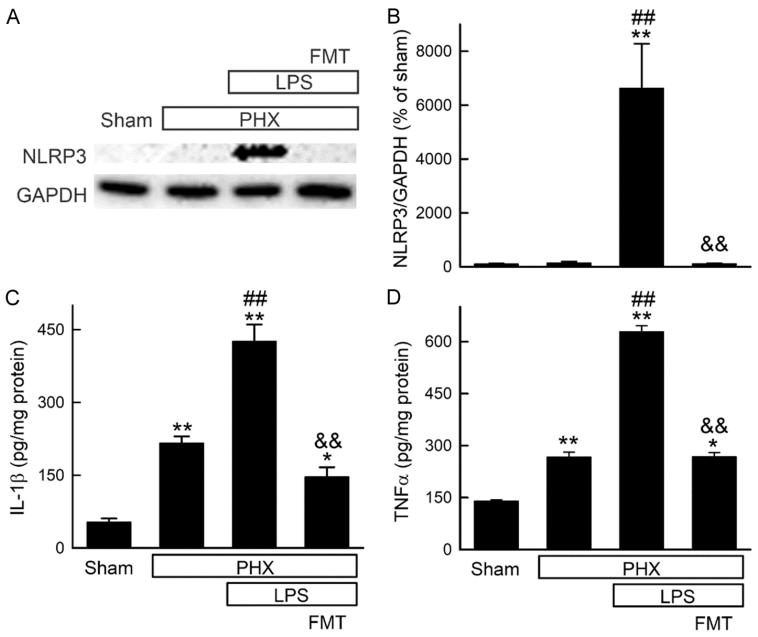

Figure 8. Inflammasome activation and proinflammatory cytokine formation increase after partial hepatectomy and endotoxin treatment: blunting by formoterol. Mice underwent sham-operation or PHX surgeries and were treated as described in **Figure 1**. Livers were harvested at 48 h after surgery. Proteins were detected by immunoblotting. A. Representative immunoblot images of NLRP3 and housekeeping protein GAPDH. B. Quantification of NLRP3 immunoblot images by densitometry. C and D. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) detected by ELISA. Values are means ± S.E.M. **, P < 0.01 vs Sham; ##, P < 0.01 vs PHX; &&, P < 0.01 vs PHX+LPS by ANOVA with Student-Newman-Keuls post-hoc test, n = 3-4 per group.

nuclei and mitochondria, disrupting the signaling pathways that normally trigger MB [63, 64]. Mitochondria are highly susceptible to oxidative stress because their inner membranes are enriched in unsaturated fatty acids [65]. mtDNA is more susceptible to oxidative damage than nDNA due to close proximity to the electron transport chain where ROS are produced, the absence of protective histones, and the lack of a nucleotide excision repair system, which limits its capacity for DNA repair in mitochondria [66]. Single- and double-strand breaks in mtDNA caused by oxidative stress suppress replication and transcription [67]. The D-loop region of mtDNA, where TFAM binds, is more susceptible to ROS attack than other regions of mtDNA [68]. Mutations in the D-loop region alter binding affinities of TFAM and other proteins, which can lead to depletion of mtDNA [68]. Thus, excessive mitochondrial oxidative stress not only causes mtDNA mutations but also alters the rate of mtDNA replication and MB. Excessive mitochondrial oxidative stress is related to aging and pathogenesis of many diseases, including diabetes, Alzheimer's disease, and cardiovascular disease [64, 69, 70]. In this study after PHX alone, DNA oxidation (8-OHdG) slightly increased in mitochondria but not in nuclei (**Figure 6**). However, 8-OHdG increased markedly in both nuclei and mitochondria after PHX+LPS (**Figure 6**). Therefore, endotoxin-induced strong oxidative stress likely contributes to both mitochondrial damage and suppressed MB (**Figure 9**).

Recent studies reveal a central role for mitochondria in the control of innate immunity and inflammatory responses, and mitochondrial dysfunction is linked to severe inflammatory disorders [71]. Mitochondria serve as a platform for assembly of protein complexes involved in immune and inflammatory signaling, such as the NLRP3 inflammasome, which leads to maturation and release of proinflammatory cytokines IL-1 and IL-18 [72]. Moreover, damaged mitochondria release proinflammatory mitochondrial damage-associated molecular patterns (mtDAMPs), such as mtDNA, into the cytosol and circulation [72, 73]. mtDNA released into the cytosol activates NLRP3 inflammasomes and the cGAS/stimulator of interferon genes (STING) pathway. mtDNA released into circulation causes systemic inflammatory responses [72]. Circulating mtDAMPs are associated with mortality in critically ill patients [74]. Oxidative stress reportedly causes release of mtDNA and triggers the activation of NLRP3 inflammasomes [74, 75]. Moreover, LPS activates toll-like receptor 4, leading to production of proinflammatory cytokines like TNFα, which enhances the expression of inflammasome components, making cells more susceptible to inflammasome activation upon encountering other stimuli [76]. In this study, we used a low dose of LPS that did not cause hepatic leukocyte infiltration when given alone (**Figure 1**). However, when LPS was

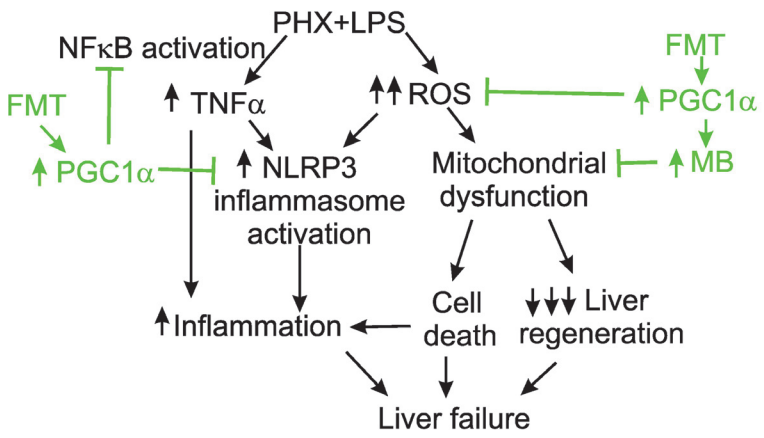

Figure 9. Potential mechanisms by which formoterol improves outcomes of liver resection complicated with endotoxemia. After liver resection, the remnant liver needs substantially higher energy production to meet the metabolic demand formerly met by the unresected liver and to support liver regeneration to recover liver mass and function. If liver resection is complicated by endotoxemia, a second hit occurs, leading to massive oxidative stress - particularly mitochondrial oxidative stress - which damages mitochondria and suppresses MB. Decreased energy supply then inhibits liver regeneration and causes cell death. Endotoxin binds to toll-like receptor 4 and activates NFκB, leading to increased production of proinflammatory cytokines, such as TNFα. Increased TNFα and mitochondrial oxidative stress together markedly increase inflammasome activation, increasing production of proinflammatory cytokines, such as IL-1 and IL-18, thus exacerbating inflammation. Increased liver injury and inflammation with suppressed liver regeneration leads to liver failure. FMT increases PGC1α expression/activation, which enhances antioxidant defenses, stimulates MB, and inhibits inflammation, thus preventing liver failure.

given after PHX, inflammation increased (leukocyte infiltration, **Figure 1**), which was associated with markedly increased mitochondrial oxidative stress, TNFα production, and NLRP3 inflammasome activation (increased NLRP3 and IL-1β, **Figure 8**). Together, mitochondrial oxidative stress and TNFα may enhance inflammasome activation, creating positive feedback that exacerbates inflammation after PHX+LPS (**Figure 9**).

Formoterol as a novel therapy for acute liver failure after liver resection complicated with endotoxemia and its potential mechanism of action

The most important new finding in this study is that FMT, a long-acting β2-adranergic receptor agonist, improves outcomes in mice subjected to liver resection and endotoxemia in association with increased PGC1α (**Figure 4**). PGC1α stimulates mitochondrial biogenesis, thus increasing liver regeneration (**Figures 2**-**5**). PGC1α also suppresses oxidative stress by increasing expression of antioxidant proteins (**Figures 6** and **7**). Moreover, PGC1α inhibits inflammatory responses (**Figure 8**). Low PGC1α activity appears to be a common feature in many organ/tissue diseases, such as in the kidney, heart, and brain, whereas strategies that increase PGC1α activity protect against injury and improve recovery [30, 77]. FMT reportedly increases MB in mouse heart, kidney, muscle, and brain [30, 78, 79]. FMT binds to β2-adrenergic receptors, which trigger a signaling cascade resulting in activation of soluble guanylate cyclase and increases of cGMP levels, thus promoting the expression of genes involved in MB, such as PGC1α [80]. Consistently in this study, FMT increased PGC1α, TFAM, and OXPHOS proteins in livers after PHX+LPS (**Figures 4** and **5**), clearly demonstrating improvement of MB by FMT. Increased MB improved mitochondrial function, thus supporting liver regeneration and recovery of liver mass and function (**Figures 1**-**3**).

FMT not only stimulates MB but also inhibits oxidative stress and inflammation (**Figures 6**-**8**). Several recent studies implicate PGC1α is a central factor connecting oxidative stress and mitochondrial metabolism with inflammatory responses [81]. In addition to its key role in regulation of MB, PGC1α also regulates expression of mitochondrial antioxidant genes, including TRX2, thioredoxin reductase, peroxiredoxin 3 and 5, manganese superoxide dismutase, and catalase, which act to suppress oxidative stress-induced mitochondrial injury and dysfunction [81, 82]. PGC1α regulates mitochondrial antioxidant gene expression, possibly through its interaction with FoxO3 and stimulation of nuclear factor erythroid 2-related factor 2 (Nrf2) expression [83, 84]. In this study, FMT increased expression of PGC1α and TRX2 and markedly decreased 8-OHdG formation after PHX-LPS (**Figures 4** and **7**), suggesting that

FMT inhibits oxidative stress by increasing PGC1α and mitochondrial antioxidants. Interestingly, the antioxidant function of PGC1α is paired with its role in increasing the mitochondrial electron transport and mitochondrial mass in cells with high energy demand [81]. Therefore, increasing PGC1α by agents like FMT is a desirable strategy to avoid the potential cytotoxic effects of ROS formation in association with increased mitochondrial metabolism (*e.g.*, increased mitochondrial fatty acid β-oxidation) during MB.

Low or lack of PGC1α also enhances inflammatory responses in many pathological situations, such as atherosclerosis, pancreatitis, chronic obstructive pulmonary disease, cerebral and renal ischemia/reperfusion injury, and aging [81, 85-87]. PGC1α interacts with the p65 subunit of NFκB, thus blocking NFκB activation and transcriptional activity toward its target genes, including those encoding proinflammatory cytokines [85]. Oxidative stress stimulates inflammation [88], whereas conversely PGC1α suppresses oxidative stress. Overexpression of PGC1α decreases but knockdown of PGC1α increases NLRP3 inflammasome activation [89]. In this study, we showed that FMT inhibited inflammasome activation and production of proinflammatory cytokines after PHX+LPS (**Figures 8** and **9**). Therefore, FMT inhibits inflammatory responses after PHX+LPS, most likely by increasing PGC1α.

In conclusion, endotoxemia compromises the outcomes of liver resection, most likely by causing excessive oxidative stress, which leads to suppression of MB and liver regeneration, as well by increasing injury and exacerbating inflammatory responses. FMT markedly improves the outcomes of liver resection complicated with endotoxemia, most likely by increasing PGC1α expression, which stimulates MB and inhibits oxidative stress and inflammation.

In addition to its potent protective effects against ALF, FMT is a widely used FDA-approved drug for therapy of asthma and chronic obstructive pulmonary disease, which is generally well-tolerated and safe [90]. Moreover, FMT has unique characteristics of rapid onset and sustained duration of action [90]. Therefore, FMT is a promising new pharmacotherapy for ALF caused by loss of liver mass complicated with endotoxemia, a situation that often occurs clinically. In this study, the protective effects of FMT were only investigated in a mouse model. Whether FMT has benefits for patients with endotoxemia after liver resection remains to be explored in future clinical trials. Additionally, FMT is usually administered as an inhalant for asthma. For treatment of liver resection complicated by endotoxemia, FMT would need to be given by injection. The pharmacokinetics of FMT after injection was not examined in this study and will need evaluation in the future.

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Disclosure of conflict of interest

None.

Abbreviations

ALF, acute liver failure; ALT, alanine aminotransferase; ASβ, ATP synthase-β; ATP, adenosine 5'-triphosphate; 5-BrdU, 5-bromo-2'-doxyuridine; CDK, cyclin-dependent kinase; cGAS, cyclic GMP-AMP synthase; CyD1, cyclin D1; DAB, 3,3'-diaminobenzidine; HPF, high power field; FMT, formoterol; H&E, hematoxylin and eosin; IL-1β, interleukin-1β; IL-18, interleukin-18; JNK, c-Jun-*N*-terminal kinase; LPS, endotoxin; MKK, mitogen-activated protein kinase kinase; mtDNA, mitochondrial DNA; ND3, NADH dehydrogenase-3; nDNA, nuclear DNA; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NIM811, N-methyl-4-isoleucine cyclosporine; NLRP3, NOD-like receptor protein 3; NRF, nuclear respi-

ratory factors; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; OXPHOS, oxidative phosphorylation; PBS-T, phosphate buffered saline with Tween 20; PCNA, proliferating cell nuclear antigen; PGC1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PHX, partial hepatectomy; PI, propidium iodide; qPCR, quantitative polymerase chain reaction/real-time PCR; ROS, reactive oxygen species; STING, stimulator of interferon genes; TFAM, mitochondrial transcription factor-A; TMRM, tetramethylrhodamine methyl ester; TNFα, tumor necrosis factor-α; TRX2, thioredoxin-2.

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Supplemental Information

5-Bromo-2'-deoxyuridine incorporation test

Cell proliferation requires synthesis of new DNA. Therefore, cell proliferation was also assessed by incorporation of 5-bromo-2'-deoxyuridine (5-BrdU) into DNA. At 2 h prior to liver harvesting, 5-BrdU (2 mg/mL in NS) was injected into mice at a dose of 20 mg/kg. Livers were harvested and processed as described in Materials and methods. Liver sections were deparaffinized and rehydrated. For antigen retrieval, liver sections were incubated in 4N HCl for 20 min, in double-distilled water for 5 min, and then in pepsin solution (Table S1) for 15 min with all steps carried out at 37°C. For blocking, liver sections were incubated with peroxidase blocking solution (Table S1) for 10 min at room temperature. After washing with phosphate buffered saline with Tween-20 (PBS-T, Table S1), liver sections were blocked with PBS-T with 1% BSA for 30 min. After blocking, sections were probed with a rabbit anti-BrdU antibody (1:200) in PBS-T with 1% BSA at room temperature for 15 min. After washing with PBS-T, liver sections were then incubated with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (Table S1, 1:1000) for 10 min. Slides were then incubated in 3,3'-diaminobenzidine (DAB, 500 µL) in the dark and DAB enhancing solution (Table S1) for 1 min each. Slides were stained with Harris' Modified Hematoxylin (1:5) for 90 sec and then dehydrated and mounted with Permount. Images of 5 random fields/slide were captured using a Zeiss AX10 microscope with a 20× objective lens, and hepatocytes with nuclear 5-BrdU staining were counted in these images.

8-hydroxy-deoxyguanosine immunohistochemical staining

After deparaffinization and rehydration, liver sections were blocked with peroxidase blocking solution (Table S1) for 5 min, rinsed with PBS-T, and then with 1% BSA in PBS-T for 30 min at room temperature. Sections were incubated with rabbit anti-OHdG antibody (1:200) in PBS-T with 1% BSA at room temperature for 1 h. After rinsing with PBS-T, sections were then probed with horseradish peroxidase conjugated secondary goat anti-rabbit antibody (1:1000) (Table S1) at room temperature for 1 h. Slides were then incubated with DAB solution (500 µL) and DAB enhancing solution (Table S1) for 5 min each at room temperature. Slides were stained with Harris' Modified Hematoxylin (1:5) (Table S1) for 90 sec. Slides were then dehydrated and mounted with Permount. Images were captured using a Zeiss AX10 microscope with a 40× objective lens.

Immunoblotting

For immunoblotting, liver tissue (~50 mg) was homogenized in lysing buffer (500 µL, Table S1) with 1% protease and phosphatase inhibitors, and the lysates were centrifuged at 15,000 g for 5 min at 4°C. Protein concentration in the supernatant was determined using a Pierce BCA protein assay kit (Table S1) according to the manufacturer's instructions. Proteins were separated by SDS-PAGE gels and transferred to a nitrocellulose membrane (Table S1). The membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) at room temperature for 1 h, incubated with specific primary antibodies for the proteins of interest at concentrations of 1:1000-3000 overnight at 4°C (Table S1) and then horseradish peroxidase conjugated secondary antibodies (Table S1) for 1 h at room temperature. The immunoblots were developed using an ECL detection kit according to manufacturer's instructions. Images of immunoblots were captured using the Chemidoc Touch Imaging System (Bio-Rad, Hercules, CA), and intensities of bands were quantified using NIH ImageJ software.

Table S1. Sources of reagents

Items	*Sources*	*Catalog #*
Alanine Transaminase Kit	Pointe Scientific, Uncoln Park, MI	A7526-150
β-Actin Antibody	Cell Signaling Technology, Danvers, MA	4967
ATP Synthase Antibody	Abcam, Waltham, MA	ab14730
Chemiluminescence Kit	Pierce Biotec., Rockford, IL	34075
BIO-RAD Clarity Max™ Western ECL Substrate Kit	Bio-Rad Laboratories, Hercules, CA	1705062
Bromodeoxyuridine	Abcam, Waltham, MA	ab152095
Cyclin D1 Antibody	Abcam, Waltham, MA	ab134175
3,3'-Diaminobenzidine Substrate Kit	Vector Lab, Newark, CA	SK-4100
3,3'-Diaminobenzidine Enhancer Solution	Vector Lab, Newark, CA	ZK0125
Mouse Interleukin-1β ELISA MAX™ Standard Set	BioLegend, San Diego, CA	432601
Mouse Tumor Necrosis Factor-α ELISA MAX™ Standard Set	BioLegend, San Diego, CA	430901
Glyceraldehyde 3-Phosphate Dehydrogenase Antibody	Cell Signaling Technology, Danvers, MA	2118
Harris' Modified Hematoxylin	Sigma-Aldrich, St. Louis, MO	HHS32
Hydrogen Peroxide 3% w/w	VWR International, Randor, PA	18I0756156
8-Hydroxydeoxyguanosine ELISA Kit	Assay Genie, Dublin, Ireland	UNFI0029
8-Hydroxyguanosine Antibody	Fisher Scientific, *Waltham, MA*	BS-1278R
Immobilon®-P PVDF Transfer Membrane	Merk Millipore Ltd., Co. Cork Ireland	0000190405
IQ-Sybr Green Mix	Bio-Rad Laboratories, Hercules, CA	1708882
iScript™ cDNA Synthesis Kit	Qiagen, Woburn, MA	1708891
LPS Elisa Kit	Assay Genie, Dublin, Ireland	UNEB0072
Lysis Buffer	Fisher Scientific, Waltham, MA	AAJ63324EQE
Myeloperoxidase Antibody	DAKO Corp, Carpinteria, California	A0398
10% Neutral Buffered Formalin	Leica Biosystems, Buffalo grove, IL	3800598
NOD-like receptor protein-3 Antibody	Abcam, Waltham,MA	ab263899
Pierce BCA Protein Assay Kit	Pierce Biotec., Rockford, IL	PI23227
Proliferating Cell Nuclear Antigen Antibody	DAKO Corp., Carpinteria, CA	M0879
Peroxidase AffiniPure Goat Anti-Rabbit IgG	Fisher Scientific, *Waltham, MA*	NC9611376
Peroxidase AffiniPure Goat Anti-Mouse IgG	Fisher Scientific, *Waltham, MA*	NC9491974
Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1α Antibody	Santa Cruz Biotech, Santa Cruz, CA	Sc-13067
1X Phosphate Buffered Saline	Fisher Scientific, *Waltham, MA*	21-040-CV
10X Tris-Buffered Saline	Bio-Rad Laboratories, Hercules, CA	1706435
Thioredoxin-2 Polyclonal Antibody	Proteintech, Rosemont, IL	13089-1-AP
Tween-20	Fisher Scientific, *Waltham, MA*	BP337-500

Table S2. Primer sequences for RT-PCR

Genes	Forward Primers	Reverse Primers
ATP Synthase-β	5'-GTC CAG TTC GAT GAG GGA TTA C-3'	5'-AGA AGG GAT TCT GCC CAA TAA G-3'
ND3	5'-CTA CAA GCT CTG CAC GTC TAC-3'	5'-GGC TAT GGT GAG GTT GAA GAA-3'
TFAM	5'-AGC CAT AGT GCC CAT CAG TTC TGT-3'	5'-AAC GCA GAG AAA CCC TGT CTC GAA-3'
PGC1α	5'-TCA CCC TCT GGC CTG ACA AAT CTT-3'	5'-TTT GAT GGG CTA CCC ACA GTG TCT-3'
HPRT	5'-GCT TTC CCT GGT TAA GCA GTA CA-3'	5'-AAA CTT GTC TGG AAT TTC AAA TC-3'

HPRT, hypoxanthine phosphoribosyltransferase 1; ND3, NADH dehydrogenase-3; PGC1α, peroxisome proliferator-activated receptor-γ coactivator-1 alpha; TFAM, mitochondrial transcription factor-A.

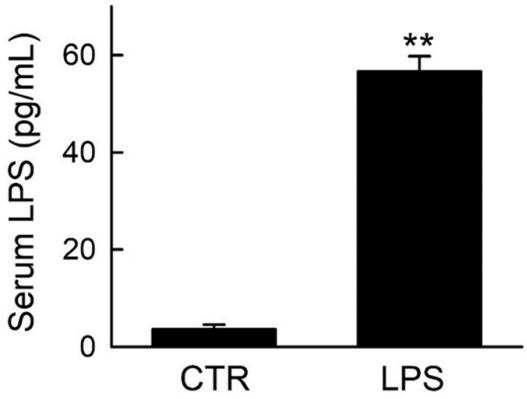

Figure S1. Intraperitoneal injection of LPS causes endotoxemia. Mice were injected with LPS (5 mg/kg, *i.p.*), and blood was collected 6 h later. LPS in the serum was detected using an ELISA kit. **, P<0.01 compared to the control (CTR) group by Student's t-test, n = 5-6/group.