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Metformin protects against infection-induced myocardial dysfunction

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Abstract

Background and Purpose—Metformin administration is associated with myocardial protection during ischemia and/or reperfusion, possibly via inhibition of inflammatory responses in the heart. Exposure to pathogens, in addition to the activation of the immune system and the associated metabolic dysfunction, often results in compromised myocardial function. We examined whether metformin administration could maintain the normal myocardial function in experimental moderate Gram negative infection, induced by lipopolysaccharide (LPS) administration.

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Disclosures

The authors have nothing to disclose.

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Author Contributions

T Tzanavari and K P Karalis formulated the original hypothesis, D V Cokkinos provided scientific advice and discussions on the clinical relevance of the project, T Tzanavari, K P Karalis and M I Kontaridis designed the experimental approach; T Tzanavari performed most of the experimental work; A Varela carried out echocardiography; S Theocharis interpreted the histological data; E Ninou provided assistance with the experimental work while the paper was being revised; A Kapelouzou performed the triglyceride content measurements; K P Karalis and D V Cokkinos supported with all reagents required; T Tzanavari analyzed the data; T Tzanavari, M I Kontaridis and K P Karalis interpreted all the data; D V Cokkinos provided feedback in the interpretation of data and layout of the manuscript; T Tzanavari, M I Kontaridis and K P Karalis wrote the manuscript

Experimental Approach—129xC57BL/6 mice were divided into control groups that received either vehicle or a single intraperitoneal (i.p.) injection of low dose LPS (5 mg/kg body wt), and metformin treated groups that received either daily metformin (4 mg/kg/animal) i.p. injections for five days prior to LPS administration [Experiment 1], or a single metformin injection following same dose of LPS [Experiment 2].

Key Results—LPS alone caused cardiac dysfunction, as confirmed by echocardiography, whereas metformin administration, either before or after LPS, rescued myocardial function. LPS caused marked reduction of the cardiac metabolism-related genes tested, including Prkaa2, Cpt1b, Ppargc1a and Ppargc1b; reduction of fatty acid oxidation, as reflected by the regulation of Ppara, Acaca and Acacb; increased glucose transport, as shown by Slc2a4 levels; reduction of ATP synthesis; significant increase of inflammatory markers, in particular IL6; and reduction of autophagy. Pretreatment with metformin normalized the levels of all these factors.

Conclusions and Implications—We show for the first time that metformin protects the myocardium from LPS-associated myocardial dysfunction mainly by supporting its metabolic activity and allowing efficient energy utilization. Metformin can be a potential cardioprotective agent in individuals susceptible to exposure to pathogens.

Keywords

Metformin; Lipopolysaccharide; Cardiac dysfunction; Bacterial infection; Fatty acid oxidation

1. Introduction

Bacterial infections initiate a systemic inflammatory response that leads to cardiac dysfunction, characterized by impaired contractility, diastolic dysfunction and reduced ejection fraction [1]. As demonstrated in a number of studies on Gram negative bacterial infection, the LPS-induced cardiac dysfunction is associated with reduced free fatty acid (FFA) and glucose oxidation, which compromise ATP availability and may eventually lead to ATP depletion [2,3].

Under physiological conditions, cardiovascular function is strongly dependent on energy availability provided by FFA oxidation (FAO) [4]. Fatty acids (FAs) account for 70%, while glucose, lactate and ketones cover for the remaining 30% of the myocardial ATP production [5]. Cardiac FAO is regulated by peroxisome proliferator-activated receptor (PPAR)α which, similar to PPARγ and PPARβ/δ [6], is activated by FAs and controls the expression of genes that regulate cardiac FAO [7]. In states of increased energy demand, such as in heart failure, glucose becomes the predominant source of energy for the heart. However, opposite to what happens in heart failure, when reduced FAO is, at least partially, compensated by increased glucose oxidation, the LPS-mediated suppression of FAO is not counterbalanced by an increase in glucose catabolism [4,8]. The enzyme 5′ AMP-activated protein kinase (AMPK), a serine–threonine kinase, is thought to act as a molecular sensor, modulating the changes in cellular energy and coordinating the enzymes involved in carbohydrate and fat metabolism to enable ATP conservation and synthesis [9].

0.15pt?>Metformin (1,1-dimethylbiguanide hydrochloride), an insulin-sensitizing biguanide that is well tolerated and has minimal adverse effects reported, is currently believed to prevent cardiovascular complications associated with type 2 diabetes [10,11]. It has been suggested that the effects of metformin are either mediated via activation of AMPK [12], which then stimulates FAO, promotes glucose transport, accelerates glycolysis, and inhibits synthesis of both triglyceride and protein [13]; or via AMPK-independent mechanisms [14]. Metformin also improves hyperglycemia and increases insulin sensitivity and FAO [13]. Emerging evidence from mouse studies highlights the protective role of metformin against myocardial damage when administered either before ischemia or at the reperfusion phase [15–17]. In addition, beneficial effects of metformin have been shown in the survival of mice following LPS-induced sepsis [18].

Based on the above, we set out to determine whether metformin could be useful as a protective agent in acute myocardial dysfunction due to typical Gram-negative infection, as simulated by a sub-lethal LPS dose, and identify the mechanisms involved.

2. Methods

2.1. Mice

Wild-type male mice of 129xC57BL/6 genetic background weighing 30 to 35 g and 10 to 14 weeks old were used. Animal housing and care were conducted according to National Institutes of Health guidelines; all experimental procedures were approved by the Animal Care and Use Committee of the Biomedical Research Foundation of the Academy of Athens in Athens, Greece. The animals were housed in plastic cages under controlled conditions (temperature 22 °C; 12-h light/dark cycle; lights on at 7 am) and were given free access to standard laboratory pellet formula and tap water. Daily inspection of cages for food spillage and monitoring of body weight and food intake were performed for the duration of the study. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [19]

Euthanasia was performed by isoflurane (IsoFlo®, Abbott) inhalation followed by cervical dislocation. The left ventricle was excised and either stored at −80 °C for RNA extraction, or fixed overnight at 4 °C in 10% formalin and embedded in paraffin.

An average of ten 2.5–3 month-old male mice per experimental group was used in all experiments and experiments were repeated to confirm the reproducibility of the findings and their statistical significance.

2.1.1. LPS-Induced Infection—Infection was induced by intraperitoneal (i.p.) injection of LPS in 0.9% NaCl at a dose of 5 mg/kg of BW. Control mice were injected with the vehicle. Echocardiograms were performed at 0 h and 24 h after LPS administration.

2.1.2. Metformin Administration—Metformin was administered i.p. either before or after LPS administration. In the first case, metformin (4 mg/kg of BW) in 0.9% NaCl was injected daily for 5 days prior to LPS administration. Last dose of metformin was administered 40 min prior to LPS administration, while 16 h past-LPS administration, mice

received one more dose of metformin [Experiment 1]. In the latter case, metformin was administered 6 h after LPS administration, as a single dose of 4 mg/kg of BW [Experiment 2]. In both cases, mice were sacrificed 24 h after LPS administration. To measure the effects of LPS on myocardial function, as well as those of metformin on LPS-induced myocardial dysfunction two control groups were also used in parallel, i.e. one treated with metformin alone (metformin-treated group), and another treated with the vehicle alone (0.9%NaCl) (control group).

2.2. Echocardiography

Two-D M-mode echocardiography examination and analysis were performed as we have previously reported [20–22]. In more detail, at selected time points, mice were sedated with an i.p. injection of ketamine hydrochloric acid [23], 100 mg/kg, and 50 mg/kg in LPS-treated mice, and heart function was evaluated by echocardiography. Short and long axis images were acquired using a digital ultrasound system (Vivid 7, GE Healthcare) with a 13 MHz linear transducer. Two-dimensional targeted M-mode imaging was obtained from the short axis view at the level of greatest left ventricular (LV) dimension. Images were analyzed using the Echopac PC SW 3.1.3 software (GE Healthcare). LV end-diastolic (LVEDD) and LV end-systolic dimension (LVESD), posterior wall thickness at the diastolic (LVPWd) and systolic (LVPWs) phase and the ejection fraction EF (%) were measured. Temporal changes between LVEDD and LVESD were used for the calculation of the percentage of LV fractional shortening [FS (%) = [(LVEDD − LVESD)/LVEDD] × 100] and of ejection fraction [EF (%) = [($LVEDD^3$ − $LVESD^3$)/$LVEDD^3$] × 100]. For heart rate (HR) calculation, five beats were averaged for each measurement. A minimum of 8 mice per experimental group of the same sex and age were used in all experiments and each experiment was repeated three times to confirm the statistical significance of the findings. Results are presented as mean ± SEM.

2.3. Histology

LV and whole heart tissues were fixed overnight in 10% formalin at 4 °C, embedded in paraffin, cut into 5 μm thick sections and stained with hematoxylin and eosin (H&E) according to standard protocols.

2.4. Quantitative Real-Time PCR (qPCR)

RNA was extracted from left heart chamber tissue using Trizol (Sigma). Two μg total RNA from each sample was treated with DNaseI (Ambion) and then reverse transcribed using the M-MLV reverse transcriptase (Invitrogen) and random hexamer primers (Life Technologies). Amplification was performed in ABI Prism 7000 (Applied Biosystems) in a 96-well plate in a final reaction volume of 20 μL using SYBR®Green/ROX™ qPCR master mix (SABiosciences) as per manufacturer's instructions. Each quantitative PCR reaction was repeated in duplicate, and the mean Ct value for each sample was used for data analysis. Each sample was normalized to 18S rRNA (housekeeping gene) value and the results expressed as fold changes of Ct value relative to controls by using the $2^{-\Delta\Delta CT}$ formula [24]. Primer pairs for individual genes are presented in Table 1.

2.5. Measurement of Metabolic Indices

Lactate concentration was measured using BM-Lactate strips (Roche) following manufacturer's instructions; ATP and ADP were measured using the ATP/ADP kit (BioVision); pyruvate concentrations were estimated using the pyruvate kit (BioVision) and triglyceride content was measured using the TG kit (Zafeiropoulos). All calculations were conducted according to manufacturers' instructions and related to mg of tissue.

2.6. Immunoblotting

LV tissue samples were homogenized on ice in RIPA lysis buffer. Protein concentration was determined by Bradford's method as per manufacturer's instructions. Samples containing 40 μg of protein were incubated at 95 °C for 5 min, resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were performed using anti-pACC, anti-LC3II, anti-total GSK3β, and anti-GAPDH antibodies (Cell Signaling), followed by incubation with the mouse/rabbit horseradish peroxidase conjugated polyclonal antibody (Vector) following manufacturer's directions. Equal protein loading and transfer were confirmed by Ponceau staining, and protein levels were related to internal standards to ensure homogeneity between samples and gels. Bands were visualized with enhanced chemiluminescence (ECL), and quantified using Photoshop 7.0.

2.7. Statistical Analysis

Data were expressed as mean values ± SEM (bars). Differences between two groups were analyzed by unpaired Student's *t*-test, while comparisons among more than two groups were assessed by one-way ANOVA coupled with Bonferroni's tests. Differences were considered as statistically significant when $P < 0.05$.

3. Results

3.1. Metformin Pretreatment Protects Mice from LPS-Induced Cardiac Dysfunction [Experiment 1]

LPS simulates a systemic Gram-negative infection, associated with myocardial inflammatory injury and tissue metabolic dysfunction [25,26]. To test the possibility that metformin can protect the myocardium from infection-induced cardiac dysfunction, we treated mice with metformin for five days before LPS administration. Echocardiographic analysis of control, metformin-treated, LPS-injected and metformin-pretreated/LPS injected mice showed that metformin protected mice from the LPS-associated cardiac dysfunction (Fig. 1). More specifically, in response to LPS alone, cardiac function deteriorated as previously reported [27], as shown by all parameters tested. LPS-injected hearts had significantly increased LVEDD and LVESD, while LVPWs and LVPWd were significantly reduced compared to control mice (Fig. 1A & B). Concomitant with these anatomic abnormalities, FS (Fig. 1C) and EF (Fig. 1D) were significantly lower, 35% compared to 45% and 72% compared to 84% respectively in LPS-injected compared to control mice. Metformin-pretreated/LPS-injected hearts had significantly reduced LVEDD and LVESD and increased LVPWs compared to LPS-injected mice (Fig. 1B). On the other hand, FS (Fig. 1C) and EF (Fig. 1D) were significantly higher, 41% compared to 35% and 80% compared

to 72% respectively, in metformin pretreated/LPS-injected as compared to LPS-injected mice. The effects of metformin on amelioration of cardiac function were specific to the LPS-injected mice, as administration of metformin alone had no impact on baseline cardiac function (Fig. 1A–1D). LPS administration obviously affected the heart (Fig. 2A) and significantly increased heart/body weight ratio, an effect compromised by metformin pre-treatment (Fig. 2B).

As shown by H&E staining (Fig. 2C), at the cellular level, LPS-treated hearts exhibited more prominent signs of inflammation and of activated fibroblasts compared to control tissues, but also as compared to the metformin pretreated/LPS-injected heart. Accordingly, no differences were detected between control and metformin-treated cardiac tissue (Fig. 2C). In addition, staining with the inflammatory marker CRP showed that LPS induced CRP expression in the heart, while in metformin pre-treated mice CRP expression was markedly reduced (Fig. 2D). Similarly, upregulated RAGE expression in the hearts of LPS-injected mice, which acts as a mediator of both acute and chronic vascular inflammation, was reduced in the hearts of metformin pre-treated mice (Fig. 2D). Along this line, we found that the mRNA expression levels of the pro-inflammatory cytokine interleukin (IL) 6 were significantly reduced in metformin-pretreated/LPS-injected, as compared to LPS-injected hearts (Supplementary Fig. 1A). However, tumor necrosis factor (TNF)α and IL1β mRNA levels were not different between LPS-injected and metformin-pretreated/LPS-injected mice (Supplementary Fig. 1A). The same was observed for circulating IL1β levels, as measured by Elisa in serum from the four groups, which showed that, as expected, IL1β levels were significantly increased in the LPS- compared to the vehicle-injected group, whereas metformin pre-treatment had a minor, though not significant, effect (Supplementary Fig. 1B). Combined these data suggest that there may only be a partial contribution, of any pro-inflammatory cytokines levels' changes in the improved cardiac function of LPS-injected mice treated with metformin.

3.2. Metformin Pretreatment Rescues the Impaired Markers of Fatty Acid Oxidation in LPS-Injected Mice [Experiment 1]

LPS-induced systemic inflammation constitutes a systemic metabolic stressor for the organism. Following LPS administration, the expression of PPARs (*Ppars*), PPARγ coactivator 1 alpha (*Ppargc1a*), and genes involved in FAO are greatly compromised [27–30]. Here, we also demonstrated significant inhibition of the expression of genes involved in energy homeostasis and lipid metabolism, including 5′ AMP-activated protein kinase α_2 (*Prkaa2*), carnitine palmitoyltransferase (*Cpt1b*), *Ppargc1a* and *Ppargc1b* in hearts from LPS-treated mice (Fig. 3A). Similarly, the expression of genes involved in FAO, including *Ppara*, acetyl-CoA carboxylase-1 (*Acaca*) and -2 (*Acacb*) were significantly reduced following LPS administration (Fig. 3B). Pretreatment with metformin significantly increased ACC phosphorylation in LPS-injected mice (Fig. 3C). Notably, metformin pretreatment rescued all the above metabolic parameters in the LPS-treated hearts (Fig. 3A & B), in agreement with the beneficial effects in cardiac function (Fig. 1A–D). No measurable effect on any of these factors in the metformin alone-treated group was detected (Fig. 3A & B).

3.3. Metformin Pretreatment Protects against LPS-Induced Attenuated Cardiac Glucose Utilization [Experiment 1]

The LPS-induced effects on carbohydrate metabolism in the heart have been previously reported [3,30,31]. Indeed, we observed reduced glucose transporter 4 (*Slc2a4*) expression in the hearts of LPS-injected mice (Fig. 4A); while we found increased levels of pyruvate dehydrogenase kinase 4 (*Pdk4*) (Fig. 4B), a mitochondrial enzyme implicated in glucose utilization by phosphorylating and inactivating the pyruvate dehydrogenase complex. Phosphoenolpyruvate carboxykinase (*Pck1*) was also significantly reduced in LPS-injected compared to control mice (Fig. 4C). The LPS effect on *Slc2a4* and *Pck1* expression was blocked by metformin treatment (Fig. 4A & C). However, although metformin seemed to have an effect on the LPS-induced changes in *Pdk4*, that change was not statistically significant (Fig. 4B). Given the effects of metformin on glucose metabolism in humans, the latter could be attributed to the short-term treatment in our studies. No measurable effect on the expression of these genes was detected by treatment with metformin alone (Fig. 4A–C).

3.4. Metformin Protects against LPS-Induced Reduction in ATP Synthesis and Autophagy [Experiment 1]

Metformin pretreatment protected mice from the expected reduction in cardiac ATP levels (Fig. 4D & E). At high doses LPS increases lactate concentration [32]. To exclude the possibility that there is such a case in our study we measured lactate levels. Lactate concentration revealed no differences between metformin-treated and non-treated mice. The effect of LPS in the dose administered did not have either any measurable effect on lactate concentration (Supplementary Fig. 2A). LPS increased pyruvate levels in heart tissue. Similar levels were found in metformin-pretreated/ LPS-injected samples (Supplementary Fig. 2B). In addition, LPS reportedly increases circulating, as well as heart tissue accumulation of triglycerides in the heart, an effect we confirmed with our experiments. Metformin, however, had no effect on this process (Supplementary Fig. 2C & D).

As previously reported, LPS induces autophagy in the heart via induction of LC3II [30]. We observed similar results in the LPS-injected group. Pretreatment with metformin protected mice from the LPS-induced autophagy induction, as significantly reduced levels of LC3II were detected. LC3II was also very low/not detected in the heart whole lysates of control and metformin-treated mice (Fig. 5).

3.5. Metformin Has a Therapeutic Effect in Acute LPS-Induced Cardiac Dysfunction [Experiment 2]

To assess the potential therapeutic effect of a single dose of metformin on LPS established acute cardiac dysfunction we administered metformin shortly (6 h) after LPS administration. As shown, by that time cardiac dysfunction has progressed close to its lower levels (Supplementary Fig.3).

Echocardiography (Fig. 6A), in LPS-injected/metformin-treated mice 24 h after LPS administration depicted significant amelioration (Fig. 6B) as compared to LPS-injected/ vehicle-treated mice, albeit to a lesser degree than the metformin pretreated mice (Fig. 1). Metformin treatment significantly decreased LVESD and LVEDD (Fig. 6B) values

compared to LPS administration, leading to significantly increased FS (Fig. 6C) and EF (Fig. 6D) values by 39% compared to 45% and 78% compared to 84%, respectively. Metformin markedly increased in LPS-induced reduction of posterior wall thickness both at systole and diastole (Fig. 6B).

Notably, expression of cardiac metabolic genes, such as increased *Prkaa2*, *Ppargc1a* and *Ppargc1b*, and reduced *Pdk4* expression, showed a trend toward normalization (Supplementary Fig. 4) although the induction/reduction was limited compared to the metformin pre-treated mice.

4. Discussion

Systemic inflammation has been associated with increased risk for cardiovascular complications, potentially leading to heart failure. LPS effects range from mild infection to endotoxemia and sepsis, all of which induce metabolic dysregulation, although of variable severity [33]. LPS administration in mice provides a good experimental model for the study of the dose-dependent effects of Gram-negative bacterial infections on the cardiovascular system.

In this study we demonstrate the protective effects of short-term pre-, as well as, post-LPS treatment with metformin, against low-moderate doses of LPS (similar to an acute infection)-induced myocardial dysfunction. We report that administration of a moderate, non-lethal, LPS dose compromised all cardiac functional echocardiographic indices (Fig. 1). We show that metformin pretreatment for 5 days prior to LPS administration, normalized all parameters assessed, including EF, posterior wall thickness, LVESD and LVEDD, and thus FS (Fig. 1B, C, D). Further, when metformin was administered 6 h after exposure to LPS, a time point at which all myocardial dysfunction indices have already reached their peak levels, it was still beneficial for cardiac function as it significantly improved all echocardiographic indices assessed. In this case, metformin substantially increased EF, LVPWd and LVPWs and decreased LVESD and LVEDD, thus improving FS (Fig. 4).

Metformin, which was recently shown to increase health span and lifespan in mice [34], is a widely used antidiabetic drug, particularly due to its effectiveness in restoring insulin sensitivity, improving hyperglycemia and increasing FAO [13]. The demonstrated antihyperglycemic properties of metformin are mediated by suppression of hepatic glucose production, and the parallel increase in tissue-specific insulin sensitivity [12,35]. As demonstrated, a major target of metformin is AMPK, a serine–threonine kinase that acts as a molecular sensor of energy availability in cells. AMPK enables ATP synthesis and energy availability, by coordinating the function of enzymes involved in carbohydrate and lipid metabolism [9]. Potential therapeutic effects of metformin against heart failure have been previously discussed. A low dose of metformin (125 μg/kg) administered either before ischemia (I) or at reperfusion (R) resulted in markedly attenuated myocardial injury in mice, whereas during the early reperfusion phase metformin augmented I/R-induced AMPK activation and increased endothelial nitric oxide (eNOS) phosphorylation [15]. Furthermore, in a murine model of heart failure, metformin administration following permanent occlusion of the left coronary artery, improved cardiac remodeling, while it attenuated heart-failure-

associated mitochondrial dysfunction through activation of AMPK and its downstream mediators eNOS and PGC1α [36]. The overall effectiveness of metformin in increasing survival in endotoxemia and sepsis remains controversial. Gras et al. reported that metformin administration at various doses, reaching levels as high as 500 mg/kg/animal, had no effect on survival rate in an animal model of *Escherichia coli*-induced sepsis [37]. On the other hand, as per another study, metformin (100 mg/kg/animal) increased mice survival rate, even when administered after endotoxemia symptoms had been established [18]. Design of combination schemes including antibiotics and cytokine antibodies with metformin may be useful in demonstrating the benefits of its administration in septic conditions.

In response to I/R or high-fat diet, compromised cardiac FAO is compensated by increased glucose metabolism, although this increase is not adequate to cover for all energy requirements. However, in response to infection, glucose metabolism is also compromised, thus causing additional decrease in energy availability and inability of the heart to adapt to the increased metabolic demands [29,38,39]. Recent studies have suggested that strategies targeting this pattern of myocardial metabolic dysfunction could potentially provide a very efficient therapeutic intervention for endotoxemia and sepsis [27,30]. Our data indicate severely compromised expression of genes mediating FAO and glucose utilization (Fig. 3C, D) in LPS-exposed mice, even at doses well below the threshold for progress to sepsis. Notably, 5 days of metformin pretreatment resulted in normalization of the expression levels of genes involved in FAO and maintenance of energy homeostasis in LPS-exposed mice. Pretreatment with metformin protected the heart from LPS-induced reduction in ATP levels. In addition, our findings show that pretreatment with metformin rescued the LPS-induced GLUT4 inhibition. Under normal conditions, in resting cells, glucose is metabolized to pyruvate via glycolysis. Pyruvate, a central molecule to metabolism, which can be converted to carbohydrates during gluconeogenesis or to fatty acids via acetyl CoA, is highly increased in disease states. Indeed, here, we found that LPS induced pyruvate production. Metformin pre-treatment, however, had no adverse effect. At high doses LPS increases lactate concentration [32]. Very few cases of severe lactic acidosis in patients with sepsis treated with metformin have been reported [40]. Following exhaustive discussions in the field the consent is that the benefits of metformin exceed by far the cautions raised by these cases. In fact, in our study measurement of lactate concentration revealed no differences between metformin-treated and not treated mice. Metformin had no effect on the LPS-induced triglyceride accumulation, as TG levels (both in the tissue and the serum) are significantly increased in LPS-injected and in metformin pre-treated/LPS-injected mice, suggesting that met-formin does not exert its cardioprotective effects via that route. A related physiological process critical in energy maintenance that is activated by stress conditions, including infection, is autophagy. Recent studies highlight the contribution of proper autophagy regulation for cardiac function [41,42]. As we report here, pre-treatment with metformin protected the heart from the LPS-induced autophagy dysregulation, an additional indication for the effects of metformin on cardiac metabolism.

Our current results offer additional insight into processes involved in infection-induced cardiac dysfunction, common complications carrying significant risks for individuals with susceptible preconditions [43,44]. We found, which is also shown by Tsoyi et al., that metformin offers protection against LPS administration. However in that study they used

much higher LPS doses (15 mg/kg/mouse) than we applied (5 mg/kg/mouse) and much higher metformin doses. In addition, they only assessed effects on survival but not changes in functional or histological cardiac indices. They showed that metformin treatment decreases the release of IL1β, IL6 and TNFα from macrophages and HMGB1 and myeloperoxidase activity in the lungs [18]. We demonstrated that metformin improved cardiac function, as assessed by echocardiography and histological evaluation. Additionally, we showed that metformin improved FA and glucose utilization and had a modest anti-inflammatory activity, as its administration led to significantly lower induction of IL6, although IL1β and TNFα levels were not different compared to LPS-injected mice. In addition, metformin administration protected mice from CRP and RAGE induction. Importantly, Ko and coworkers have stressed the dominant effect of IL6 on inflammation-induced glucose metabolism reduction through suppression of AMPK in the heart [45]. These findings suggest that the beneficial effects of metformin on infection-induced cardiac dysfunction are most likely mediated through mechanisms targeting the compromised metabolic function rather than the associated cytokine increase. Our findings are in line with those of Drosatos and coworkers who showed beneficial effects against a similar dose of LPS (5 mg/kg of BW) using a PPAR agonist that significantly improved FA and glucose oxidation, while it had no effect on inflammatory cytokines. The authors concluded that therapeutic approaches in septic patients should probably include a combination of anti-inflammatory treatment alongside with stimulation of cardiac energy production [30].

Importantly, we also showed that metformin exerted its beneficial effects even when administered after LPS at a stage when LPS-induced cardiac dysfunction had already been established. This finding suggests that apart from its potential prophylactic actions, metformin could be added as a therapeutic agent in patients with infection-induced cardiac dysfunction [Experiment 2].

All the above collectively suggest that metformin pretreatment has a significant effect on the main factors involved in the LPS-induced impaired lipid and carbohydrate myocardial metabolism, including ATP synthesis, FA and glucose oxidation and autophagy mechanisms. Throughout this study we found similar expression of metabolism- and inflammation-related indices in control and metformin-treated mice (Fig. 3), an observation that ruled out the possibility for non-specific, and potentially toxic, metformin effects. This finding stands in further support of its potential usefulness in the prevention from cardiac dysregulation often accompanying systemic infection. Our findings are in accordance with the well described effects of metformin, in other settings, in the maintenance of homeostasis [34,36].

In summary, we demonstrate that acute, mild Gram-infection, as mimicked by LPS administration, has a significant impact on the cardiovascular function. As previously reported, this is the outcome of significant changes in cardiac metabolic homeostasis, and, to a lesser extent, of the associated inflammatory process [30]. We provide evidence that metformin can prevent infection-associated cardiovascular dysfunction and rescue mice from the corresponding cardiac specific changes, mainly by preserving myocardial metabolic activity and energy utilization. The demonstrated metformin-induced changes in energy utilization should be further verified in future ex vivo experiments in isolated cell populations. Based on these findings and on the negligible side effects of metformin in

humans [46], we propose that further larger-scale studies should address the potential of metformin administration in inflammation-associated myocardial dysfunction, apart from its already mentioned anti-ischemic effects [15]. Our findings highlight the possibility for a novel indication for metformin, as a short-term, prophylactic cardioprotective treatment for individuals at risk of exposure to pathogens and point to the critical role of the tissue-specific metabolic dysfunction in this process.

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Abbreviations

ACC	Acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
Cpt	Carnitine palmitoyltransferase
EF	Ejection fraction
eNOS	Endothelial nitric oxide
FAO	Fatty acid oxidation
FS	Fractional shortening
GLUT	Glucose transporter
IL	Interleukin
LPS	Lipopolysaccharide
LV	Left ventricle
PDK	Pyruvate dehydrogenase kinase
PEPCK	Phosphoenolpyruvate carboxykinase
PGC	Peroxisome proliferator-activated receptor γ coactivator
PPAR	Peroxisome proliferator-activated receptor
TNF	Tumor necrosis factor

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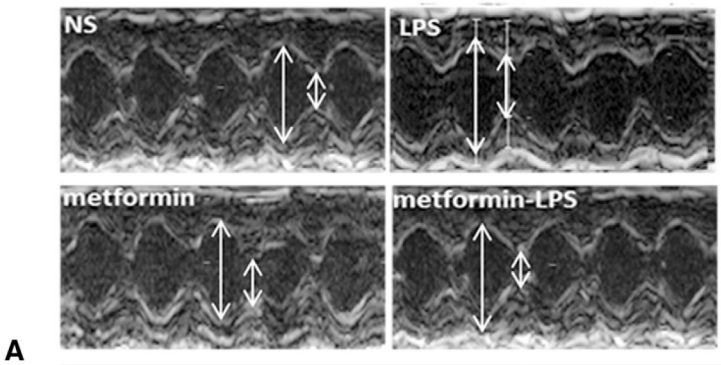


A

	Ctl	Metformin	LPS	Metformin-LPS
HR (bpm) ± SEM	628 ± 10	628 ± 24	446 ± 16 ***	514 ± 29
LVESD (mm)	1.68	1.62	2.16 **	1.79 †
LVEDD (mm)	3.08	3.04	3.27 *	3.06 †
LVPWD (mm)	0.77	0.77	0.69 ***	0.71
LVPWS (mm)	1.25	1.24	1.11 ***	1.19 †††

B

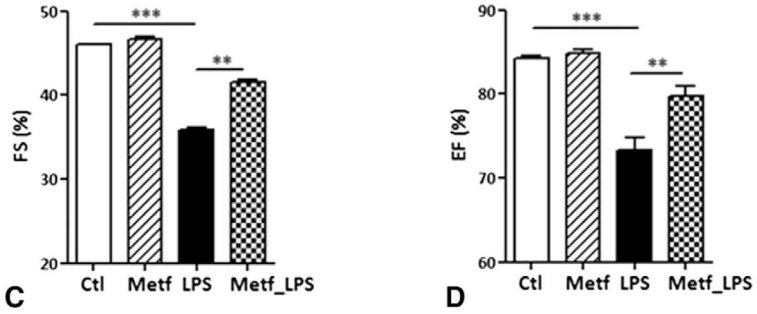


Fig. 1.

Pretreatment with metformin protects the heart from the LPS-induced functional changes. Two-D M-mode echocardiography showed that in mice treated with metformin prior to LPS administration, myocardial function was significantly less affected by LPS. Two-headed arrows indicate left ventricle chamber size at diastole and systole (A). Myocardial function indices including HR, LVESD, LVEDD, LVPWS and LVPWD [* indicates statistical comparison between ctl (vehicle-treated) and LPS-injected; † indicates statistical comparison between LPS-injected and metformin-pretreated/LPS-injected mice] (B), FS (C) and EF values (D) of ctl, metformin-treated, LPS-injected and metformin-pretreated/LPS-injected mice. Metformin alone had no effect on myocardial function indices. Control mice were injected with normal saline (0.9%). Bars are mean ± SEM. Differences between two groups were analyzed by unpaired Student's *t*-test, while comparisons among more than two groups were assessed by one-way ANOVA coupled with Bonferroni's tests (n = 30/group, 5 independent experiments). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

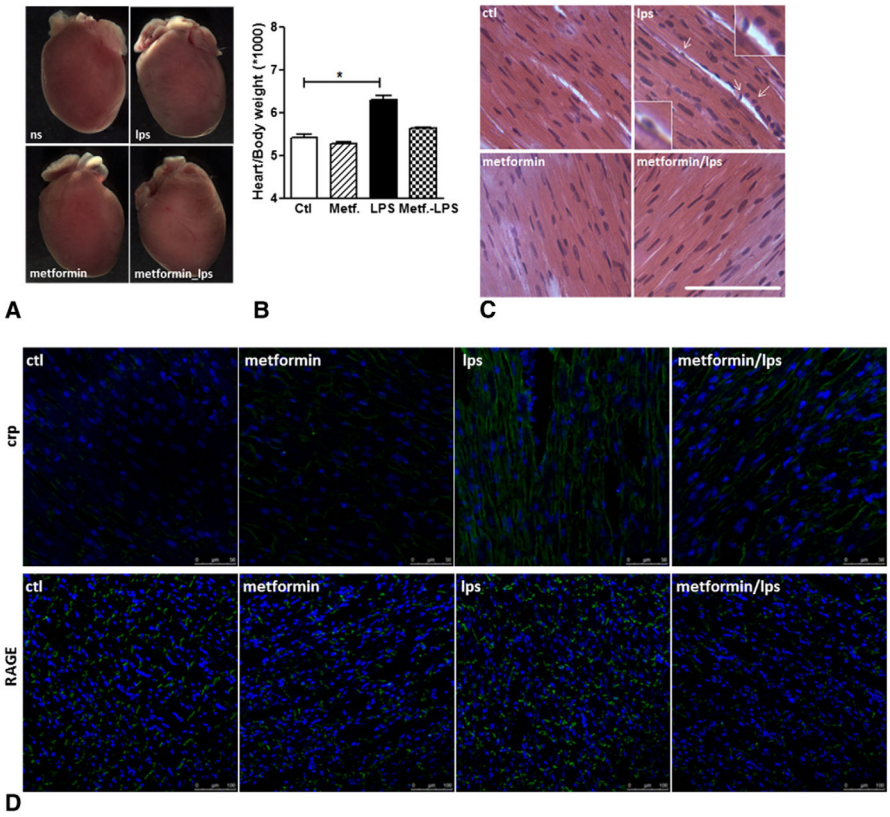


Fig. 2.

Whole hearts (1×) (representative picture of n = 10 hearts/ group) (A); heart per body weight (n = 12/ group, 2 independent experiments) (B). H&E staining (white bar 100 μm) of paraffin-embedded longitudinal hearts sections (representative photos of n = 24/ group, 3 independent experiments). Note the more prominent interstitial fibroblasts (indicated by white arrows and magnified in insets) (C). anti-CRP and anti-RAGE immunofluorescence staining of cryosections from ctl, metformin-treated, LPS-injected and metformin pretreated/ LPS-injected mice (n = 6/group, 2 independent experiments) (D). Bars are mean ± SEM. $*P < 0.05$.

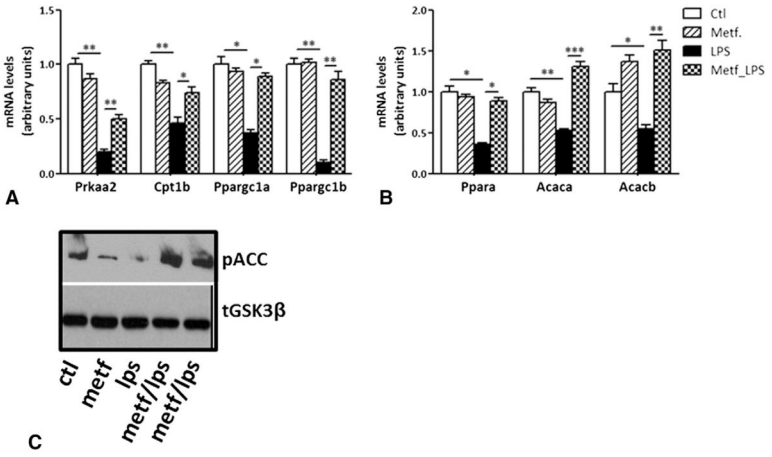


Fig. 3.

Fatty acid metabolism markers Prkaa2, Cpt1β, Ppargc1a, and Ppargc1b (A); fatty acid oxidation markers Ppara, Acaca and Acacb (B); and ACC phosphorylation levels (C) in control, metformin-treated, LPS-injected, and metformin pretreated/LPS-injected mice. Control mice were injected with normal saline (0.9%). Bars are fold change + positive standard error as calculated using the $^{\Delta\Delta}$CT method. Comparisons were assessed by one-way ANOVA coupled with Bonferroni's tests (n = 12/group, 2 independent experiments). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

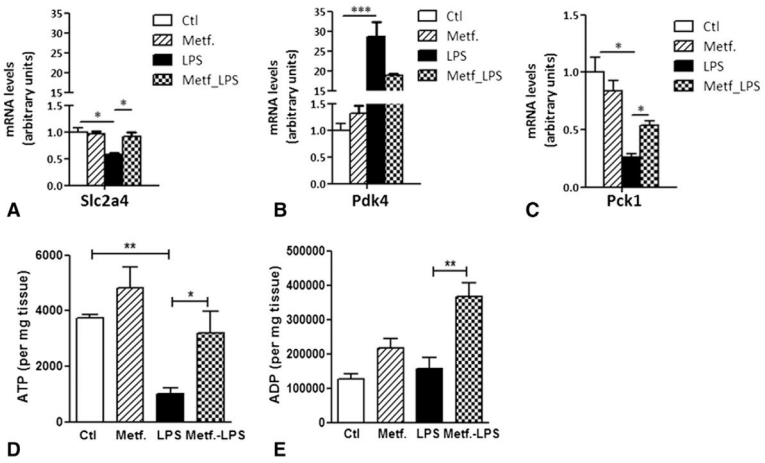


Fig. 4.

Glucose metabolism markers Slc2a4 (A) and Pdk4 (B); Pck1 (C); ATP (D) and ADP levels (E) in control, metformin-treated, LPS-injected, and metformin pretreated/LPS-injected mice. Control mice were injected with normal saline (0.9%). Bars are fold change + positive standard error as calculated using the $^{\Delta\Delta}$CT method. Comparisons among more than two groups were assessed by one-way ANOVA coupled with Bonferroni's tests (n = 12/group, 2 independent experiments). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

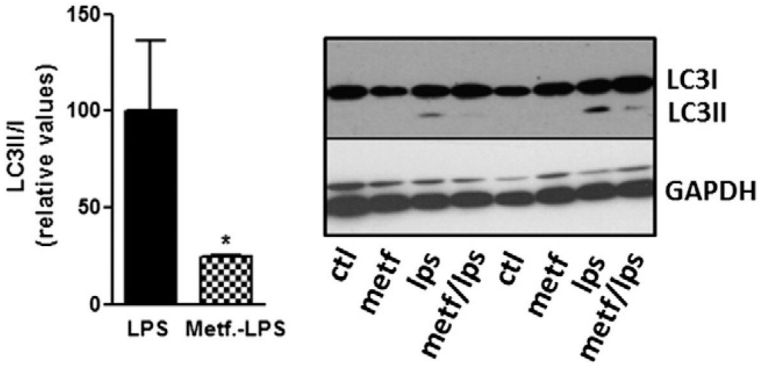
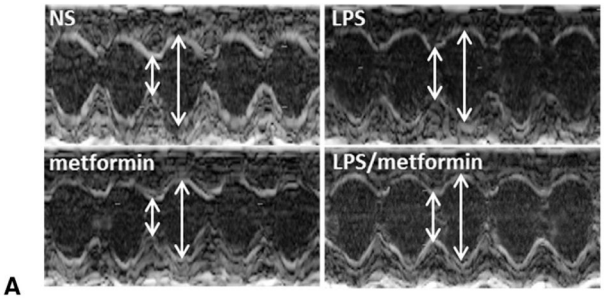


Fig. 5.

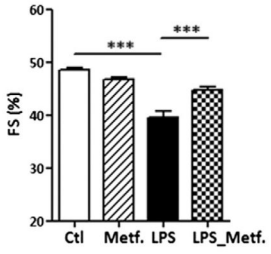
LC3II/I levels in control, metformin-treated, LPS-injected, and metformin pretreated/LPS-injected hearts. Bars are mean ± SEM (n = 10/group, 2 independent experiments). Comparisons were assessed by unpaired Student's *t*-test. **P* < *0.05*.



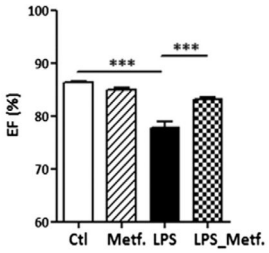
A

	Ctl	Metformin	LPS	LPS/Metformin
HR (bpm) ± SEM	616 ± 6.5	648 ± 10.5	541 ± 17.8	571 ± 12.1
LVEDD (mm)	2.93	2.96	3.23 **	3.05 †
LVPWd (mm)	0.79	0.8	0.72 ***	0.76 †
LVESD (mm)	1.51	1.57	1.96 ***	1.7 ††
LVPWs (mm)	1.3	1.29	1.21 ***	1.26 ††

B



C



D

Fig. 6.

Treatment with metformin 6 h after LPS administration protects the heart from the LPS-induced functional changes. Two-D M-mode echocardiography results showed that in mice treated with metformin 6 h after LPS administration, myocardial function was significantly improved compared to LPS-injected mice. Two-headed arrows indicate left ventricle chamber size at diastole and systole (A). Myocardial function indices including HR, LVESD, LVEDd and LVPWs (B) [* indicates statistical comparison results between ctl (vehicle-treated) and LPS-injected, while † indicates comparisons between LPS-injected and LPS-injected/metformin-treated mice], FS (C) and EF values (D). Control mice were injected with normal saline (0.9%). Bars are mean ± SEM. Differences between two groups were analyzed by unpaired Student's *t*-test, while comparisons among more than two groups were assessed by one-way ANOVA coupled with Bonferroni's tests (n = 12/group, 2 independent experiments). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Table 1

Real-time PCR primer pairs for individual genes.

Gene	Sense	Antisense
Acaca	TAACAGAATCGACACTGGCTGGCT	ATGCTGTTCCTCAGGCTCACATCT
Acacb	ACCCACTGTCTTCCAATGACACCT	TCAGCTGTCTCTTGATGTGTGCCT
Prkaa2	ATTGTGTTCTGCTCTGCACCTTGC	ACAGGTAAGCAACCAGACTCAGCA
Cpt1b	AGGCAAGTTCTGCCTGACTTACGA	TCGGAGCTGCGATATGGTACAGAA
Slc2a4	TCGTGGCCATATTTGGCTTTGTGG	TAAGGACCCATAGCATCCGCAACA
IL1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
IL6	ATCCAGTTGCCTTCTTGGGACTGA	TAAGCCTCCGACTTGTGAAGTGGT
Pdk4	AGTGACTCAAAGACGGGAAACCCA	ACACAATGTGGATTGGTTGGCCTG
Pck1	ATCTTTGGTGGCCGTAGACCT	GCCAGTGGGCCAGGTATTT
Ppargc1a	TCACCCTCTGGCCTGACAAATCTT	TTTGATGGGCTACCCACAGTGTCT
Ppargc1b	ATTGAACAAAGCTGCTTCCGTCCG	GTGGCAGTGGAACATCAACAGCAT
Ppara	AAGAACCTGAGGAAGCCGTTCTGT	GCAGCCACAAACAGGGAAATGTCA
TNFα	TCTCATGCACCACCATCAAGGACT	ACCACTCTCCCTTTGCAGAACTCA
18S rRNA	AACTTTCGATGGTAGTCGCCG	CCTTGGATGTGGTAGCCGTTT