Role of IRF4 in the Protection of Metformin-Mediated Sepsis Myocarditis

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Abstract

**Aims:** Metformin has been shown to play a protective role in diabetes. However, we found that metformin could mediate myocardial protection. Given that protein kinase C epsilon (PKCe) and interferon regulatory factor 4 (IRF4) are critical for cardioprotection signaling. And measurement of fluorescence resonance energy transfer (FRET) efficiency can be used to determine whether 2 fluorophores are within a certain distance of each other. So we sought to determine whether metformin promotes PKCe/IRF4 activation by FRET.

**Methods and Results:** The study built a mouse septic myocarditis model by intraperitoneal injection of *Escherichia coli*; thus, it provides valuable experimental data for the diagnosis and treatment of septic myocarditis. And cellular model of cardiomyocyte damage from adult rat cardiomyocytes or H9c2 cells was induced by lipopolysaccharide employed to examine PKCe by molecular, biochemical, and cellular imaging analysis. Life span of septic myocarditis mouse was significantly prolonged by metformin. Metformin also decreased transforming growth factor β level and increased interleukin-10 productions. The FRET analysis in H9c2 cells suggested that there is prominent FRET signal for PKCe along in mitochondrial by metformin.

**Conclusion:** We demonstrate that metformin promotes rapid association of PKCe with IRF4 at mitochondrial microdomain of cardiac myocytes and PKCe via direct molecular interaction with IRF4. This regulatory mechanism may play an important role in cardioprotection.

**Keywords**
metformin, PKCe, IRF4, mitochondria, FRET

Introduction

Sepsis constitutes a major cause of death following trauma and a persistent problem in surgical patients. The injury or infection mechanism of sepsis to the human body is well known, including suppression of inflammatory cytokine secretion and inhibition of polymorph nuclear leukocyte recruitment,1-4 which may lead to the serious injury of the body organs and the life of the crisis. These symptoms characterize systemic inflammatory response syndrome and evolve to septic shock when hypotension and loss of conscience appear and to multi-organ failure when organ dysfunction develops, organ failure in sepsis affect significant organs of the body, including the heart. Myocardial dysfunction is a well-described complication of severe sepsis and endotoxemia, which includes both systolic and diastolic dysfunction.

A number of mechanisms have been proposed to be involved in myocardial dysfunction in this syndrome. Protein kinase C (PKC) exists as a family of at least 12 isoforms. Protein kinase C epsilon (PKCe) plays important role in the myocardial protection. Upon activation, PKC isoforms can translocate to multiple subcellular localizations,5-7 such as plasma membrane, mitochondria, or nucleus. Although the translocation of specific PKCs to the mitochondria is believed to be a critical step in the signal transduction of myocardial...
protection, the precise mechanism underlying PKC-mediated cardioprotection remains largely unknown.

The interferon regulatory factor (IRF) family is a group of transcription factors, which comprises 9 members in both humans and mice. The IRFs are originally described as downstream regulators of interferon signaling and play important roles in the regulation of innate and adaptive immune response. The IRF4 contains a highly conserved N-terminal DNA-binding domain with a signature tryptophan pentad and a carboxy-terminal portion of an IRF-associated domain just like all other IRF members, which regulates the transcriptional activity of IRFs by mediating protein–protein interactions. However, whether IRF4, as a new transcription factor, plays a role in the heart, especially response to chronic pressure overload, has never been examined thus far. Hence, it will be very interesting to determine the consequence of overexpression and ablation of IRF4 in the septic myocarditis.

Metformin is a first-line drug for controlling circulating insulin levels in diabetes. Over the past decade, researchers have found that metformin has a cardiovascular protective effect, which can significantly reduce the patient’s cardiovascular events. But its mechanism is still unclear. Studies have shown that metformin has the effect of improving energy metabolism, and whether it can improve the cardiac function via improving the mitochondrial energy metabolism of heart failure after myocardial infarction remains unclear.

Fluorescence resonance energy transfer (FRET), resonance energy transfer (RET), or electronic energy transfer is a mechanism describing energy transfer between 2 light-sensitive molecules (chromophores). A donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor chromophore through nonradiative dipole–dipole coupling. The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, making FRET extremely sensitive to small changes in distance. Here, we performed FRET measurements in H9c2 cells to study PKCε translocate to mitochondrial under metformin stimulation. And this study established the model of septic myocarditis induced by *Escherichia coli* to investigate the mechanism of energy metabolic disorder in heart failure after myocardial infarction, and further study the protection of metformin on PKCε-IRF4 signaling pathway in mitochondrial after myocardial infarction and its mechanism, so as to provide new treatment targets for energy metabolism of septic myocarditis.

**Materials and Methods**

**Materials and Complementary DNA Constructs**

Mouse or goat immunoglobulin G (IgG) directed against PKCε, caveolin-3, IRF4, and rabbit IgG directed against PKCε were obtained from Santa Cruz Biotechnology (Santa Cruz, California). Rabbit IgG purchased from Cell Signaling Technology (Danvers, Massachusetts) were used to against GAPDH and PKCε. Trypan blue was purchased from Fisher Scientific (Pittsburgh, Pennsylvania). Caveolin-3-CFP was provided by Dr Jefferey R Martens (University of Michigan, Ann Arbor, Michigan), and PKCe-YFP was obtained from Dr Ralf Kubitz (Heinrich-Heine University of Düsseldorf, Düsseldorf, Germany). The full-length caveolin-3 was ligated into pECFP-C1 and PKCε was ligated into pEYFP-N1. Electrochemiluminescence (ECL) was purchased from Thermo Pierce (Thermo, USA).

**Cell Culture and Transfection**

Rat heart-derived H9c2 cells were cultured in Dulbecco’s modified Eagle’s medium/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin streptomycin. H9c2 cells were transfected with complementary DNAs using Fugene 6 according to the manufacturer’s instructions. Experiments were carried out 48 to 72 hours after transfection.

**Isolation of Cardiomyocytes**

Adult rat ventricular myocytes were isolated from Sprague-Dawley rats (250-300 g) by enzymatic dissociation. In brief, hearts were excised and retrogradely perfused via the aorta with gas (5% CO2-95% O2) Tyrode’s solution containing (mM) NaCl 126, KCl 5.4, NaH2PO4 0.33, HEPES 10, CaCl2 1.0, MgCl2 1.0, and glucose 10. The steps of heart perfusion and subsequent operation were performed at 37°C. The perfusate was then changed to a Tyrode’s solution that is nominally Ca2+ free, but otherwise has the same composition. Therefore, perfusion with the same solution was added 0.06% (w/v) crude collagenase for 20 minutes. Softened ventricular tissues were removed, cut into small pieces, and mechanically dissociated by trituration.

**Purification of Membrane or Mitochondrial Fractions**

Caveolin-rich fractions from adult rat cardiomyocytes or H9c2 cells were prepared by a previously described detergent-free method with some modification. Briefly, cells were pretreated with or without hypoxic preconditioning and then were spin down and resuspended in 0.5 M sodium carbonate (pH 11.0; 2 mL). Subsequently, the cell homogenates were subjected to centrifugation at 4 g, and the supernatant was received. The supernatant was adjusted to 45% sucrose by addition of an equal volume of 90% sucrose in 4-morpholineethanesulfonic acid (MES)-buffered saline (MBS; 25 mM MES, pH 6.5/0.15 M NaCl, pH 6.5) and placed at the bottom of an ultracentrifuge tube to be centrifuged. A 5% to 35% discontinuous sucrose gradient (in MBS containing 250 mM sodium carbonate) was formed above, by overlaying with 4 mL of 35% sucrose (prepared in MBS with 250 mM sodium carbonate) and then 4 mL of 5% sucrose (again prepared in MBS with 250 mM sodium carbonate). After adding continuous layers to the tubes, the tubes were centrifuged at 39 000 g for 18 to 20 hours in an SW41 rotor. Twelve 1-mL distinct membranous fractions were seen from the top to the bottom of the gradient for subsequent
analysis by Western blot. Caveolin-rich fractions (fractions 4-6) contain caveolin but exclude most other cellular proteins. In order to concentrate the caveolar to a pellet, we centrifuged the tubes at 40,000 g for 2 hours. Caveolin-rich fractions (fractions 4-6) were then suspended in lysis buffer and sonicated. To immunoprecipitate the caveolar, samples were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane, and analyzed by probing with various antibodies. The relative purity of caveolar or cellular fractions was evaluated by antibody against the marker proteins caveolin-3 or GAPDH, respectively.

**Western Blot and Co-immunoprecipitation**

Immunoblot analysis was carried out as described in detail previously. Briefly, the cellular and caveolar fractions were lysed and denatured in a sample buffer. Equal amounts of proteins were separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS), immunoblotted with primary antibodies in TBS, 0.1% Tween 20 for 2 hours at room temperature or overnight at 4°C. After washing, the blots were reacted with peroxidase-conjugated secondary antibodies for 45 minutes and the protein concentrations were determined by the ECL detection system.

Immunoprecipitation experiments were performed according to the previous report. The cells were lysed and centrifuged to get supernatant. Following the 2 hours at 4°C, incubation supernatant and antibody against PKCε or IRF4 complex were captured with r-protein-G agarose. Agarose beads slurry were washed 4 times with solubilization buffer before removal of bound proteins by boiling at 100°C for 5 minutes in SDS sample buffer. Samples were loaded in duplicate and separated by SDS-PAGE. Resolved proteins were transferred onto nitrocellulose membranes, blocked, incubated with primary and secondary antibodies, then analyzed by the ECL detection system.

**Analysis of FRET**

H9c2 cells were transfected with PKCδ-YFP/caveolin-3-CFP and PKCε-YFP/mitochondria-CFP. Images were acquired sequentially through cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and FRET filter channels as we described previously. Filter sets used were the donor CFP, the acceptor YFP, and FRET. A background value was determined from a region in each image without any cells. The background value was subtracted from the raw images before carrying out FRET calculations. Corrected FRET (FRETc) was calculated for entire images or selected regions of images, such as cell peripheral regions, using the equation: FRETc = FRET - (0.5 × CFP) - (0.5 × YFP), where FRET, CFP, and YFP correspond to background-subtracted images of cells coexpressing CFP and YFP acquired through the FRET, CFP, and YFP channels, respectively. The 0.5 values are the fractions of bleed through of CFP or YFP fluorescence, estimated from cells expressing either CFP- or YFP-fusion proteins. Mean FRETc values were calculated from mean fluorescence intensities for each selected subregion. All calculations were performed using Meta-Morph (version 2.1). The FRETc images are presented as a quantitative pseudocolor image.

**Data Analysis**

Group data were presented as mean ± standard error. Unpaired t test was used to compare between groups. Multiple group means were compared by analysis of variance followed by least significant difference post hoc test. Statistical significance was defined as P < .05 (*) and P < .01 (**).

**Results**

**Metformin Significantly Protects the Mice Heart From Lethal Dose of Infection and IRF4 is Responsible for the Protective Effect in Heart**

First of all, we established the inflammation lethal model by intraperitoneal (ip) injection of 1 mL E. coli (3 × 10^8 CFU/mL) in mice. As expected, all the mice received E. coli died within 72 hours following ip injection. We also set up metformin treatment group (MF) that the mice received lethal E. coli for 6 hours and then injected Metformin. And the life spans were significantly prolonged compared with lethal model. Various time points (1, 2, 3, 4, 6, 12, 24, 48, and 72 hours) were further evaluated.

Hematoxylin and eosin staining results showed that there was heart tissue in lethal model group compared to control group. In the MF group, the heart damages were largely reversed (Figure 1C). Meanwhile, the lethal model induced transforming growth factor β (TGF-β) was remarkably increased in peripheral blood. And metformin decreased TGF-β level and increased interleukin-10 (IL-10) productions (Figure 1B). Similar trend was obtained in IRF4 protein expressions when analyzed by Western blot (Figure 1D). From the results, we could see that IRF4 expressions were increased during 1 to 24 hours in sepsis group. The MF upregulated IRF4 expression during 1 to 24 hours.

**Protein Kinase C Epsilon Involvement in Metformin Protective Effect**

To investigate metformin could induce PKC isoform translocation from cytosol to cell membrane or not. We employed whether PKCε colocalizes with caveolin-3 by confocal microscopy. Figure 2A shows fluorescence images from adult rat ventricular myocytes with or without lipopolysaccharide (LPS). The antibody against caveolin-3 (green) or PKCε (red) demonstrated a prominent surface sarcolemmal punctate staining area. Merged images showed significant punctuate areas of colocalization for PKCε and caveolin-3 along the plasma membrane. These data suggest that metformin treatment could
induce PKC\(\varepsilon\) translocate to cell membrane and localized in caveolin-3-associated membrane domains.

We also used PKC\(\varepsilon\) to test in H9c2 cells whether YFP fused to the C-terminus of PKC\(\varepsilon\) is sufficiently close to CFP fused to mitochondria to yield FRET. H9c2 cells were transfected with Mito-CFP or PKC\(\varepsilon\)-YFP or both. As shown in Figure 2B, FRET\(^C\) was minimal in all regions of cells that were not subjected with metformin, indicating that there was no significant amount of PKC\(\varepsilon\) localized in mitochondria. Treatment of the cells with metformin treatment led to a small amount of translocation of PKC\(\varepsilon\) to the mitochondria micro-domains and exhibited FRET signals due to energy transfer from Mito-CFP to YFP at the C-terminus of PKC\(\varepsilon\) and fluorescence intensity measured along the line. To compare FRET efficiencies, the FRET\(^C\) was normalized to the intensity of PKC\(\varepsilon\)-YFP after background subtraction. Our results suggest that metformin promoted interaction between Mito-CFP and PKC\(\varepsilon\)-YFP.

It is generally believed that the activated PKC\(\varepsilon\) are translocated to the cell membrane and mitochondrial under stimulated condition.\(^{16}\) Therefore, we hypothesized that the PKC\(\varepsilon\) is translocated from cytosol to membrane and mitochondrial under metformin treatment. To test this hypothesis, we first separated the cell plasma membrane fraction from cytosol and then detected the mitochondrial distribution in these 2 fractions in adult rat cardiomyocytes. GAPDH and Na\(^+\)/K\(^+\)-ATPase are

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**Figure 1.** Effect of metformin on mice. A, The survival curves. Each line represents survival of mice in a group, 8 mice were in each group. Kaplan-Meier analysis was performed to test the difference. The mice injected with the lethal dose of \(E\ coli\) (3 × 108 CFU), then treated with metformin at different time points, *versus saline. B, ELISA assay of TGF-\(\beta\)/IL-10 in peripheral blood of mice at different time point. \(*P < .01\) (Student \(t\) test). C, Heart tissue of mice detected by H&E staining. D, Expression of IRF4 in total cellular lysates of mice cardiomyocyte. Representative Western blots of total cellular from 4 independent experiments. IRF4 indicates interferon regulatory factor 4.
used as cytosol and membrane markers, respectively. As shown in Figure 2C, under control condition, there is a minimum expression of PKCε on the cell membrane while the majority is distributed in the cytosol. However, metformin triggers the significant translocation of PKCε from cytosol to cell plasma membrane in cardiomyocytes. Meanwhile, we found metformin triggers the significant translocation of PKCε from cytosol to mitochondrial in cardiomyocytes (Figure 2D).

**Effect of Metformin Treatment on the Association of PKCε with IRF4**

To determine whether the activated IRF4 is localized to mitochondria by metformin treatment, we performed confocal microscope for IRF4 in H9c2 cells. From the results, we could see that the IRF4 inside the cell is localized in the cytosol at the normal condition. With the MF stimulation, the colocalization of IRF4 in the mitochondria was dramatically increased. And MF group could also increase IRF4 in the mitochondria in Figure 3A.

And we performed co-immunoprecipitation for PKCε and IRF4 in the mitochondrial fractions from H9c2 cells pretreated with or without metformin treatment. Mitochondrial fractions containing equal amounts of total proteins were immunoprecipitated with anti-PKCε antibody. The immune complex was collected with protein G beads and analyzed by immunoblotting. As shown in Figure 3B, there was a minimum association of IRF4 with PKCε under control condition in the mitochondria fractions. Treatment with metformin treatment could significantly increase the association of IRF4 with PKCε. The same experiments were repeated 3 times. The proteins detected in the PKCε immunoprecipitates were not...
detected in the lysates that were immunoprecipitated with control IgG. These results demonstrate that metformin treatment promotes the rapid association of IRF4 with PKCε in the mitochondria fractions of H9c2 cells.

**Discussion**

In fluorescence microscopy, FRET is a useful tool to quantify molecular dynamics in biophysics and biochemistry, such as protein–protein interactions and protein conformational changes. For monitoring the complex formation between 2 molecules, one of them is labeled with a donor and the other with an acceptor. The FRET efficiency is measured and used to identify interactions between the labeled complexes. There are several ways of measuring the FRET efficiency by monitoring changes in the fluorescence emitted by the donor or the acceptor.

The inflammatory component of the pathophysiology of sepsis is complex, involving activation of plasmatic and cellular systems. The innate immune response leads to a strong activation of the cytokine system, which effects on cardiac tissue lead to changes in vascular permeability and endothelial function. It has been observed that patients with sepsis with myocardial dysfunction have a 3-fold increase in mortality compared with those without cardiovascular impairment. Experimental approaches using in vivo animal models, isolated hearts, and cultured cells, and clinical studies have demonstrated that decreased contractility and impaired myocardial compliance are major factors in myocardial dysfunction in sepsis.

Heart requires large amounts of energy to sustain contractile function and is the major consumer of energy in the body on a weight basis. It has been shown that during heart failure, the myocardium has low adenosine triphosphate (ATP) content due to decreased ability to generate ATP by oxidative metabolism, and thus it is unable to effectively transfer chemical energy to contractile work. Speculatively, persistence of structural alterations may, among others, contribute to the morbidity, decreased health-related quality of life, and increased mortality observed in hospitalized patients with sepsis. Patients who died from sepsis showed myocarditis, disruption of the contractile apparatus, increased amounts of interstitial collagen, and damaged mitochondria.

In adult cardiomyocytes, mitochondria largely exist as discrete rounded organelles packed together between myofibrils. They are also clustered within the perinuclear and subsarcolemmal regions, but in no instance do they normally form interconnected networks. During sepsis-induced inflammation, cell survival requires the full support of energy metabolism, and damage to mitochondria may initiate apoptosis or trigger necrosis or both, by several mechanisms. It has been informed that mitochondrial abnormalities may decrease at longer periods after LPS challenge.

The effect of metformin in diabetes has been known, but it can prevent the occurrence of excessive inflammation of the body. We report here that metformin could cure septic myocarditis through activating PKCε-IRF4 signaling pathway in mitochondria of cardiomyocyte. In our previous work, we set up the dental mice model with *E. coli*. Based on this mice model, we constructed model of mice for septic myocarditis. The experimental data show that metformin significantly protects the mice heart from lethal dose of infection and PKCε-IRF4 signal pathway is responsible for the protective effect in the heart.

Metformin can regulate the mitochondrial function in septic myocarditis model, and its mechanism needs to be further studied. We found that under basal condition the level of reactivity
for PKCe in mitochondrial was minimal, but enhanced significantly following metformin stimulation. Our data also show that IRF4 is associated with PKCe. These observations provide the novel evidence that metformin induces the translocation and targeting of PKCe to mitochondrial. To address further the molecular interaction that may confer PKCe translocation to mitochondrial, we used PKCe as an example and studied the effect of metformin on direct molecular interaction between PKCe and IRF4. Our observation with FRET analysis revealed that there was no significant interaction between PKCe-YFP and mitochondrial-CFP as evidenced by minimum FRET signal. However, metformin induced substantial increase in FRET signal between PKCe-YFP and mitochondrial-CFP, indicating that metformin increased translocation of PKCe to mitochondrial. We performed co-immunoprecipitation for PKCe and IRF4 in the mitochondrial fractions and found that there was a minimum association of IRF4 with PKCe under control condition in the mitochondria fractions. Treatment with metformin treatment could significantly increase the association of IRF4 with PKCe.

In summary, our data demonstrate that metformin promotes PKCe targeting of IRF4 in mitochondrial of cardiomyocyte. This finding provides new mechanistic insight into our understanding of the role of metformin mediates protection of septic myocarditis.

**Declaration of Conflicting Interests**
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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