Berbamine Protects the Heart From Ischemia/Reperfusion Injury by Maintaining Cytosolic Ca\textsuperscript{2+} Homeostasis and Preventing Calpain Activation

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Background: Berbamine, a natural compound from Barberry, was reported to protect myocardium from ischemia/reperfusion (I/R) injury, but the underlying mechanisms are largely unknown.

Methods and Results: Berbamine pretreatment from 10 to 100 nmol/L concentration-dependently improved post-ischemic myocardial function. Similar protection was confirmed in isolated cardiomyocytes characterized by the attenuation of I/R-induced intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) overloading and the depression of cell shortening and Ca\textsuperscript{2+} transients, which were partially mimicked but not augmented by calpain inhibitor calpeptin and abolished by mitochondrial ATP-sensitive potassium (mitoK\textsubscript{ATP}) channel inhibitor 5-hydroxydecanoate (5-HD) and phosphoinositide 3-kinase (PI3K) inhibitor wortmannin. Consistently, I/R-induced increase of calpain activity and decrease of sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA2) activity; and protein expression of SERCA2a, desmin, calpastatin and Akt was significantly attenuated by berbamine. In addition, I/R-decreased Akt protein was reversed by calpeptin. Moreover, berbamine further increased I/R-enhanced phosphorylation of Akt and glycogen synthase kinase-3\beta (GSK3\beta). These protections were abolished by wortmannin. Furthermore, berbamine significantly attenuated I/R-induced lactate dehydrogenase release, infarct size and contractile dysfunction, and such cardioprotective actions were abolished by wortmannin and 5-HD or mimicked by glycogen synthase kinase-3\beta (GSK3\beta) inhibitor SB216763 but without additive effect.

Conclusions: These findings suggest that berbamine confers cardioprotection against I/R injury by attenuating [Ca\textsuperscript{2+}] overloading and preventing calpain activation through the activation of the PI3K-Akt-GSK3\beta pathway and, subsequently, opening of the mitoK\textsubscript{ATP} channel. (Circ J 2012; 76: 1993–2002)

Key Words: Berbamine; Ca\textsuperscript{2+} overloading; Calpain; Ischemia/reperfusion; PI3K-Akt-GSK3\beta

Myocardial infarction resulting from coronary atherosclerosis is the leading cause of death in modern society. Reperfusion is an essential treatment to salvage ischemia myocardium from necrosis, but the reperfusion can also lead to additional damage.\textsuperscript{1} Ischemic preconditioning (IPC) is a powerful way to initiate intrinsic adaptive responses protecting the heart from subsequent severe ischemia/reperfusion (I/R) injury.\textsuperscript{2} The IPC can be mimicked by pharmacological preconditioning.\textsuperscript{3–5} Because the pharmacological preconditioning is easy to apply without intrusion, exploration of effective medicines to protect the heart from I/R injury is one of the objectives of cardiovascular research. So far, however, few agents are clinically available for patients with ischemic heart disease.

Currently, there is growing interest in exploring effective cardioprotective components used in traditional Chinese medicine (TCM) because it has been practiced for thousands of years, providing a vast source of pharmaceutical materials. Berbamine is a type of bisbenzylisochinoline alkaloid derived from the roots, bark and stems of Barberry, which has been used as a medicinal plant in TCM. Berbamine has anti-tumor, immunomodulatory and cardiovascular effects.\textsuperscript{6–8} We recently found that it has a mild positive inotropic effect on the rat heart.\textsuperscript{9} It has also been shown to improve post-ischemic myocardial function and reduce the infarct size and arrhythmia rate,\textsuperscript{8,10,11} but the mechanisms are largely unknown.
Intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) plays a crucial role in regulating cardiomyocyte function in both physiological and pathophysiological conditions. A rise of [Ca\(^{2+}\)]\(_{i}\) due to abnormal Ca\(^{2+}\) handling, such as the decrease of sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase (SERCA2) function, is one of the major factors involved in I/R injury,\(^{12-14}\) resulting in contractile dysfunction and triggering the mechanisms of calcium-dependent injury.

Calpain, a Ca\(^{2+}\)-dependent thiol protease, is activated due to Ca\(^{2+}\) overload during I/R and proteolyzes its substrate, such as myoflament (eg, troponin I and T) and cytoskeletal proteins (eg, desmin),\(^{15}\) leading to plasma membrane rupture and contractile dysfunction. Inhibition of calpain activity attenuates the I/R-induced myocardial damage,\(^{16}\) but it is unclear whether berberine confers cardioprotection by inhibiting calpain activity through maintaining [Ca\(^{2+}\)]\(_i\) homeostasis.

Extensive evidence demonstrates that activation of phosphoinositide 3-kinase (PI3K) and protein serine/threonine kinase, protein kinase B (PKB/Akt) and the downstream inhibition of the glycogen synthase kinase-3β (GSK3β) signaling pathway confers myocardial protection against I/R injury by activating the anti-apoptosis pathway, opening the mitochondrial ATP-sensitive potassium (mitoK\(_{ATP}\)) channel and inhibiting mitochondrial permeability transition pore opening.\(^{17,18}\) The activation of the extracellular regulated kinase 1/2 (ERK1/2)\(^{19,20}\) or the signal transducer and activator of transcription 3 (STAT3) also mediates myocardial protection.\(^{21,22}\) Whether these signaling pathways contribute to the berberine-conferring cardioprotection, however, remains unknown.

To address these issues, the purpose of the present study was to: (1) characterize the concentration-dependent effects of berberine pretreatment on the post-ischemic myocardial function; (2) clarify the effects of berberine on the calpain activity and the protein degradation during I/R; (3) examine whether berberine regulates the calpain activity by maintaining [Ca\(^{2+}\)]\(_i\) homeostasis during I/R; and (4) determine the role of the PI3K-Akt-GSK3β signaling pathway in the berberine-induced cardioprotection. The present data provide new insight into the mechanism of berberine-induced cardioprotection and suggest the potential of berberine in the protection of the heart against I/R injury.

**Methods**

An expanded description of the methods involved in heart perfusion, total protein preparation, preparation of SR vesicles, western blot, determination of SERCA2 activities, calpain activity assay, measurement of [Ca\(^{2+}\)]\(_i\) and cell shortening, and simulated I/R in isolated cardiomyocytes is provided in Data S1.

**Heart Perfusion**

Male Sprague-Dawley rats (250–300 g; Shanghai Laboratory Animal Center, SIBS, CAS, Shanghai, China) were anesthetized with sodium pentobarbital (45 mg/kg i.p.). The hearts were isolated at 37°C using the Langendorff technique at a coronary perfusion pressure of 80 mmHg as previously described.\(^{23,24}\) Left ventricular (LV) pressure was monitored using a water-filled latex balloon connected to a pressure transducer (AD Instrument, Bella Vista, NSW, Australia) and inserted into the LV cavity, achieving an LV end-diastolic pressure (LVEDP) between 0 and 8 mmHg. LV developed pressure (LVDP), LVEDP, maximum rates of pressure development over time (dP/dt\(_{max}\)) and pressure decay over time (–dP/dt\(_{max}\)), and heart rate were monitored with PowerLab system (AD Instrument, Australia).

All experimental procedures on rats conformed to the guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996), and were approved by the Institutional Review Board of the Institute of Health Sciences, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine.

**Experimental Protocols in Isolated Hearts**

All hearts were equilibrated with KHB for 15 min before the application of experimental protocols described herein. I/R hearts were subject to 30 min of no-flow ischemia followed by 45 min or 120 min of reperfusion.

**Protocol A** To determine the concentration-response relationship of berberine on the recovery of post-ischemic myocardial function, berberine (Sigma), dissolved in deionized distilled water, was added to perfuse at final concentrations of 0, 10, 30, 100 and 300 nmol/L for 5 min prior to ischemia.

**Protocol B** To investigate the role of the PI3K-Akt-GSK3β pathway and mitoK\(_{ATP}\) channel in the cardioprotection of berberine, the PI3K-specific inhibitor wortmannin (300 nmol/L, Upstate Biotechnology)\(^{25}\) or GSK3β-specific inhibitor SB216763 (1 μmol/L, Sigma)\(^{26}\) was applied for 5 min followed by 5 min washout or 10 min prior to ischemia with or without berberine (100 nmol/L) treatment; and the mitoK\(_{ATP}\) channel-specific inhibitor 5-hydroxydecanoate (5-HD, 100 μmol/L, Sigma)\(^{27}\) was applied for 20 min prior to ischemia with or without berberine or SB216763 treatment. There were no hearts excluded because of ventricular fibrillation.

**Measurement of Lactate Dehydrogenase (LDH)**

LDH was spectrophotometrically measured in the coronary effluent before ischemia and at 5 and 45 min of reperfusion as previously described.\(^{24}\)

**Infarct Size Estimation**

The hearts subjected to 30 min of ischemia followed by 120 min of reperfusion were frozen and cut into 2-mm-thick slices as previously described.\(^{28}\) Sections were then incubated in 1% triphenyltetrazolium chloride for staining. Infarct size was measured on planimetry (Image Pro Plus, Media Cybernetics) and represented as a percentage of the LV area at risk.

**Protein Preparation and Western Blot**

The LV tissue was immediately frozen in liquid N\(_2\) at the end of perfusion experiments. Total protein and SR protein preparation were performed using modified methods as described previously,\(^{12,23,25}\) and the protein contents of SERCA2a in SR protein preparation\(^{13}\) and desmin, calpastatin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ERK1/2 (total and phosphorylated at Thr202/Thr204), Akt (total and phosphorylated at Ser473), GSK3β (total and phosphorylated at Ser9) and STAT3 (total and phosphorylated at Tyr705) in total protein preparation were analyzed on standard western blotting. Equal loading of SR protein samples was demonstrated by staining membranes subjected to western blotting with Coo-massie brilliant blue (Figure S1).

**Determination of SR Ca\(^{2+}\)-ATPase Activity**

The SR vesicles were prepared and SR Ca\(^{2+}\)-ATPase activity was determined as previously described.\(^{23}\)

**Real-Time Polymerase Chain Reaction (Q-PCR)**

SYBR green (TOYOBO) Q-PCR for Akt1 was performed using cDNA generated from total RNA extracted from LV
tissue. Akt1 primers were as follows: sense, 5’-TTTATTGGCTACAAGGAACG-3’; antisense, 5’-AGTCTGAATGGCGGTGGT-3’.

Calpain Activity Assay
Myocardial total protein was prepared and the calpain activity was determined using InnoZyme™ Calpain 1/2 Activity Assay Kit (Cat. No. CBA054, Calbiochem) as previously described.26

Isolation of Ventricular Myocytes
LV myocytes were isolated from adult male Sprague-Dawley rat hearts using a standard enzymatic method as previously described,3 and ≥85% of isolated rod-shape myocytes were Ca^{2+} tolerant.

Measurement of [Ca^{2+}]: and Cell Shortening
For measuring [Ca^{2+}], cells were incubated with Ca^{2+} indica-
tor indo-1 AM (5 μmol/L, Molecular Probes) at 25°C for 10 min. Cell shortening and \([\text{Ca}^{2+}]_i\) were simultaneously monitored through a fluorescence camera (Olympus, Tokyo, Japan) and a myocyte contraction and \([\text{Ca}^{2+}]_i\) recording system (IonOptix; Milton, MA, USA) as previously described. 3,12

Simulated I/R in Isolated Cardiomyocytes and Experimental Protocols
To measure \([\text{Ca}^{2+}]_i\) and cell shortening during I/R, a cellular model of simulated I/R was used as previously described. 3,12 Freshly isolated adult rat cardiomyocytes were exposed to 20 min of simulated ischemia followed by 30 min reperfusion through a fluorescence camera (Olympus, Tokyo, Japan) and a myocyte contraction and \([\text{Ca}^{2+}]_i\) recording system (IonOptix; Milton, MA, USA) as previously described. 3,12

Results
Berbamine Pretreatment Improves Post-Ischemic Recovery of Myocardial Performance and Survival
To characterize the cardioprotective effects of berbamine against I/R injury, we perfused the isolated rat hearts with berbamine at concentrations from 10 to 300 nmol/L for 5 min prior to ischemia. In control hearts the contractile function and relaxation of LV were severely suppressed during 45 min reperfusion following 30 min ischemia (Figures 1A–E). Berbamine from 10 to 100 nmol/L, having a mild positive inotropic effect on baseline cardiac function (Figure 1A) as we previously reported, 9 concentration-dependently improved the post-ischemic myocardial performance including increase of the recovery of LVDP, +dP/dt\(_{\text{max}}\), and –dP/dt\(_{\text{max}}\), and attenuation of I/R-induced elevation of LVEDP (Figures 1B–E). These effects became weaker, however, when berbamine reached 300 nmol/L, although berbamine did not affect the heart rate at both the pre-
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ischemic or I/R stages within the concentration range examined (Figure 1F).

Next, we investigated whether berbamine affects cell survival by detecting LDH release, an indicator of myocardial injury, and myocardial infarct size in isolated hearts suffering 30 min of ischemia followed by 120 min of reperfusion. LDH release was hardly detected in the coronary effluent before ischemia in both control and berbamine groups, but it was markedly induced at 5 and 45 min of reperfusion and the increases were significantly suppressed by 100 nmol/L of berbamine pretreatment (Figure 2A). Concordantly, berbamine pretreatment also significantly attenuated the I/R-induced myocardial infarction (Figure 2B) and suppression of myocardial contractile function (Figure 2C) without affecting the heart rate (Figure 2D).
Berbamine Improves Suppressed Cell Contraction and Ca\(^{2+}\) Transients and Prevents \([\text{Ca}^{2+}]_i\) Overload During I/R

To determine whether berbamine confers cardioprotection through its direct action on the cardiomyocytes, berbamine was applied to cardiomyocytes for 5 min prior to simulated I/R. Berbamine up to 100 nmol/L did not affect pre-ischemic cell shortening and Ca\(^{2+}\) transients, but it concentration-dependently improved post-ischemic cell shortening and Ca\(^{2+}\) transients from 3 nmol/L to 30 nmol/L (Figure 3; Figure S2). I/R markedly depressed the amplitude and maximum velocity of upstroke and decay velocity (±dL/dt\(_{\text{max}}\) and ±d[Ca\(^{2+}\)]/dt\(_{\text{max}}\)) of both cell shortening and Ca\(^{2+}\) transients, while those suppressions were significantly attenuated by 30 nmol/L of berbamine pretreatment (Figure 3). In addition, berbamine treatment did not affect the resting \([\text{Ca}^{2+}]_i\) before ischemia but it markedly reduced I/R-induced \([\text{Ca}^{2+}]_i\) overload (Figures 3A, 4).

To clarify the mechanism underlying berbamine-maintained Ca\(^{2+}\) homeostasis during I/R, the protein expression of SERCA2a and activity of SERCA2 were examined. I/R significantly decreased protein content of SERCA2a and activity of SERCA2, while these alterations were attenuated by berbamine pretreatment (Figure 5).

Berbamine Attenuates I/R-Increased Calpain Activation

Increasing evidence suggests that SERCA2a is degraded by calpain during I/R.\(^{14}\) We then determined whether berbamine provides cardioprotection via limiting calpain activation. I/R resulted in a marked increase of calpain activity compared with that of non-ischemia hearts, whereas the increased calpain activity was significantly attenuated by berbamine (Figure 6A). Moreover, I/R induced marked decreases of calpastatin and desmin, 2 sensitive substrates of calpain,\(^{15,27}\) while such alterations were suppressed by berbamine pretreatment (Figure 6B).

Next, we examined whether calpain inhibition contributes to the cardiac protection afforded by berbamine by using a calpain selective inhibitor, calpeptin. Calpeptin at 10 \(\mu\)mol/L partially mimicked berbamine-induced improvement of cell shortening and Ca\(^{2+}\) transients (Figure 3) and attenuation of Ca\(^{2+}\) overload (Figure 4) in simulated-I/R cardiomyocytes, but the combination of calpeptin and berbamine did not have additive protective effects compared with that of berbamine alone. These results suggest that the cardioprotection of berbamine is at least partially mediated via inhibition of calpain activation due to the maintenance of \([\text{Ca}^{2+}]_i\) homeostasis during I/R.

Berbamine Maintains \([\text{Ca}^{2+}]_i\) Homeostasis and Improves Myocardial Survival and Performance Through Opening of the MitoK\(_{\text{ATP}}\) Channel

To further determine whether the cardioprotection conferred
by berbamine occurs through the maintaining of mitochondria function via opening of the mitoK\(_{ATP}\) channel, the mitoK\(_{ATP}\) inhibitor 5-HD (100\,\mu\text{mol/L}) was applied to isolated cardiomyocytes and hearts. 5-HD alone did not affect the pre-ischemic or post-ischemic values compared with the corresponding control, but it abolished the berbamine-aided improvement of cell shortening and Ca\(^{2+}\) transients (Figure 3), and attenuation of [Ca\(^{2+}\)] overload in isolated cardiomyocytes (Figure 4). Consistently, the berbamine-improved LDH release, LV infarct size and contractile function were also abolished by 5-HD in I/R hearts (Figure 2). These results suggest that berbamine maintains [Ca\(^{2+}\)] homeostasis and improves myocardial survival and performance through opening of the mitoK\(_{ATP}\) channel.

Berbamine Confers Cardioprotection via PI3K/Akt Pathway

The reperfusion injury salvage kinase (RISK) pathway consisting of PI3K-Akt-GSK3\(\beta\) and ERK1/2 signaling events and the survivor activating factor enhancement (SAFE) pathway involving the activation of tumor necrosis factor-\(\alpha\) and the JAK-STAT3 axis have been implicated in myocardium protection against I/R injury.\(^{21,28}\) To determine whether the cardioprotection of berbamine is mediated through the RISK or SAFE pathways, we next examined ERK1/2, Akt, GSK3\(\beta\) and STAT3 phosphorylation on western blotting. I/R induced an increase in the phosphorylation of ERK1/2 (Thr202/Thr204) and STAT3 (Tyr705) compared with non-ischemia hearts, while it was not further increased by 100\,\mu\text{mol/L} of berbamine pretreatment (Figure 7A), suggesting that the ERK1/2 and STAT3 pathways are not involved in the cardioprotection of berbamine. The phosphorylation of Akt (Ser473) and GSK3\(\beta\) (Ser9) was also increased in I/R myocardium compared with that of non-ischemia hearts and such increase was further enhanced by berbamine pretreatment (Figure 7B). Further analysis showed a decrease of Akt protein due to I/R and such decrease was significantly attenuated by pretreatment of berbamine and calpeptin (Figures 7B,8A), but the combination of berbamine and calpeptin did not have an additive effect. In addition, Akt1 mRNA remained unchanged in I/R myocardium with or without berbamine pretreatment (Figure 8B).

To further determine whether berbamine protects the heart thought the activation of the PI3K-Akt-GSK3\(\beta\) signaling pathway, the selective PI3K inhibitor wortmannin or GSK3\(\beta\) inhibitor SB216763 was applied before the addition of berbamine. Wortmannin at 300\,\mu\text{mol/L} alone did not affect the pre-ischemic and post-ischemic values, while it abolished berbamine-improved post-ischemic LDH release, LV infarct size, and myocardial contractile function (Figure 2). Additionally, wortmannin abolished the berbamine-confirmed improvement of cell shortening and Ca\(^{2+}\) transients (Figure 3) and attenuation of [Ca\(^{2+}\)] overload after I/R (Figure 4). Moreover, the berbamine-attenuated increase of calpain activity, reduction of calpastatin, desmin, and SERCA2a contents and SERCA2 activity due to I/R, and I/R- or berbamine-increased Akt and GSK3\(\beta\) phosphorylation were abolished by wortmannin (Figures 5,6,7B). Furthermore, we determined the relationship between the GSK3\(\beta\) inactivation and the mitoK\(_{ATP}\) channel opening. SB216763, a GSK3\(\beta\)-specific inhibitor, at 1\,\mu\text{mol/L} mimicked but not augmented the berbamine-induced improvement of myocardial survival and contractile function during I/R, while its protective effects were abolished by a mitoK\(_{ATP}\) channel-specific inhibitor 5-HD, although all of those inhibitors did not affect the heart rate (Figure 2). Taken together, these results indicate a critical role of the PI3K-Akt-GSK3\(\beta\) pathway and, subsequently, opening of the mitoK\(_{ATP}\) channel, in the cardioprotection of berbamine.

Discussion

It has been almost 20 years since the first report about the cardioprotective effect of berbamine against I/R injury,\(^{10}\) but we know little about its mechanisms. In this study, we not only determined the concentration-dependent cardioprotection of berbamine, but also identified new mechanisms responsible for the cardioprotection conferred by berbamine. We found that (1) berbamine improves cell contraction and Ca\(^{2+}\) transients, prevents [Ca\(^{2+}\)] overload and conserves SERCA2a protein and SERCA2 function during I/R; (2) berbamine ex-
erts cardiac protection by attenuating I/R-induced activation of calpain and reductions of myocardial proteins, such as desmin, calpastatin, and Akt; and (3) the cardioprotection of berbamine is dependent on the activation of the PI3K-Akt-GSK3β signaling pathway and subsequently the opening of the mitoKATP channel to maintain [Ca^{2+}]_i homeostasis and to prevent calpain activation during I/R. These findings provide insight into the mechanisms underlying the cardioprotection conferred by berbamine.

**Cardioprotection of Berbamine Against I/R Injury**

We observed that berbamine pretreatment from 10 to 100 nmol/L improves post-ischemic myocardial function in a concentration-dependent manner and attenuates myocyte damage, while the improvement diminished at the higher concentration of 300 nmol/L in perfused hearts or 100 nmol/L in isolated cardiomyocytes (Figure S2). Those observations are consistent with the previous reports that berbamine could protect myocardium from I/R damage characterized by reducing creatine kinase release and promoting the recovery of cardiac functions in isolated perfused rabbit or rat hearts. The difference in the concentrations between the present study and previous reports may be due to the purity of berbamine and/or different species used. Moreover, the concentrations for berbamine-avoided cardioprotection are much lower than those causing a negative inotropic effect in the isolated hearts, as demonstrated recently. Further, the present data show that the improvement of post-ischemic myocardial performance by berbamine is related to its direct action on the cardiomyocytes. These data suggest a potential therapeutic role of berbamine in cardioprotection within an optimal concentration range.

**Berbamine Maintains [Ca^{2+}]_i Homeostasis During I/R via Preserving SERCA2 Function**

Intracellular Ca^{2+} overload plays a prominent role in I/R-induced cardiomyocyte damage by inducing excessive enzyme activation leading to cytoskeletal and sarcolemmal fragility, and mitochondrial permeability transition. Although berbamine has been shown to attenuate I/R-increased total Ca^{2+} content in the dried heart tissue of rat after I/R on atomic absorption spectrophotometry, the precise dynamic changes of [Ca^{2+}]_i during I/R in living myocytes remain unknown. The present data show that berbamine significantly improves cell contraction and Ca^{2+} transients, and suppresses [Ca^{2+}]_i overloading during both simulated ischemia and reperfusion by measuring the effect of berbamine on the beat-to-beat real-time alternations of contrac-

![Figure 7. Effect of berbamine (BM) on the activation of the PI3K-Akt-GSK3β signaling pathway. (A) Western blotting for phosphorylated and total ERK1/2 and STAT3; (B) western blotting for phosphorylated and total Akt and GSK3β. BM, berbamine (100 nmol/L); Con, control; I/R, ischemia/reperfusion; WM, wortmannin, a PI3K-specific inhibitor (300 nmol/L). n=3–4 hearts. *P<0.05, **P<0.01 vs. baseline control; †P<0.05 vs. I/R control; #P<0.05, ##P<0.01 vs. I/R hearts pretreated with BM alone.](attachment:image.png)
tion and [Ca\(^{2+}\)]: simultaneously in single isolated cardiomyocytes during I/R.

Opening of the mitoK\(_{\text{ATP}}\) channel is suggested to contribute to the maintaining of mitochondrial Ca\(^{2+}\) homeostasis and the preserving of mitochondrial function for providing adequate ATP during I/R, which is essential to maintain cytosolic Ca\(^{2+}\) homeostasis. Therefore, the opening of the mitoK\(_{\text{ATP}}\) channel by berbamine may explain its effect on inhibiting [Ca\(^{2+}\)] overloading during I/R. Decrease of Ca\(^{2+}\) handling proteins such as SERCA2a leads to a disturbance in cellular Ca\(^{2+}\) homeostasis during I/R. Hence, the berbamine-preserved SERCA2a content and SERCA2 activity during I/R also explain the berbamine-maintained [Ca\(^{2+}\)] homeostasis during I/R. Because the Na\(^{+}/\text{Ca}^{2+}\) exchanger (NCX) also plays a crucial role in the regulation of [Ca\(^{2+}\)] and is dysfunctional during I/R, \(^{13}\) whether the NCX participates in the berbamine-maintained Ca\(^{2+}\) homeostasis during I/R needs to be investigated.

Berbamine Protects I/R Hearts by Attenuation of I/R-Increased Calpain Activation

Extensive studies have demonstrated that a rise of [Ca\(^{2+}\)] activates calpain and the suppression of [Ca\(^{2+}\)] increase inhibits calpain activation.\(^ {30,31}\) Calpain is activated during I/R due to intracellular Ca\(^{2+}\) overload.\(^ {14,36}\) Activation of calpain cleaves a series of intercellular proteins, such as desmin and SERCA2a, which contributes to cardiac dysfunction and cell death after I/R insult.\(^ {12,14,15}\) Berbamine-maintained Ca\(^{2+}\) homeostasis during I/R is expected to depress I/R-induced calpain activation. This is supported by the observation that I/R-induced increase of calpain activity and the reductions of calpastatin, desmin, SERCA2a proteins are attenuated/abolished by berbamine pretreatment. Therefore, berbamine may protect the heart from I/R insult via the reduction of calpain-induced specific proteolysis.

Calpastatin is an endogenous inhibitor of calpains and also a sensitive substrate of calpain; I/R-induced calpain activation proteolyzes calpastatin and reduces its inhibition to calpains.\(^ {27}\) In addition, desmin, a predominant intermediate filament protein in mature muscle, forms a 3-D scaffold in the cell, which contributes to the integration of contraction and to cell integrity.\(^ {15}\) Therefore, as discussed here, berbamine-conferred inhibition of calpain activation and subsequent prevention of the decreases of those proteins appears to be a crucial mechanism of berbamine-conferred cardioprotection. This is further supported by the observation that the calpain inhibitor calpeptin partially mimicked the protection conferred by berbamine in the simulated I/R cardiomyocytes but without the additive effect of berbamine.

Involvement of the PI3K-Akt-GSK3\(\beta\) Pathway in the Cardioprotection of Berbamine

Pharmacological and genetic studies have shown that the PI3K-Akt-GSK3\(\beta\) axis of the RISK pathway is essentially involved in myocardium protection against I/R injury.\(^ {20,32}\) Whether ERK1/2 contributes to cardiac protection, however, remains controversial.\(^ {33,34}\) Here, the increase of phosphorylated Akt and GSK3\(\beta\), but not ERK1/2 or STAT3 by berbamine may contribute to the berbamine-affected cardioprotection by maintaining [Ca\(^{2+}\)] homeostasis, subsequently preventing calpain activation and proteins reduction, and resulting in the improvement of post-ischemic myocardial function. This is confirmed by the abolishment of berbamine-induced cardioprotection when the PI3K-specific inhibitor wortmannin was applied prior to the berbamine treatment, and by the mimicked cardioprotection of the GSK3\(\beta\)-specific inhibitor SB216763 without the additive effect of berbamine. These findings further confirmed that PI3K-Akt-GSK3\(\beta\) is a critical signaling pathway mediating acute cardioprotection against I/R injury as documented previously.\(^ {18,20,32}\) Although it remains controversial whether inactivation of GSK3\(\beta\) through phosphorylation (Ser 9) reduces apoptosis and enhances cell survival via opening of the mitoK\(_{\text{ATP}}\) channel,\(^ {18,35}\) the present results provide evidence that the activation of the PI3K-Akt-GSK3\(\beta\) pathway is upstream of the opening of the mitoK\(_{\text{ATP}}\) channel in berbamine-induced cardioprotection.

Another interesting finding is that Akt protein is decreased after I/R injury, but this decrease is reversed by berbamine. Considering that Akt mRNA remains unchanged during I/R with or without berbamine, berbamine might attenuate the decrease of Akt by inhibiting the calpain activity. This is supported by the attenuation of Akt decrease during I/R using calpain inhibitor, and that there is no additive effect by combining calpain inhibitor and berbamine. The conserved Akt protein would in turn further contribute to the protection of berbamine through the PI3K-Akt-GSK3\(\beta\) pathway. This possibility needs to be further investigated.

Conclusions

Berbamine concentration-dependently improves post-ischemic myocyte performance from 3 nmol/L to 30 nmol/L and myo-
cardial performance from 10 nmol/L to 100 nmol/L. Such cardioprotection is at least partially mediated by the suppression of IR-increased calpain activation and protein degradation due to the maintenance of [Ca^2+]-homeostasis through the activation of the PI3K-Akt-GSK3β signaling pathway and, subsequently, opening of the mitoKATP channel but not the ERK1/2 or STAT3 pathway.

References


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Supplementary Files

Supplementary File 1

Data S1. Methods

Figure S1. Representative Coomassie-stained membrane showing equivalent SR protein loading.

Figure S2. Effect of berbamine on the cell shortening and Ca^2+ transients in isolated cardiomyocytes subjected to 20 min simulated ischemia followed by 30 min reperfusion.

Please find supplementary file(s); http://dx.doi.org/10.1253/circj.CJ-11-1431

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