Original Research

Sparstolonin B attenuates hypoxia–reoxygenation-induced cardiomyocyte inflammation

Qing Liu[1,2], Junfeng Wang[1,3], Qiaoli Liang[4], Dawei Wang[2], Yi Luo[2], Jianping Li[1], Joseph S Janicki[1] and Daping Fan[1]

[1]Department of Cell Biology and Anatomy, University of South Carolina School of Medicine, Columbia, SC 29209, USA; [2]The Second Clinical School of Medicine, Guangzhou University of Chinese Medicine, Guangzhou, 510405, China; [3]Centre for Stem Cell Research and Application, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, China; [4]School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210046, China
Corresponding author: Daping Fan. Email: daping.fan@uscmed.sc.edu

Abstract

Myocardial ischemia–reperfusion (MIR) injury is characterized by a rapid increase in cytokines and chemokines and an infiltration of inflammatory cells. Toll-like receptors (TLRs) 2 and 4 mediate these inflammatory responses. Herein we investigated the ability of Sparstolonin B (SsnB), a new selective TLR2/4 antagonist, to inhibit the TLR2/4-mediated inflammatory responses during cardiomyocyte hypoxia–reoxygenation injury as well as the responsible mechanisms. Lactate dehydrogenase (LDH) assay was performed to measure the cytotoxicity of SsnB on H9c2 cardiomyocytes. Quantitative real-time PCR (qRT-PCR) confirmed that TLR2 and TLR4 expression was elevated during hypoxia–reoxygenation, and that their up-regulation in cardiomyocytes was significantly inhibited by SsnB ($P < 0.05$). Both the mRNA and protein levels of monocyte chemotactic protein-1 and high mobility group box 1 were up-regulated during hypoxia–reoxygenation and were significantly attenuated by SsnB ($P < 0.05$). Next we found that extracellular signal-regulated kinase 1 or 2 (ERK1/2) and c-Jun NH2-terminal kinase (JNK) signaling pathways were activated during hypoxia–reoxygenation and SsnB significantly inhibited their activation ($P < 0.05$). Moreover, transwell migration assays revealed that the migration of mouse macrophages to hypoxia–reoxygenation injured cardiomyocytes was significantly reduced by SsnB ($P < 0.05$). In conclusion, our data indicate that the new selective TLR2 and TLR4 antagonist, SsnB, can substantially attenuate hypoxia–reoxygenation-induced inflammation of cardiomyocytes via inhibiting ERK1/2 and JNK signaling pathways. Accordingly, SsnB has the potential to serve as a therapeutic agent for the prevention of MIR injury.

Keywords: Myocardial ischemia–reperfusion injury, Sparstolonin B, inflammation, Toll-like receptor, macrophage

***Experimental Biology and Medicine* 2014; 239: 376–384. DOI: 10.1177/1535370213517620**

Introduction

Myocardial infarction (MI) leads to an acute loss of viable myocardium due to hypoxia. The early restoration of blood flow and thus oxygen supply to ischemic myocardium is a common treatment strategy aimed at limiting myocardial infarct size. However, during the hypoxia and the subsequent reperfusion, a large amount of proinflammatory cytokines and chemokines are produced by compromised cardiomyocytes in response to first hypoxic and then oxidative stress. The high local concentration of cytokines and chemokines lead to a rapid influx and retention of circulating leukocytes. The accumulated leukocytes escalate the myocardial inflammation and cause additional cardiomyocyte death and paradoxically increased infarct size during the reperfusion, a situation referred as myocardial ischemia–reperfusion (MIR) injury. Therefore, limiting an ischemia–reperfusion (I/R) induced myocardial inflammatory response is expected to improve long-term survival and quality of life.[1]

MIR occurs in a sterile environment and results in a sterile inflammation. Nevertheless, the MIR-induced inflammatory response shares many phenotypic parallels with activation of a host immune response directed toward invading micro-organisms.[2] This sterile inflammation is mainly triggered by the interactions between toll-like receptors (TLRs) and their endogenous ligands generated in ischemic myocardium, such as apoptotic cell debris, fibrinogen, high mobility group box (HMGB) 1, and heat shock proteins.[3] The activation of TLRs including TLR2 and TLR4 on local endothelial cells and cardiomyocytes results in the expression of adhesion molecules and secretion of cytokines and chemokines.[4,5] A detrimental role of TLRs

in MIR injury has been well documented in animal models,[6] therefore blocking the signaling initiated by TLRs, especially TLR2 and TLR4 is a promising strategy for the treatment of MI.

Chinese herbal medicine has been widely used in traditional Chinese medicine for the treatment of MI for hundreds of years. Recently we isolated a new compound, designated Sparstolonin B (SsnB), from the Chinese herb *Sparganium stoloniferum*, and characterized it as a selective TLR2 and TLR4 antagonist.[7] In addition, we have shown that SsnB potently inhibits macrophage inflammatory responses to multiple TLR2 and TLR4 ligands *in vitro* and *in vivo*,[7] and blocks lipopolysaccharide-induced endothelial cell activation.[8] The purpose of this study was to determine if SsnB could similarly inhibit the inflammatory response of cardiomyocytes to hypoxia–reoxygenation, which mimics I/R, and thus may be developed as a new therapy for MI.

Materials and methods

Cell culture, induction of hypoxia–reoxygenation

The H9c2 rat ventricular cell line (CRL1446, ATCC, Manassas, VA) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS, Invitrogen) at 37°C and 5% CO_2. The medium was replaced every 2 days. When cells were 80% confluent, they were subcultured and subjected to experimental procedures at 80–90% confluence. Hypoxia was induced by exposing cells to a 1% O_2/94% N_2/5% CO_2 environment in serum-free low-glucose DMEM for 4 or 16 h. In the reoxygenation phase, the medium was changed to standard DMEM with 10% FBS and cells were cultured in a normoxia environment with 5% CO_2 for 4 h.

SsnB treatment

The isolation and structural determination of SsnB was described previously.[7] SsnB powder was dissolved in dimethyl sulfoxide as a stock solution of 50 mg/mL (186.6 mM) and then diluted into appropriate media to desired concentration. The solubility of SsnB in most of the cell culture media is ~150 μg/mL (0.56 mM). SsnB-containing medium was added during ischemia and/or reperfusion treatments. A commercially available TLR-2 antagonist, CU-CPT22 (Calbiochem, Billerica, MA), was used as a control.

LDH assay

LDH is normally retained in the cytosol until the sarcolemmal membrane is ruptured, after which it is free to diffuse into the surrounding media. To determine the amount of cell injury induced by hypoxia–reoxygenation, both the culture medium and cell lysate were collected and immediately assayed for LDH activity using a LDH Cytotoxicity Detection Kit according to the manufacturer's instruction (Clontech, Mountain View, CA) with a Spectra Max M5 Microplate Reader (Molecular Devices, Sunnyvale, CA) at Optical density (OD) 490 nm. Cell viability was calculated as the ratio of LDH amount in the cell lysate to the total LDH amount from both the medium and cell lysate.

Enzyme-linked immunosorbent assay

HMGB1 and monocyte chemotactic protein-1 (MCP1) protein concentrations in the conditioned medium were measured by Enzyme-linked immunosorbent assay (ELISA) (MyBioSource, San Diego, CA and Pierce, Rockford, IL, respectively) according to the manufacturer's instructions. After experimental treatment, culture medium of cardiomyocytes was collected and centrifuged at 1000*g* for 15 min to pellet the cell debris. The supernatant was collected and stored at −80°C prior to the measurement. After reactions, 96-well microplates were read using a Spectra Max M5 Microplate Reader at OD 450 nm.

Real-time reverse-transcription polymerase chain reaction (qPCR)

Total RNA was extracted and purified from H9c2 cells using TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions before performing reverse transcription using a First-strand cDNA Synthesis Kit (Bio-Rad, Hercules, CA) on the cDNA Synthesis System (Bio-Rad). qPCR analyses were carried out using iQ SYBR Green Supermix (Bio-Rad) on an Eppendorf Realplex2 Mastercycler (Bio-Rad). The primers (Integrated DNA Technologies, Coralville, IA) used in qRT-PCR are listed as follows: rat 18 S RNA (internal control) 5′- TGAGGCCATGATTAAGAGGG-3′ (forward) and 5′-AGTCGGCATCGTTTATGGTC-3′ (reverse); rat TLR2, 5′-GCTCCTGTGAACTCCTGTCCTT-3′ (forward) and 5′-GCTTTCTTGGGCTTCCTCTTGG-3′ (reverse); rat TLR4, 5′- ACCAGGAAGCTTGAATCCCTG-3′ (forward) and 5′-CCAGCCACTGAAGTTGTGAGA-3′ (reverse); rat HM GB1, 5′- TCCTTCGGCCTTCTTCTTGT-3′ (forward) and 5′-CGGCCTTCTTTTCATAGGGC-3′ (reverse); rat MCP1, 5′-CCAATGAGTCGGCTGGAGAACT-3′ (forward) and 5′-AGTGCTTGAGGTGGTTGTGGAA-3′ (reverse). Samples were amplified using the following program, 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 58°C for 15 s, and 72°C for 20 s, then a melting curve analysis from 65 to 95°C every 0.2°C. The abundance of each gene product was calculated by relative quantification, with values for the target genes normalized with 18 S RNA.

Western blot

After experimental treatment, H9c2 cells were subjected to the Lysis buffer (Cell signaling, Danvers, MA) on ice for 15 min, and the lysates were clarified by centrifugation at 4°C for 10 min at 13,000 rpm. After quantitation of protein concentration by Lowry assay, 30 μg [for extracellular signal-regulated kinase 1 or 2 (ERK1/2)] or 60 μg [for c-Jun NH2-terminal kinase (JNK)] of total protein were loaded onto 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels for electrophoresis at 100 V for 1.5 h. The size-separated proteins were then transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature with 5% nonfat dry

milk, then incubated with primary antibodies against p-ERK1/2 and t-ERK1/2 (Millipore, Billerica, MA), p-JNK and t-JNK (Cell signaling, Danvers, MA), and β-actin (1:500 ~ 1:1000) overnight at 4°C. After three washings with PBST, the membranes were incubated with corresponding Horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Signal of target proteins was detected with an ECL kit (Pierce). The gray density of the bands in the obtained images was quantified by the software Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD).

Macrophage migration assay

Primary peritoneal macrophages were obtained from wild-type C57Bl/6 mouse. Firstly, 3 mL of 3% thioglycollate was injected into mice intraperitoneally. After 3 days, 10 mL of PBS was injected into the peritoneal cavity to collect cells. Then the cells were seeded in a 60-mm dish in DMEM with 10% FBS for 1 h and washed with PBS 3 times to remove unattached cells. The attached macrophages were then transferred to the Transwell inserts of a 24-well plate (Corning, Corning, NY) in serum-free DMEM medium, and further cocultured for 1 h with H9c2 cells that have undergone hypoxia for 16 h followed by reoxygenation for 4 h. The inserts were then taken out and fixed in 4% formaldehyde for 20 min. Cells were swabbed from the upper well using cotton buds and then stained in 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) for 1 min, followed by washing twice with PBS for 2 min. For each sample 10 fields of the stained cells were photographed using an inverted wide-field fluorescence microscope (Eclipse E600, Nikon, Japan) at 100 × magnification. The number of cells in the obtained images was counted using Image-Pro Plus 6.0.

Statistical analysis

GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used to carry out all statistical analysis. One-way analysis of variance was used for multiple group comparisons. If the data followed a Gaussian distribution, then Bonferroni's multiple comparisons were used as a posttest. Otherwise, the nonparametric Kruskal–Wallis test and Dann's multiple comparison posttest were used to analyze the data. When only two groups were compared, Student's *t*-test was performed. A P value of < 0.05 was considered statistically significant.

Results

Cytotoxicity measurement of SsnB on H9c2 cells

Before investigating the biological activity of SsnB on H9c2 cells, LDH assay was performed both in normoxia and hypoxia conditions to evaluate the cytotoxicity of SsnB. Cell viability was calculated by the ratio of LDH amount in the cell lysate to the LDH amount from both the medium and cell lysate. In normoxia, cell viability was not decreased by treatment of SsnB at concentrations up to 10 μM; however, SsnB at 100 μM significantly reduced the cell viability (Figure 1(a)). In hypoxia, SsnB at concentrations from 0.1 to 10 μM did not reduce cell viability; but it significantly

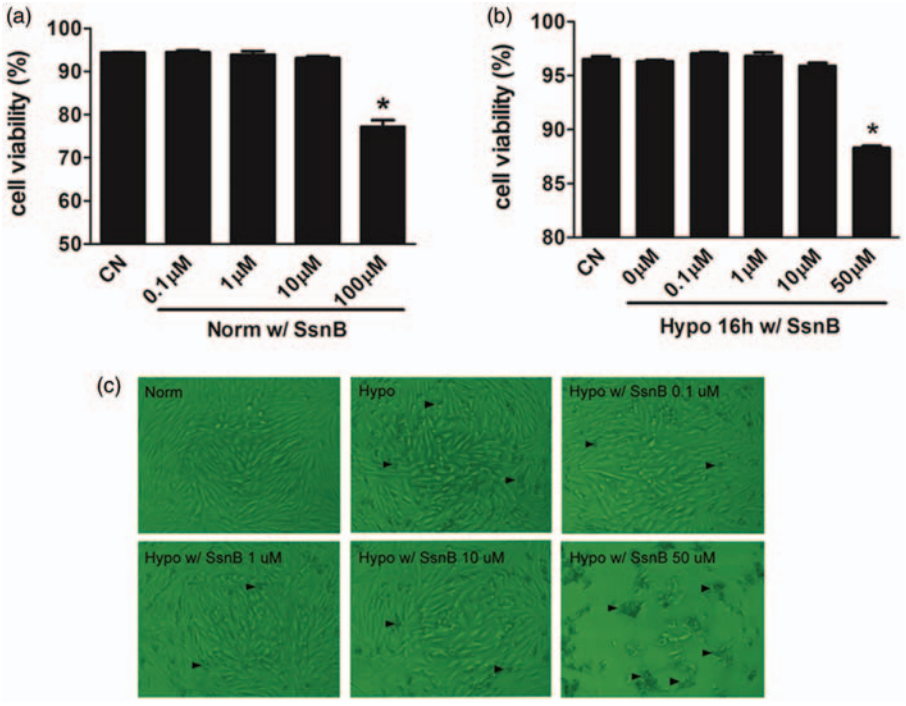

Figure 1 Cytotoxicity measurement of SsnB on H9c2 cells. (a) Cell viability of H9c2 treated with SsnB at indicated concentrations in normoxia (19% O_2) for 16 h, as determined by LDH assay. Data are presented as mean ± SEM, $n = 3$, $^*P < 0.05$ vs SsnB 0 μM (CN) group. (b) Cell viability of H9c2 treated with SsnB at indicated concentrations in hypoxia (1% O_2) for 16 h, as determined by LDH assay. Data are presented as mean ± SEM, $n = 3$, $^*P < 0.05$ vs CN (complete DMEM medium and normoxia group). (c) Images of H9c2 cells treated with SsnB at indicated concentrations in hypoxia for 16 h. Representative images were taken using a light microscope. Black arrows indicate dead cells. Magnification: 100 ×. (A color version of this figure is available in the online journal.)

reduced cell viability at 50 μM (Figure 1(b)). Therefore, SsnB was used in the following experiments at 10 μM concentration.

SsnB inhibited the up-regulation of TLR2 and TLR4 in H9c2 cells induced by hypoxia–reoxygenation

To investigate how TLR2 and TLR4 expression is changed during hypoxia and reoxygenation conditions, and if SsnB has any effects on that, qPCR was performed to measure mRNA levels of TLR2 and TLR4 in H9c2 cells. As shown in Figure 2(a), hypoxia for 4 and 16 h significantly induced TLR2 mRNA levels (4- and 20-fold, respectively) and TLR4 mRNA levels (1.6- and 3.4-fold, respectively). SsnB treatment did not significantly affect the TLR2 and TLR4 expression increases in the cells that were subjected to hypoxia for 4 h; however, when SsnB was used during the hypoxia for 16 h, the increases in TLR2 and TLR4 expression were significantly attenuated by 3.5- and 1.8-fold, respectively. In another set of experiments, the cells were treated with hypoxia for 16 h and then reoxygenation in normoxia condition for 4 h, with SsnB added to the medium either only during the reoxygenation period or during both hypoxia and reoxygenation periods. We found, while hypoxia–reoxygenation dramatically increased TLR2 and TLR4 expression in H9c2 cells by 20- and 2.3-fold, respectively, SsnB significantly attenuated TLR2 and TLR4 expression with the effects on TLR2 being more dramatic. In the case of TLR4, treatment with SsnB during both hypoxia and reoxygenation periods produced more profound reduction in mRNA levels compared to when treatment of SsnB was limited to the reoxygenation period (Figure 2(b)).

SsnB inhibited hypoxia-induced inflammatory response in H9c2 cells

To test if hypoxia triggers an inflammatory response in H9c2 cells and if SsnB can inhibit the response, we examined the expression of MCP1 and HMGB1 in the cells during hypoxia with or without the addition of SsnB in the cell culture medium. qRT-PCR and ELISA were performed to measure the mRNA and protein levels, respectively. qPCR data showed that MCP1 mRNA levels increased by 3- and 16-fold after 4 and 16 h of hypoxia, respectively, compared to normoxia control. SsnB treatment reduced the MCP1 mRNA levels by 5- and 2-fold, respectively, compared to nontreatment controls (Figure 3(a), left panel). The changes in HMGB1 mRNA levels were not as dramatic. While hypoxia induced HMGB1 mRNA levels by 2.3- and 1.4-fold at 4 and 16 h, respectively, SsnB treatment significantly reduced HMGB1 mRNA at 4 h hypoxia time point, but only slightly reduced the level at 16 h time point (Figure 3(a), right panel). ELISA results were mostly consistent with qPCR data. Hypoxia increased MCP1 protein levels in the conditioned medium by 2.5-and 5.6-fold at 4 and 16 h, respectively, while SsnB treatment substantially suppressed the MCP1 protein level increases (Figure 3(b), left panel). The protein levels of HMGB1 were increased in the conditioned medium during hypoxia, and SsnB treatment slightly but significantly diminished the increases (Figure 3(b), right panel). When we used CU-CPT22, a TLR2 antagonist, as a positive control, we observed that the inhibitory effects of SsnB on the expression of MCP1 and HMGB1 were more substantial than that of CU-CPT22 at the same concentration (10 μM) (Figure 3(c)).

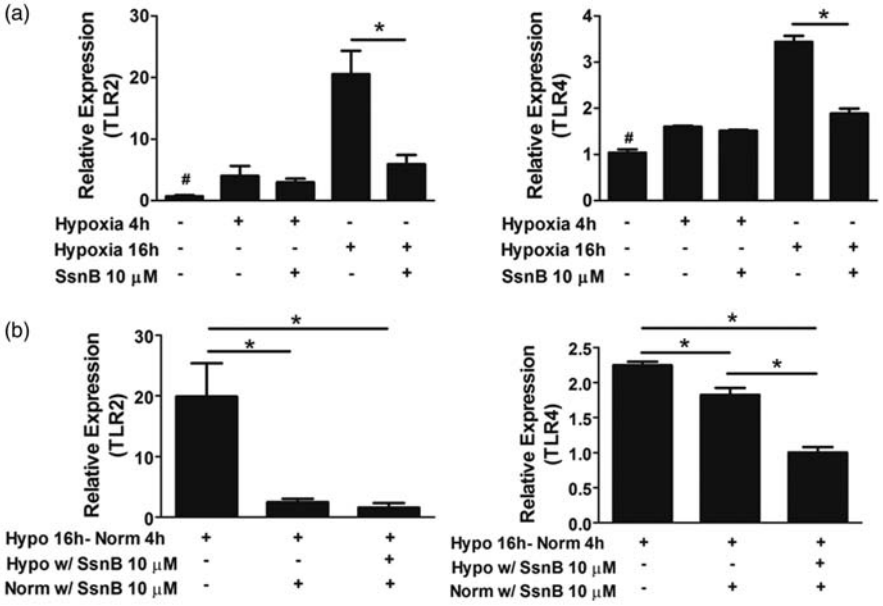

Figure 2 SsnB inhibited hypoxia–reoxygenation-induced myocardial TLR2 and TLR4 up-regulation. (a) qPCR results of TLR2 and TLR4 mRNA levels in H9C2 induced by hypoxia in the absence or presence of SsnB (10 μM) for 4 or 16 h. The ratio of target gene expression level to 18 s RNA expression level at normoxia condition was set to a value of 1. The relative expression of target genes at altered conditions was normalized accordingly. Data are presented as mean ± SEM, $n = 3$, $^{*}P < 0.05$ between two groups; $^{\#}P < 0.05$ vs all other groups. (b) qPCR results of TLR2 and TLR4 mRNA levels in H9C2 induced by hypoxia 16 h–reoxygenation 4 h in the absence or presence of SsnB (10 μM). The relative expression of target genes was normalized as in (a). Data are presented as mean ± SEM, $n = 3$, $^{*}P < 0.05$

These data indicate that SsnB could significantly inhibit hypoxia-induced inflammatory responses in H9c2 cells.

SsnB attenuated hypoxia–reoxygenation-induced inflammation in H9c2 cells

To further investigate whether SsnB could inhibit the expression of MCP1 and HMGB1 under the condition of hypoxia followed by reoxygenation, we did another set of experiments and measured the mRNA and protein levels of MCP1 and HMGB1. H9c2 cells were cultured under hypoxic condition for 16 h and then returned to a normoxia condition for an additional 4 h. Medium was collected for ELISA assay and the cells were collected for total RNA extraction. Two groups were treated with SsnB. In one group, SsnB was only added in the medium during the 4 h normoxia period. In the other, SsnB was added both in the 16 h hypoxia period and 4 h normoxia period. Figure 4(a) shows that SsnB treatment, either only at the reoxygenation period or during the whole hypoxia and reoxygenation period, significantly reduced the MCP1 and HMGB1 mRNA expression in H9c2 cells, compared to the nontreatment group. ELISA assay data using the conditioned medium was consistent with the qPCR data. For MCP1, SsnB inhibited both mRNA and medium protein levels by more than twofold, while the inhibitory effects on HMGB1 were not as profound. Taken together, these data indicate that SsnB inhibited hypoxia–reoxygenation-induced inflammation in H9c2 cells.

SsnB inhibited the activation of ERK1/2 and JNK signaling in H9c2 cells induced by hypoxia–reoxygenation

The mitogen-activated protein (MAP) kinase superfamily of serine/threonine kinases has emerged as an important component of cellular signal transduction pathways which are activated in MIR injury. Two important MAP kinase families, ERK and JNK, have been shown to be activated by specific cascades responsible for certain stimuli or stress and eventually induce a variety of cell responses.[9,10] Both of the signaling pathways can be activated by TLR2

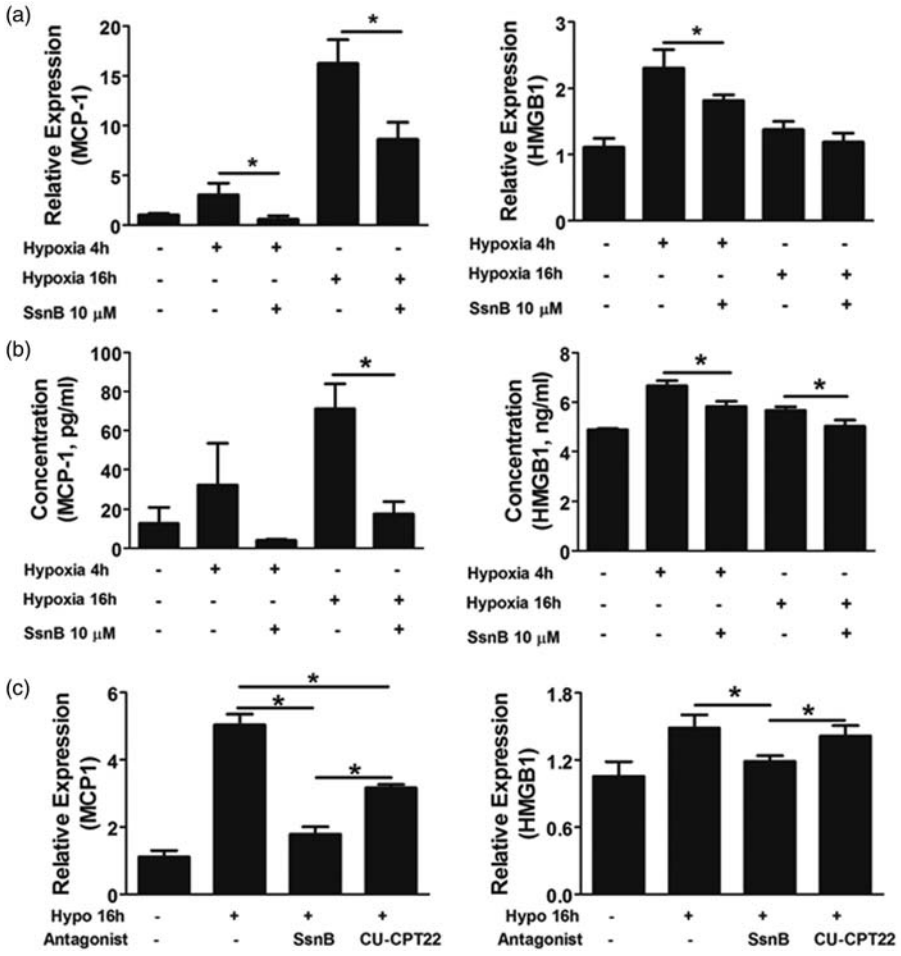

Figure 3 SsnB inhibited hypoxia-induced myocardial inflammation. (a) qPCR results of MCP1 and HMGB1 mRNA levels in H9C2 induced by hypoxia in the absence or presence of SsnB (10 μM) for 4 or 16 h. Data are presented as mean ± SEM, $n = 3$, $^*P < 0.05$. To normalize the relative expression of target genes, the ratio of target gene expression level to 18 s RNA expression level at normoxia condition was set to a value of 1. (b) ELISA results of MCP1 and HMGB1 protein levels in the conditioned medium of H9C2 under hypoxia in the absence or presence of SsnB (10 μM) for 4 or 16 h. Data are presented as mean ± SEM, $n = 3$, $^*P < 0.05$. (c) qPCR results of MCP1 and HMGB1 mRNA levels in H9C2 induced by hypoxia in the absence or presence of SsnB (10 μM) and CU-CPT22 (a TLR2 antagonist, 10 μM) for 16 h. Data are presented as mean ± SEM, $n = 3$, $^*P < 0.05$

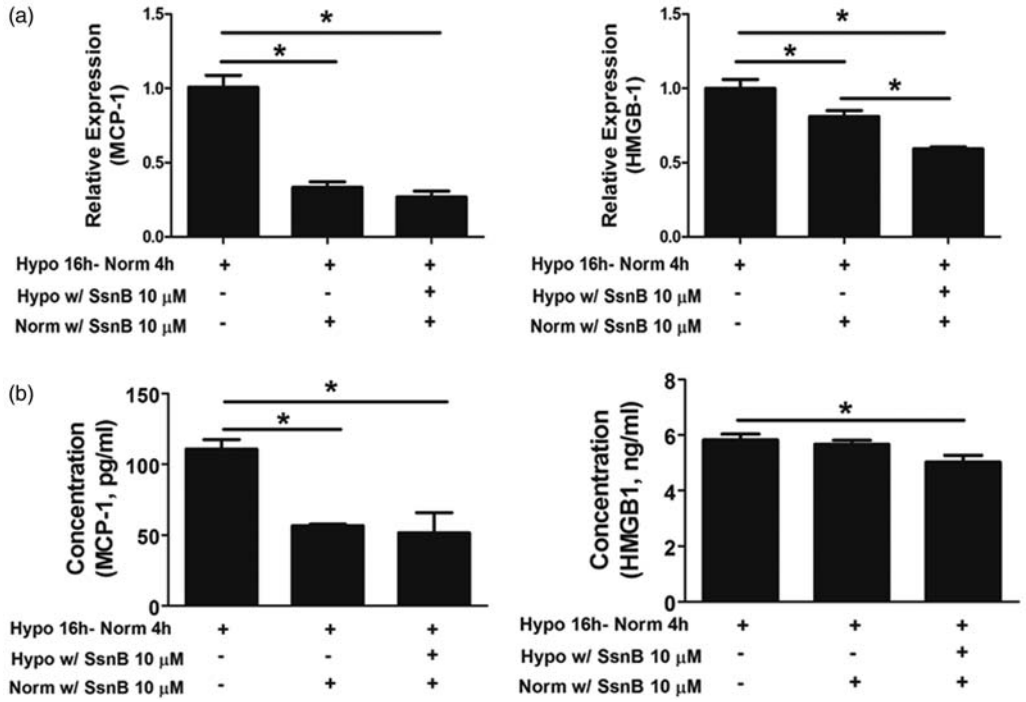

Figure 4 SsnB inhibited hypoxia–reoxygenation-induced myocardial inflammation. (a) qPCR results of TLR2 and TLR4 mRNA levels in H9C2 induced by hypoxia 16 h–reoxygenation 4 h in the absence or presence of SsnB (10 μM). To normalize the relative expression of target genes, the ratio of target gene expression level to 18 s RNA expression level at hypoxia 16 h–normoxia 4 h condition was set to a value of 1. Data are presented as mean ± SEM, $n = 3$, $^*P < 0.05$. (b) ELISA results of MCP1 and HMGB1 protein levels in the conditioned medium of H9C2 under hypoxia 16 h–reoxygenation 4 h in the absence or presence of SsnB (10 μM). Data are presented as mean ± SEM, $n = 3$, $^*P < 0.05$

and TLR4, and we have shown previously that SsnB can inhibit ERK1/2 and JNK phosphorylation induced by TLR2 and TLR4 ligands.[7] For this reason, we detected the changes of phosphorylation of ERK1/2 and JNK in hypoxia–reoxygenation, and tested if SsnB can inhibit their activation.

Firstly, we found phosphorylation of both ERK1/2 and JNK progressively increased by hypoxia 16 h, and hypoxia 16 h plus reoxygenation 4 h (Figure 5(a) and (b)). This result confirmed that ERK1/2 and JNK signaling pathways were both involved in the inflammatory response of H9c2 cells to hypoxia–reoxygenation injury. In another set of experiments, H9c2 cells were cultured under hypoxia condition for 16 h followed by normoxia condition for 4 additional hours. SsnB was either added to the medium at the reoxygenation period or during the entire hypoxia and reoxygenation period. The effects of SsnB on phosphorylation of ERK1/2 and JNK were determined. Figure 5(c) and (d) shows that in both scenarios, SsnB significantly attenuated the activation of ERK1/2 and JNK. These data suggest that SsnB may attenuate inflammatory responses of H9c2 cells to hypoxia–reoxygenation by inhibiting ERK1/2 and JNK activation.

SsnB inhibited macrophage migration toward hypoxia–reoxygenation insulted H9c2 cells

Since the previous results have indicated that MCP1 production and secretion were dramatically increased in H9c2 cells during hypoxia–reoxygenation, SsnB significantly inhibited these changes, and MCP1 is the main factor that attracts monocyte/macrophage infiltration into myocardium during MIR injury, we tested whether SsnB can reduce macrophage migration toward H9c2 cells undergoing hypoxia–reoxygenation. H9c2 cells were subjected to hypoxia for 16 h followed by reoxygenation for 4 h, with SsnB added either during the reoxygenation period or during the entire hypoxia and reoxygenation period. At the end of the reoxygenation phase, macrophages were added to the upper chamber of transwell, and macrophages were allowed to migrate through the transwell membrane for 1 h. The results indicated the number of migrated macrophages toward the hypoxia–reoxygenation injured H9c2 cells was increased by 70% compared to control, and SsnB significantly diminished the increase at both treatment scenarios (Figure 6).

Discussion

Our recent exciting discovery of a newly isolated Chinese herb-derived single compound, SsnB, to be a selective TLR2 and TLR4 antagonist prompted us to explore its potential therapeutic value for inflammatory diseases. Inflammation is a key detrimental component of MIR injury,[11] in which TLR2 and TLR4 signaling in myocardial cells, including microvascular endothelial cells, cardiomyocytes, and recruited monocytes/macrophages, plays a crucial pathological role.[5] Since our previous studies have shown that SsnB could attenuate inflammatory responses in macrophages and endothelial cells, in the current study we

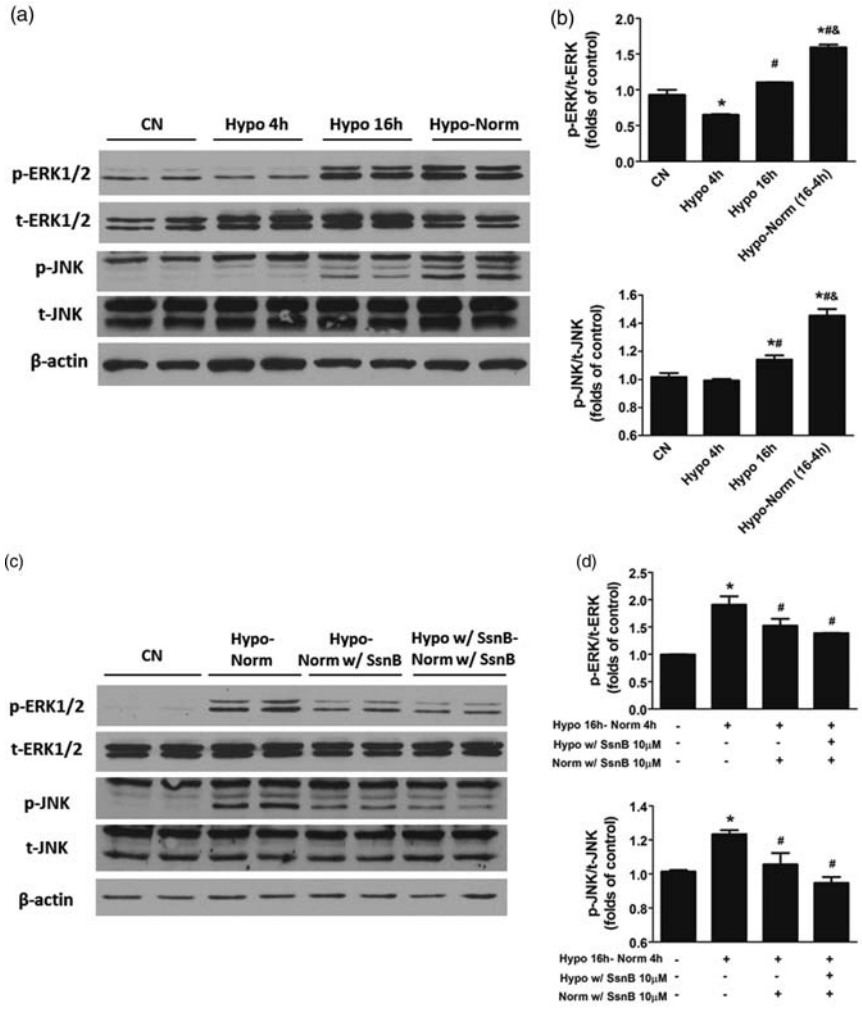

Figure 5 SsnB suppressed ERK1/2 and JNK activation in H9c2 cells. (a) Western blot of phosphorylated (p-) and total (t-) expressions of ERK1/2 and JNK protein levels in H9C2 induced by hypoxia 4, 16, and reoxygenation 4 h. (b) To quantify the phosphorylation levels of the signaling protein, the ratio of the gray density of the phosphorylated form to that of the total protein at control condition was set to a value of 1. Quantitative data are presented as mean ± SEM, $n = 4$, $^{*}P < 0.05$ vs CN (normoxia control), $^{\#}P < 0.05$ vs hypoxia 4 h, $^{\&}P < 0.05$ vs hypoxia 16 h. (c) Western blot of phosphorylated (p-) and total (t-) expressions of ERK1/2 and JNK protein levels in H9C2 induced by hypoxia 16 h–reoxygenation 4 h in the absence or presence of SsnB (10 μM). (d) Quantitative data are presented as mean ± SEM, $n = 4$, $^{*}P < 0.05$ vs CN (normoxia control), $^{\#}P < 0.05$ vs hypoxia 16 h–reoxygenation 4 h without SsnB group

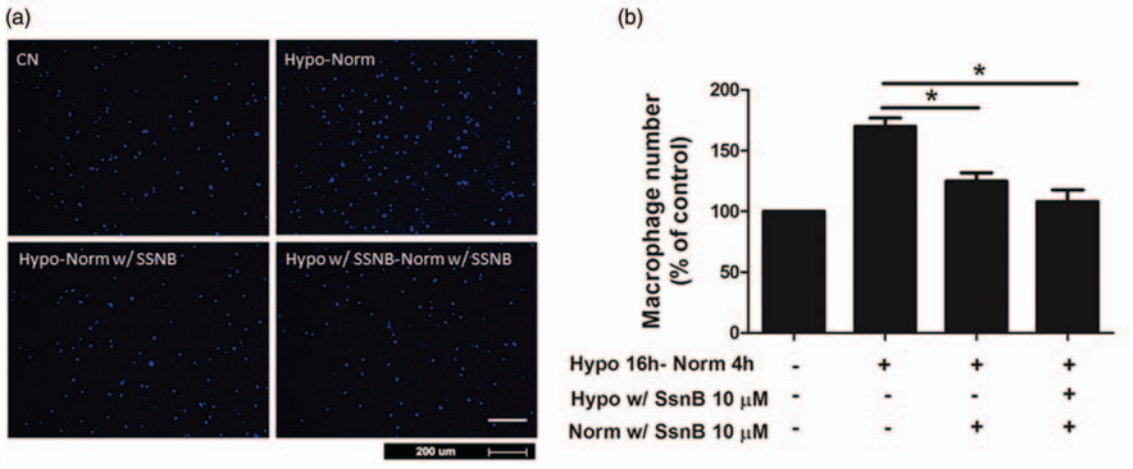

Figure 6 SsnB inhibited macrophage migration toward hypoxia–reoxygenation injured cardiomyocytes. (a) DAPI staining of migrated macrophages toward hypoxia 16 h–reoxygenation 4 h injured H9C2 in the absence or presence of SsnB (10 μM). Representative images were taken under a wide-field fluorescence microscope (×100). CN, complete medium-normoxia; Hypo-Norm, hypoxia 16 h–reoxygenation 4 h; Hypo-Norm w/SSNB, SSNB was added only in reoxygenation 4 h; Hypo w/ SSNB-Norm w/SSNB, SSNB was added both in hypoxia 16 h and reoxygenation 4 h. (b) Quantitative data are presented as mean ± SEM, $n = 3$, $^{*}P < 0.05$. (A color version of this figure is available in the online journal.)

focused our efforts on cardiomyocytes. We employed an H9c2 cell hypoxia–reoxygenation model, which is a commonly used *in vitro* model for MIR, to test if SsnB could suppress cardiomyocyte inflammation.

We first evaluated the cytotoxicity of SsnB on H9c2 cells, and found at 10 μM concentration, SsnB did not cause any toxicity to H9c2 cells either at normoxia conditions or hypoxia conditions for 16 h. Given our previous finding that SsnB at 10 μM concentration could effectively suppress endothelial cells and macrophage inflammation, we chose to use SsnB at 10 μM concentration throughout the study.

It has been shown that TLR2 and TLR4 are expressed in myocardium during I/R injury[12–17] and their activation by local endogenous ligands contributes to the up-regulation of their own expression. It has been well documented that multiple mechanisms mediate a positive feedback loop between TLRs and their downstream signaling[18]; therefore, blockage of TLR2/4 signaling could halt the vicious inflammatory cycle. Our data showed that hypoxia and reoxygenation induced TLR2 and TLR4 expression in H9c2 cells. SsnB significantly suppressed the up-regulation of both TLR2 and TLR4 in the cells that were subjected to hypoxia for 16 h or hypoxia for 16 h followed by reoxygenation for 4 h, but did not obviously affect their expression in the cells that were subjected to hypoxia for only 4 h. However, SsnB could significantly reduce MCP1 and HMGB1 expression in the cells after 4 h of hypoxia. These data suggested that SsnB effectively suppressed inflammatory responses of H9c2 cells to hypoxia and reoxygenation. At the early time point (4 h of hypoxia), SsnB exerted inhibitory effects on TLR2 and TLR4 signaling thereby suppressing MCP1 and HMGB1 expression. At 16 h of hypoxia and during reoxygenation, SsnB not only continuously suppressed MCP1 and HMGB1 expression by blocking TLR2 and TLR4 signaling, but also suppressed the up-regulation of TLR2 and TLR4 induced by prolonged hypoxia and subsequent reoxygenation, by blocking the positive feedback between the TLR2/4 signaling and TLR2/4 expression, and thus further diminishing the expression of MCP1 and HMGB1 induced by hypoxia and reoxygenation. We would like to mention that even though SsnB, as an effective TLR2/4 inhibitor, significantly diminished TLR2/4, MCP-1, and HMGB1 expression induced by hypoxia and reoxygenation, it did not eliminate the myocyte inflammatory responses to hypoxia–reoxygenation. This could be due to the incomplete blockage of TLR2/4 signaling by SsnB, and also reflects the fact that myocyte inflammatory responses to hypoxia–reoxygenation are complex and involve many signaling pathways including Reactive oxygen species (ROS), TLRs, and other pattern recognition receptors.[2]

We used MCP1 and HMGB1 as biomarker for H9c2 cell inflammation because these two molecules are of particular importance in MIR injury. HMGB1 as a damage associated pattern molecule is produced in cardiomyocytes and secreted immediately by the stressed cells.[19,20] We found that HMGB1 gene expression and protein levels in the medium reached a peak at the 4 h time point of hypoxia and declined as the hypoxia condition continued. This indicates HMGB1 may serve as an initial inflammatory stimulus in the hypoxic myocardium by activating TLR2 and TLR4 located on cardiomyocytes.[21] The fact that SsnB effectively suppressed the HMGB1 gene expression as well as protein secretion suggests that SsnB can blunt the initiation of myocardial inflammation during MIR injury. MCP1 is the major chemoattractant molecule responsible for monocyte/macrophage recruitment to the myocardium during MIR.[22–24] Expression and secretion of MCP1 by cardiomyocytes and then also by the recruited monocytes/macrophages is contributory to the vicious cycle of myocardial inflammation.[25,26] Recruited monocytes/macrophages express and secrete a large amount of other chemokines and cytokines and thus cause cardiomyocyte apoptosis and myocardial remodeling.[27] SsnB dramatically suppressed MCP1 expression and secretion by H9c2 cells throughout the course of hypoxia and reoxygenation, suggesting that SsnB is effective in attenuating myocardial inflammation during the full course of MIR. In fact, macrophage migration assays showed that under hypoxia–reoxygenation conditions, SsnB treated H9c2 cells attracted significantly fewer macrophages, indicating that MCP1 expression suppression by SsnB in cardiomyocytes would reduce monocyte/macrophage infiltration into the myocardium following I/R.

There are various intracellular pathways that mediate TLR2 and TLR4 signaling, and ERK1/2 and JNK pathways have been demonstrated to be involved in the MIR-induced inflammation.[10,28–31] We confirmed that ERK1/2 and JNK signaling were activated during hypoxia and following reoxygenation.[32,33] We further found that SsnB indeed suppressed the activation of these two pathways during hypoxia and reoxygenation. However, we did not exclude other TLR2 and TLR4 associated intracellular pathways that may also contribute to the inhibitory effects of SsnB on H9c2 cells.

In summary, we have demonstrated that the Chinese herb-derived compound SsnB, a new selective TLR2 and TLR4 antagonist, can suppress TLR2 and TLR4 expression and inhibit TLR2/4-mediated MCP1 and HMGB1 production and release in cardiomyocytes, as well as reduce macrophage-recruiting capacity of injured cardiomyocytes, in the inflammatory response during hypoxia–reoxygenation. These anti-inflammatory effects of SsnB on cardiomyocytes are at least partially the result of suppressing ERK1/2 and JNK signaling pathways. Together with our previous findings that SsnB effectively inhibits TLR2 and TLR4 mediated macrophage and vascular endothelial cell inflammation, our studies have provided initial evidence that SsnB has potential to be used as a new therapeutic agent for MIR injury. However, additional *in vivo* studies are required to determine the therapeutic efficacy of SsnB in MI.

Author contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; QL, JW, and JL conducted the experiments, QL, JSJ, and DF wrote the manuscript.

ACKNOWLEDGEMENTS

This work was supported by grants from national Institute of Health R21AT006767, R01HL116626, and P20GM103641 (to DF).

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(Received July 31, 2013, Accepted October 28, 2013)