Original Article

Simvastatin protects cardiomyocytes from doxorubicin cardiotoxicity by suppressing endoplasmic reticulum stress and activating Akt signaling

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Abstract: Doxorubicin is a widely used anti-cancer chemotherapy drug with high cardiotoxicity. Previous studies have reported the pleiotropic beneficial effects of simvastatin on cardiovascular system, it is still unknown whether it protects cardiomyocytes against doxorubicin cardiotoxicity. The present study aimed to investigate the role of simvastatin in doxorubicin induced cardiomyocyte apoptosis and the possible mechanisms. We found that simvastatin reduced serum cardiac enzymes such as LDH and CK and reversed the reduction of heart function induced by doxorubicin in mouse models. Mechanistically, we showed that simvastatin was able to inhibit ROS generation and apoptosis upon doxorubicin treatment, which is accompanied by reduction of proteins in endoplasmic reticulum (ER) stress, suggesting that suppression of ER stress associated death (ERAD) may confer the protective effect of simvastatin. Furthermore, we noted that the pro-survival Akt signaling was also activated by simvastatin. In summary, our work revealed for the first time that simvastatin alleviates doxorubicin cardiotoxicity by attenuating ER stress and activating Akt pathway, and simvastatin administration could be useful in the prevention of cardiotoxicity in cancer patients receiving doxorubicin treatment.

Keywords: Endoplasmic reticulum stress, apoptosis, cardiomyocyte, doxorubicin, simvastatin, Akt

Introduction

The cardiotoxicity of the wide-spectrum chemotherapy drug doxorubicin has been well characterized clinically, and previous experimental studies have proposed several possible mechanisms of doxorubicin induced cardiomyopathy [1, 2]. However, the underlying mechanisms remain largely unknown. Moreover, the clinical interventions for doxorubicin induced cardiomyopathy are undeveloped. Understanding the molecular basis of doxorubicin cardiotoxicity would be essential to prevent cardiac events in cancer patients receiving doxorubicin treatment.

Simvastatin belongs to a family of inhibitors of hydroxymethylglutaryl CoA (HMG-CoA) reductase that is commonly administrated for hypercholesterolemia. A large number of studies have proved that simvastatin exerts beneficial actions on cardiovascular system [3, 4]. In addition, studies have shown simvastatin is able to attenuate cardiac remodeling and reduce the extent of cardiac fibrosis after myocardial infarction [5, 6]. However, it is seldomly investigated whether simvastatin act directly on cardiomyocytes. Given the cardiac beneficial effects demonstrated by the previous works, we assumed that simvastatin might potentially promote the survival of cardiomyocyte in the presence of doxorubicin.

Endoplasmic reticulum is a membrane system that is critical for proper folding, processing and trafficking of proteins. Importantly, endoplasmic reticulum is one of the central organelles that control intracellular homeostasis. Upon various stimuli, a stress response that is referred to as endoplasmic reticulum stress (ER stress) is activated due to the sustained production of unfolded proteins in the lumen of ER [7-9]. ER stress associated death (ERAD) has been widely implicated in cardiac syn-
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dromes [10-13]. In view of the recent proposal that implicates ERAD as a target of neurodegenerative disease [14], it might be feasible to study its similar role in cardiovascular system.

In the current work, we investigated the potential effects of simvastatin on the survival of cardiomyocytes exposed to doxorubicin. We found that simvastatin greatly benefited the heart function after doxorubicin administration in vivo. Importantly, we report that simvastatin treatment significantly attenuated ER stress induced apoptotic response in cardiomyocytes, which is accompanied by augmented pro-survival Akt signaling. Our study identified for the first time that simvastatin alleviates doxorubicin cardiotoxicity by activating Akt pathway and attenuating ER stress and might give a hint in the management of cancer patients receiving doxorubicin treatment.

Materials and methods

Animal studies and heart function test

Eight-week-old male C57BL/6 mice were used in this study. Simvastatin (Sigma-aldrich, St. Louis, MO, USA) were intragastrically administrated at the dose of 0.4 mg/g/day for 7 consecutive days. The mice in control group were administrated with the same volume of saline. Then, all the mice were injected with doxorubicin (Sigma-aldrich) at the dose of 20 mg/kg or the same volume of saline. Weeks after the treatment, the mice were anaesthetized and subjected to echocardiographic study using an ultrasound machine (Vivid 7 GE Medical) to obtain the parameters of ejection fraction and fractional shortening.

LDH and CK measurement

After the above described treatment, mice were sacrificed. 2ml blood sample was collected for each mouse. The serum level of LDH and CK were measured using the commercially available kit from Nanjing Jiancheng Biotechnology Company (Nanjing, China) according to the instructions provided by the manufacturer.

Primary culture of mice cardiomyocytes

To culture the cardiomyocytes in vitro, we dissected the heart from new born mice. The heart tissues were cut into pieces and digested with 0.25% trypsin solution for several times. The cell suspensions were collected and passed through a 75 μm strainer. The cardiac fibroblasts were removed by a 2-hour differential plating in a flask. Then the cardiomyocytes were plated in 96-well plates for MTT assay and ROS detection and 6-well plates for protein assay. Further experiments were followed 48 hours after plating. The culture medium for cardiomyocytes is DMEM supplemented with 10% FBS.

MTT assay

We employed an MTT assay to detect the cell viability under various conditions with an MTT Cell Viability Assay Kit (Abnova, Taipei, Taiwan). After appropriate incubation with MTT reagent for 4 hours, the formazan were formed and visualized according to the kit instruction. The cell viability was measured at 570 nm using a spectrophotometer.

ROS detection

The ROS detection kit was purchased from Beyotime biotechnology (Shanghai, China). After the desired treatment, cells were treated with DCFH-DA provided in the kit. Cells were then subjected to a series wash for 3 times with DMEM with no FBS to remove the DCFH-DA that did not enter cells. The ROS levels were measured with a fluorescent microplate reader, the excitation wave length was 488 nm.

Western blot

Cells grown in 6-well plates were lysed with RIPA buffer (Beyotime) and the whole cell lysates were denatured at 100°C in SDS loading buffer for 3 min followed by electrophoresis on a SDS-PAGE gel. The seperated proteins on the gel were then transferred on to a PDVF membrane and labeled with specific primary antibodies. HRP conjugated secondary antibodies were used on the following day after 3 times wash with PBS-tween20. The protein bands were detected with an ECL chemiluminescence kit (Beyotime). Protein quantification was performed using the Quantity One software (BioRad, Richmond, CA, USA). The source of primary antibodies were as follows: antibiot-
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Proteins for Bcl-2, CHOP, Caspase-12, GRP78 and β-actin were from Cell Signaling Technology, Inc (Beverly, MA, USA); antibodies for Bax, p-Akt and Akt were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

Statistical analysis

Data were expressed by means±SEM. One way analysis of variance (ANOVA) was used to test the significance among multiple groups. Pairwise comparison was performed using Bonferroni test. A p value no less than 0.05 was considered to have no statistically significance.

Results

Simvastatin alleviates doxorubicin induced cardiotoxicity in vivo

To understand whether simvastatin exerts beneficial actions on doxorubicin induced cardiotoxicity, we established a mouse model by doxorubicin injection. Simvastatin was administrated daily one week before doxorubicin treatment. We observed that cardiac enzymes such as LDH and CK were significantly induced after doxorubicin treatment, and mice pretreated with simvastatin exhibited lower levels of LDH and CK in their serum (Figure 1A and 1B). In addition, simvastatin reversed the adverse effect of doxorubicin on heart function parameters such as ejection fraction and fractional shortening (Figure 1C and 1D). These results confirmed the protective effect of simvastatin against doxorubicin induced cardiotoxicity in vivo.

Simvastatin attenuates doxorubicin induced ROS generation and apoptotic cell death

We next treated primary cultured cardiomyocytes with various dose of simvastatin. MTT results showed that medium to high dose of simvastatin was able to attenuate cell death induced by doxorubicin (Figure 2A). ROS is one of the main reasons for cardiomyocyte apoptotic death, consistent with the MTT results, ROS level was also decreased in a dose dependent manner upon simvastatin treatment (Figure 2B). We also found that apoptosis related proteins Bax and Bcl-2, which are critical for apoptosis initiation, were significantly altered after simvastatin treatment (Figure 2C). Bax/Bcl-2 ratio is a reliable indicator for apoptosis; after a careful analysis of the western blot

Figure 1. Simvastatin alleviates doxorubicin induced cardiotoxicity in vivo. Mice were administrated with 0.4 mg/g/d simvastatin (SIM) for 7 consecutive days before doxorubicin (DOX) injection (20 mg/kg). 2 weeks later, the blood samples were collected for measuring the serum level of lactate dehydrogenase (LDH) and creatine kinase (CK) (A and B); the heart parameters EF (ejection fraction) and FS (fractional shortening) was measured by echocardiography before bleeding (C and D). *P<0.05 versus control (Ctrl); tP<0.05 versus DOX, n=5 in each group.

Figure 2. Simvastatin attenuates doxorubicin induced ROS generation and apoptotic cell death.
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results we found that the Bax/Bcl-2 ratio was significantly downregulated after medium to high dose simvastatin treatment (Figure 2D).

Inhibition of ER stress associated apoptosis is involved in the protective effect of simvastatin

We further investigated the mechanism of the anti-apoptotic action on doxorubicin induced apoptosis. ER stress is commonly induced after certain stimuli. To test the possible involvement of ER stress in our system, we detected several protein markers for ER stress. As shown in Figure 3A, doxorubicin treatment significantly induced upregulation of CHOP and GRP78. And consequently, ER stress specific apoptotic pathway was activated as evidenced by increased cleavage of Caspase-12. High dose (8 μM) simvastatin reduced all these effects (Figure 3B-D). Together with the results in Figure 2, these results suggested that simvastatin...
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Simvastatin could inhibit apoptosis of cardiomyocytes via suppression of ER stress.

Simvastatin activates the pro-survival Akt signaling in cardiomyocytes

Previous studies have demonstrated that PI3K/Akt signaling is critical for the surviving of cardiomyocytes upon ischemia reperfusion injury [15]. We further noted the positive effect of simvastatin on this survival pathway. As shown in Figure 4A and 4B, doxorubicin treatment remarkably impaired the phosphorylation of Akt, whereas simvastatin partially reversed Akt activation, suggesting PI3K/Akt signaling pathway might also confer the protective effect of simvastatin against doxorubicin cardiotoxicity.

Discussion

Doxorubicin induced cardiomyocyte loss is one of the most severe adverse effects in cancer patients receiving chemotherapy, and it represents a major challenge to mitigate its cardiotoxicity. Statins are a class of HMG-CoA reductase inhibitors that are broadly utilized to inhibit cholesterol production. High cholesterol is one of the risk factors of cardiovascular disease [16], and recent clinical evidences also support statins to be the primary prevention of cardiovascular complications [4]. However, whether statins are effective to prevent doxorubicin induced cardiomyocyte death is still yet to be clarified. In this study, we have revealed for the first time that simvastatin show beneficial effects towards doxorubicin cardiotoxicity.

Figure 3. Inhibition of ER stress associated apoptosis is involved in the protective effect of simvastatin. Cardiomyocytes were treated with doxorubicin with or without the presence of high dose (8 μM) simvastatin. ER stress markers CHOP and GRP78 and marker for endoplasmic reticulum associated death Caspase-12 were measured by western blot (A), the statistical data were shown in (B-D). *P<0.05 versus (Ctrl); #P<0.05 versus DOX alone, n=5 in each group.
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Figure 4. Simvastatin activates the pro-survival Akt signaling in cardiomyocytes. Cardiomyocytes were treated with doxorubicin with or without the presence of high dose (8 μM) simvastatin. Phosphorylation of Akt was detected by western blot (A and B). *P<0.05 versus (Ctrl); #P<0.05 versus DOX alone, n=5 in each group.

found that simvastatin improved heart function and reduced cardiotoxicity in vivo, and our in vitro studies suggested that simvastatin inhibited doxorubicin induced ER stress and activated the pro-survival Akt signaling. Our data not only suggest the potential application of simvastatin to treat doxorubicin cardiotoxicity but also reveal a novel mechanism underlying the effects of simvastatin and doxorubicin. The cytotoxicity of doxorubicin has been well characterized by previous studies [1, 2]. Multiple biological events such as ROS generation, calcium dyshomeostasis and mitochondria dysregulation may account for doxorubicin induced cardiomyocyte death [17-19]. Particularly, recent findings showed that doxorubicin specifically act on cardiomyocytes by delete cardiac gene Top2b [20]. Theses studies suggested that doxorubicin induced cardiotoxicity is a readout of multiplex biological processes. Recent studies have highlighted the importance of ER stress response in the protein quality control and cell survival, and interplay between ER stress and other biological processes is broadly incorporated into the pathogenesis of neurodegenerative and cardiovascular disease [12, 21]. Therefore, targeting ER stress might be promising in this scenario. In the present work, we found that ER stress is significantly induced by doxorubicin treatment, which is in agreement with previous studies on H9C2 cells and doxorubicin rat models [22, 23]. We observed a pronounced upregulation of Bax/Bcl-2 ratio and activation of Caspase-12, suggesting that doxorubicin induced ER stress associated death. The protective effect of statins has been demonstrated by several studies. Rosuvastatin reduced the sudden death of chronic heart failure [24], and simvastatin improved electrophysiology in a rabbit model of cardiac hypertrophy [25]. More recently, simvastatin has been shown to inhibit post infarction fibrosis in mice [6]. Although these studies demonstrated the favorable action of simvastatin in vivo or in cardiac fibroblasts little is known whether simvastatin show direct effect on apoptosis of cardiomyocytes; in addition, the role and mechanisms of simvastatin on doxorubicin toxicity has not been fully uncovered. In the present work, we observed attenuated serum cardiac enzymes, which reflect the severity of cardiomyopathy, and the heart function was also improved, as exemplified by increased EF and FS. In exploring the mechanism by which simvastatin inhibits doxorubicin cardiotoxicity, we observed decreased ROS generation and Bax/Bcl-2 ratio, which suggested that modulation of the apoptotic machinery may be required for the protective action of simvastatin. Furthermore, upregulation of GRP78, CHOP and cleaved-Caspase-12 by doxorubicin was significantly inhibited by simvastatin, supporting our hypothesis that simvastatin functions against doxorubicin cardiotoxicity via signaling pathways implicated in ER stress associated cell death. However, it is still unknown whether these effects depend on HMG-CoA reductase activity, future investigations may be required to clarify this issue. One of the intriguing findings in the present study is that we observed reversed Akt phosphorylation after simvastatin treatment. Our data is in consistent with previous studies on rat model of heart ischemia reperfusion injury and insulin resistance [26, 27]. The PI3K/Akt signaling, which is broadly involved in nutrient metabolism, calcium homeostasis, autophagy, apoptosis and nitric oxide generation, represents the master switch.
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for the control of cell survival in cardiomyocytes [28, 29]. Qin L et al. reported that inhibition of Akt/mTOR signaling acts downstream of ER stress to inhibit autophagy [30]. Hu C et al. suggested in the recent work that, constitutive activation of Akt inhibits ER stress [31]. These evidences suggested that a possible positive feedback loop, which involves PI3K/Akt/mTOR and ER stress pathway, is activated after simvastatin treatment. The relationship between ER stress and Akt signaling in this scenario needs to be determined in future works. The essential requirement for the application of simvastatin in patients undergoing doxorubicin treatment is that simvastatin should at least not reduce the anti-cancer efficacy of doxorubicin and other anticancer treatment strategies. We noted that simvastatin act synergistically to exert pro-apoptotic functions or to enhance chemo- and radio-therapy efficiency in several cancers [32-37]. Therefore simvastatin may possibly enhance the efficacy of doxorubicin and inhibit cardiotoxicity at the same time, even though the mechanism of the differential effects of simvastatin on apoptosis between normal cells and cancer cells is unclear.

In summary, we demonstrate here in the present work that suppression of ER stress and activation of Akt signaling is critically involved in simvastatin induced cardiac protective effect against doxorubicin toxicity. Since simvastatin possesses both anti-cancer and cardioprotective actions, administration of simvastatin could be one of the promising approaches to prevent doxorubicin cardiotoxicity.

Disclosure of conflict of interest

None.

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References

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