Role of salubrinal in protecting cardiomyocytes from doxorubicin-induced apoptosis

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ABSTRACT. We determined whether salubrinal can protect cardiomyocytes from doxorubicin-induced apoptosis and explored the related mechanisms to provide experimental evidence for exploring novel drug candidates to decrease cardiac toxicity. Neonatal rat cardiomyocytes were isolated, cultured *in vitro*, and pretreated with salubrinal (10, 20, or 40 µM) to observe their response to doxorubicin-induced cell apoptosis. Lactate dehydrogenase assay, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling staining, and flow cytometry were used to assess the extent of cardiomyocyte apoptosis. Fluorescent probes conjugated with 2',7'-dichlorofluorescein diacetate and a chemiluminescence assay were used to detect the production of reactive oxygen species. Western blotting was employed to quantify expression levels of cleaved caspase-3, cytosolic cytochrome c, and B-cell lymphoma-extra large (Bcl-xL). The mechanisms of salubrinal-related functions were also explored. Salubrinal effectively inhibited doxorubicin-induced reactive oxygen species production and

nicotinamide adenine dinucleotide phosphate oxidase activation, decreased the levels of cleaved caspase-3 and cytosol cytochrome c, and increased Bcl-xL expression, thereby protecting cardiomyocytes from doxorubicin-induced apoptosis. Furthermore, salubrinal was found to protect cardiomyocytes by decreasing the dephosphorylation of eukaryotic translation initiation factor 2α (eIF2α). Salubrinal can protect cardiomyocytes from doxorubicin-induced apoptosis through its effects on eIF2α. It possibly ameliorates cardiac toxicity and can be used in clinical practice.

Key words: Salubrinal; Doxorubicin; Cardiac toxicity; Cell apoptosis; eIF2α

INTRODUCTION

With advances in diagnostic and treatment methods, the survival rate of cancer patients has increased significantly in recent years. Data show that 83% of children with newly diagnosed cancers survive more than 5 years (Silverman et al., 2010; Tarver, 2012). However, despite such tremendous progress in cancer treatment, medication side effects occur in many patients. For example, anthracycline, which is often used in treating hematological and solid tumors, has been reported to cause impairments in other organs in addition to effectively killing tumor cells (Lipshultz et al., 2008). A study showed that 74% of patients using anthracycline drugs developed chronic organ damage, and 43% of cancer survivors had more severe conditions (Oeffinger et al., 2006). The statistical data from some studies have led researchers to postulate cardiac toxicity as one of the most important causes of death or disability in cancer survivors (Lipshultz and Adams, 2010; Tukenova et al., 2010). Therefore, exploring the possible elimination of cardiac toxicity is necessary.

Studies have shown that endoplasmic reticulum-activated cell apoptosis plays an important role in the development of cardiac toxicity (Chua et al., 2006; Wang et al., 2012). Therefore, one possible approach to eliminating cardiac toxicity is through the regulation of endoplasmic reticulum-activated apoptosis. Salubrinal is a recently discovered selective inhibitor of the dephosphorylation of eukaryotic translation initiation factor 2α (eIF2α) (Boyce et al., 2005). It protects against endoplasmic reticulum-activated cell apoptosis or cardiomyocyte apoptosis (Dalal et al., 2012). Therefore, salubrinal could work as a new drug candidate for treating cardiac toxicity. Doxorubicin is a drug used in cancer chemotherapy. Its most serious side effect is life-threatening heart damage. However, the detailed mechanism by which doxorubicin induces cardiac toxicity has not been well established.

This study utilized an *in vitro* model of doxorubicin-induced cardiac toxicity using primary cultured rat cardiomyocytes to evaluate the protective effect of salubrinal on cardiomyocytes. We also elucidated the possible mechanisms involved by assaying the level of phosphorylation in eIF2α.

MATERIAL AND METHODS

Primary culture of rat cardiomyocytes

Rats were used for all experiments, and all procedures were approved by the Animal

Ethics Committee of The Third Xiangya Hospital of Central South University, Hunan, China. The primary culture of cardiomyocytes from neonatal rats followed the methods described previously (Chen et al., 2008). In brief, the ventricle of neonatal (postnatal day 1-2) Sprague Dawley rats was separated and cut into approximately 1-mm^3 pieces. The tissue samples were then digested with 0.25% type IV collagenase for 1 h, spread evenly in complete medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum and streptomycin), and cultured in 5% CO_2 at 37°C in an incubator for 2-4 days. Adherent and fused cells were sub-cultured, collected, and frozen at -80°C for future use.

Drug treatment

Cardiomyocytes collected using the above method were cultured in the complete medium also described above. They were then divided into different drug pretreatment groups: 1) 4-h treatment with different concentrations of salubrinal (10, 20, and 40 μM); 2) 24-h incubation with 1 μM doxorubicin; and 3) 24-h treatment with doxorubicin following 4-h culture with different concentrations of salubrinal (10, 20, and 40 μM). A further control group was cultured without drugs. Assays were performed on all treated cells as described in the following sections.

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining

TUNEL staining was performed using a TUNEL assay kit (Roche, Basel, Switzerland) following the manufacturer instructions. Five fields in which cells with dark brown granular signals were defined as having positive signals were randomly selected using a microscope at high magnification. The rate of cell apoptosis was calculated using the formula:

Rate of cell apoptosis = total number of positive cells in the five fields / total number of all cells in the five fields x 100%.

Lactate dehydrogenase assay

A test kit (Jiancheng Corp., China) was used to determine the level of lactate dehydrogenase in the cultured cardiomyocytes following the manufacturer instructions.

Assay of cytosolic reactive oxygen species, superoxides, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity

The detection of reactive oxygen species followed the protocol described previously (Eruslanov and Kusmartsev, 2010). In brief, 30 μM 2',7'-dichlorofluorescein diacetate fluorescent dye was added to the cells of the different drug pretreatment groups and incubated for 1 h. Treated cells were then digested and washed, and fluorescence intensity was measured to quantify the level of reactive oxygen species. The numbers of cells in the different groups were also counted to calibrate the fluorescent signal strength. The superoxides assay followed the method reported elsewhere (Wung et al., 1997).

The measurement of NADPH oxidase activity also followed a previously reported method (Juan et al., 2007). In brief, the microbodies in the cardiomyocytes were separated using an ultracentrifuge and dissolved in 150 μL reaction buffer (50 mM potassium phosphate

buffer, pH 7, containing 1 mM ethylene glycol tetraacetic acid, 150 mM sucrose, 5 μM lucigenin, and 100 mM NADPH). The fluorescence intensity was measured using an Orion microplate reader every 60 s. The protein concentration was also quantified using a BCA Protein Assay Kit (Pierce Corp., USA). The activity of NADPH oxidase was calculated as the ratio of the relative fluorescence intensity to the protein concentration.

Western blot analysis of protein expression

Cardiomyocytes were lysed by cell lysis buffer (Biyuntian Corp., China) and centrifuged at 12,000 rpm for 10 min. The supernatant was quantified for BCA protein concentration using the BCA protein assay kit (Pierce Corp.), as mentioned above. In brief, protein solutions were mixed with appropriate volumes of load buffers and boiled at 100°C for 5 min. The protein sample was separated by sodium dodecyl sulfonate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane by transmembrane reaction. The membrane was blocked by 5% skimmed milk powder in TBST buffer (Tris-buffered saline and Tween 20) for 1 h, followed by 4°C overnight incubation using primary antibody. After washing with TBST three times, the sample was incubated with secondary antibody conjugated with horseradish peroxidase at room temperature for 1 h. After washing with TBST three more times, the membrane was exposed on an X-ray film using the chromogenic substrate (Pierce Corp.). The relative expression level of protein was quantified using the Image J software (NIH, USA). All antibodies used in this study, including those against cleaved caspase-3, p-eIF2α, eIF2α, cytochrome c, and B-cell lymphoma-extra large (Bcl-xL), were commercially purchased (Santa Cruz, US). Actin was included as the internal reference protein for the Western blot.

Statistical analysis

SPSS v11.5 (IBM Corp., USA) was used to analyze all collected data. All results are reported as means ± SE. The comparison among multiple groups was accomplished by analysis of variance. Statistical significance was defined as $P < 0.05$.

RESULTS

Pretreatment with salubrinal can protect cardiomyocytes against doxorubicin-induced cell apoptosis

We first determined whether salubrinal had any side effects on neonatal rat cardiomyocytes by measuring the level of lactate dehydrogenase. The results showed no significant change in the level of lactate dehydrogenase in neonatal rat cardiomyocytes, even after pretreatment with 40 μM salubrinal (Figure 1A). A further TUNEL assay revealed that salubrinal protects against doxorubicin-induced cardiomyocyte apoptosis: the number of TUNEL-positive cells after pretreatment with 20 or 40 μM salubrinal was significantly lower than that observed without salubrinal treatment (Figure 1B; $P < 0.05$ in both cases). A decrease in the TUNEL-positive cell number also occurred in cardiomyocytes pretreated with 10 μM salubrinal, although the difference was not statistically significant (Figure 1B; $P > 0.05$).
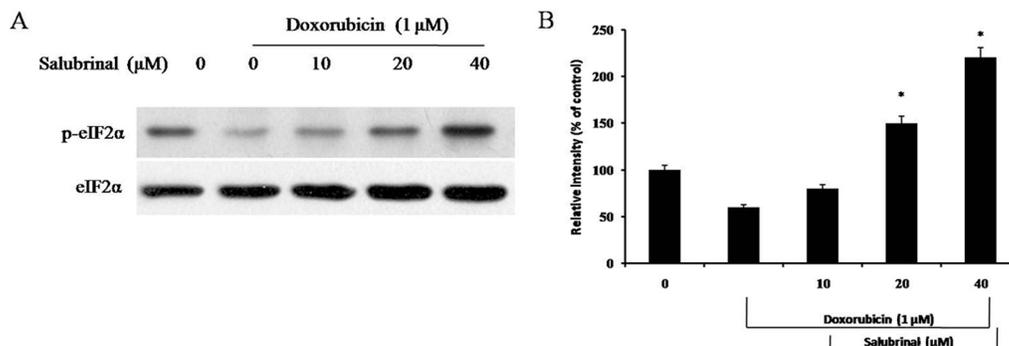
Figure 1. Doxorubicin-induced cardiomyocyte apoptosis following salubrinal pretreatment. **A.** Effect of salubrinal pretreatment on lactase dehydrogenase level; **B.** terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end-labeling (TUNEL)-positive cardiomyocytes undergoing salubrinal pretreatment and doxorubicin-induced apoptosis. *$P < 0.05$.

Effect of salubrinal pretreatment on cardiomyocyte apoptosis-related protein levels

The western blot showed that cleaved caspase-3 and cytochrome c, which are both apoptosis-related proteins, had decreasing expression levels after pretreatment with increasing concentrations of salubrinal (Figure 2A and B). The cells that had undergone salubrinal pretreatment had significantly lower protein levels compared with those without salubrinal ($P < 0.05$ in both the 20 and 40 μM salubrinal groups). However, the level of apoptosis-related protein Bcl-xL increased significantly with increasing concentrations of salubrinal compared with those cells without salubrinal pretreatment ($P < 0.05$ in both 20 and 40 μM salubrinal groups; Figure 2C).
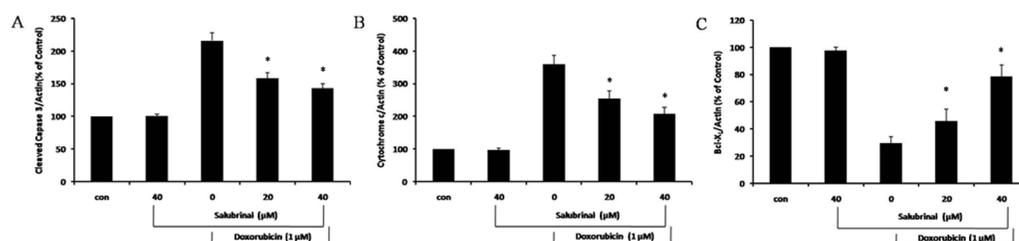

Figure 2. Analysis of doxorubicin-induced cardiomyocyte apoptosis following salubrinal pretreatment. **A.** Relative change of cleaved caspase-3 protein expression level; **B.** relative change of cytochrome c protein expression level; **C.** relative change of B-cell lymphoma-extra large (Bcl-xL) protein expression level. *$P < 0.05$.

Effect of salubrinal pretreatment on reactive oxygen species level and NAPDH oxidase activity

To further evaluate the function of salubrinal in doxorubicin-induced apoptosis in neonatal rat cardiomyocytes, the level of reactive oxygen species and the activity of NADPH oxidase were further quantified. The results showed a significantly lower level of reactive oxygen species in neonatal rat cardiomyocytes after pretreatment with 20 or 40 μM salubri-

nal compared with cells that had not received pretreatment and had undergone doxorubicin incubation alone (P < 0.05 in both cases; Figure 3A). The cytosolic peroxide concentration was also measured and the results showed a significantly lower peroxide level in neonatal cardiomyocytes following salubrinal pretreatment (20 or 40 μM) than in those cells that had received doxorubicin treatment only (P < 0.05 in both cases; Figure 3B). Furthermore, the NADPH oxidase activity of the intracellular microsomes was significantly decreased following pretreatment with 20 or 40 μM salubrinal (P < 0.05 in both cases compared with those cells receiving doxorubicin but no salubrinal; Figure 3C).
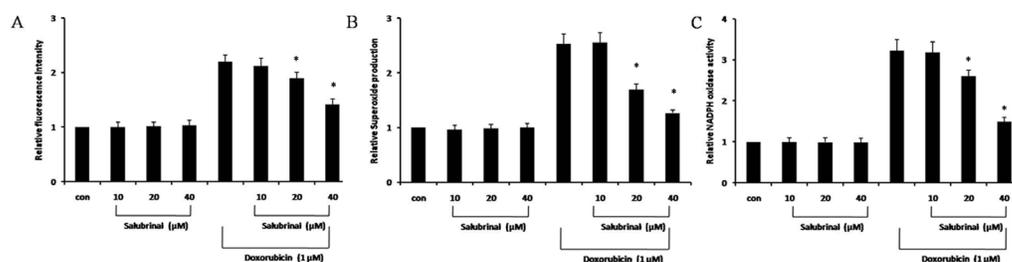

Figure 3. Measurement of reactive oxygen species production (**A**); superoxide generation (**B**); and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (**C**). *P < 0.05.

Salubrinal protected neonatal rat cardiomyocytes by inhibiting eIF2α dephosphorylation

To study the molecular mechanism by which salubrinal protects neonatal rat cardiomyocytes, we measured the phosphorylation level of eIF2α. The results showed that following pretreatment with 20 or 40 μM salubrinal, the level of dephosphorylation in eIF2α in the cardiomyocytes was significantly lower than that in the control group without salubrinal (P < 0.05 in both cases; Figure 4A and B). Cells pretreated with 10 μM salubrinal showed a minor decrease in eIF2α dephosphorylation, although at a statistically insignificant level (P > 0.05; Figure 4A and B).
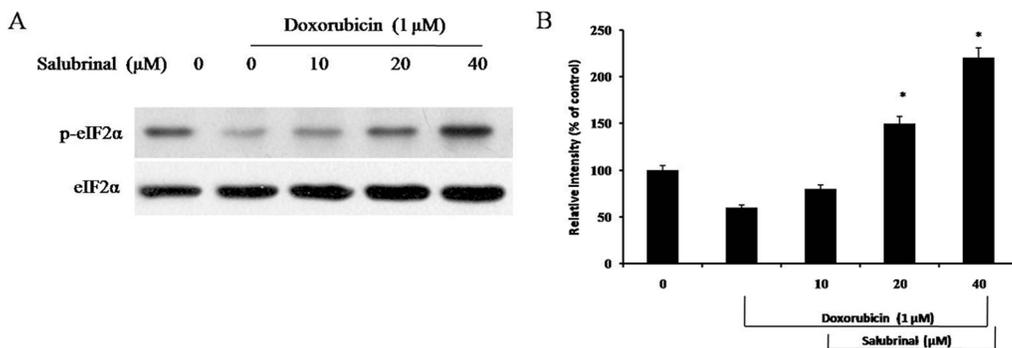

Figure 4. Protection of cardiomyocytes against doxorubicin-induced cell apoptosis by salubrinal. **A.** Level of phosphorylation of eukaryotic translation initiation factor 2α (eIF2α); **B.** relative intensity produced by phosphorylated eIF2α (p-eIF2α). *P < 0.05.

DISCUSSION

This study aimed to determine whether salubrinal pretreatment protects against doxorubicin-induced cell apoptosis in primary-cultured neonatal rat cardiomyocytes. Our results showed a significantly reduced number of doxorubicin-induced apoptotic cardiomyocytes following salubrinal pretreatment. Furthermore, the production of reactive oxygen species and the activity of NADPH oxidase were significantly reduced following salubrinal pretreatment. Regarding the functional mechanism, we discovered a significantly decreased dephosphorylation level in eIF2α in neonatal rat cardiomyocytes following salubrinal pretreatment. These data all suggest that salubrinal protects cardiomyocytes against doxorubicin-induced cell apoptosis.

As a selective inhibitor of eIF2α, salubrinal has been reported to protect rat neural cell line PC-12 against tunicamycin-induced endoplasmic reticulum stress response (Paschen, 2003). A further study determined the potency of salubrinal in ameliorating neurotoxicity produced by cyclosporine-induced endoplasmic reticulum stress response in rats (Gasparetto et al., 2004). Furthermore, salubrinal has been used in the treatment of patients with urinary disease (Katsoulieris et al., 2009) and diabetes (Cnop et al., 2007). A more recent study has shown that salubrinal can effectively inhibit cardiomyocyte apoptosis induced by tunicamycin or hypoxia, possibly by its regulation of the pERK-eIF2α signaling pathway (Liu et al., 2012). These studies support the hypothesis of the protective role of salubrinal against doxorubicin-induced cardiomyocyte apoptosis, which was the focus of our study.

Cardiomyocytes that have been treated with doxorubicin for 16 h show the typical characteristics of cell apoptosis, such as staggered DNA bands under gel electrophoresis (Negoro et al., 2001). In this study, we utilized a similar *in vitro* model to that reported by Negoro et al. (2001) to determine if pretreatment with salubrinal of different concentrations can protect cardiomyocytes from doxorubicin-induced cell apoptosis. Furthermore, reactive oxygen species play an important role in doxorubicin-induced cardiomyocyte apoptosis. Consistent with previous reports (Park et al., 2011), our study demonstrated the inhibitory function of salubrinal in the production of reactive oxygen species. NAPDH oxidase, which is one of the enzymes involved in the production of superoxide, is mainly expressed in blood vessels, and participates in multiple pathogeneses including cardiac fibrosis (Yisireyili et al., 2013), malfunctions in endothelial cells (Galán et al., 2014), and atherosclerosis (Gray et al., 2013). Additionally, the genetic polymorphism of three subunits of NADPH oxidase is related to doxorubicin-induced cardiac toxicity. Our research found that salubrinal pretreatment could significantly reduce NADPH oxidase activity, which, at high levels, along with the production of reactive oxygen species, is believed to be the major cause of doxorubicin-induced cardiac pathology (Deng et al., 2007).

Because some reports have suggested that salubrinal exerts its protective functions mainly by its regulatory effects on eIF2α (Boyce et al., 2005; Wiseman and Balch, 2005), we also measured the expression pattern of eIF2α, which showed a lower dephosphorylation level after salubrinal pretreatment.

In summary, this study suggests that salubrinal pretreatment can effectively protect cardiomyocytes against doxorubicin-induced apoptosis by inhibiting eIF2α dephosphorylation. However, some limitations exist as no *in vivo* study has been performed to substantiate this working model. Further studies are therefore required to determine the potency of salubrinal in ameliorating cardiac toxicity in the body.

Conflicts of interest

ACKNOWLEDGMENTS

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