Antioxidant Effect of Human Selenium-containing Single-chain Fv in Rat Cardiac Myocytes

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Abstract Reactive oxygen species(ROS) plays a key role in human heart diseases. Glutathione peroxidase(GPX) functions as an antioxidant as it catalyzes the reduction of hydroperoxide. In order to investigate the antioxidant effect of human selenium-containing single-chain Fv(Se-scFv-B3), a new mimic of GPX, a model system of hydrogen peroxide(H_2O_2)-induced rat cardiac myocyte damage was established. The cardiac myocyte damage was characterized in terms of cell viability, lipid peroxidation, cell membrane integrity, and intracellular H_2O_2 level. The Se-scFv-B3 significantly reduced H_2O_2-induced cell damage as shown by the increase of cell viability, the decline of malondialdehyde(MDA) production, lactate dehydrogenase(LDH) release, and intracellular H_2O_2 level. So Se-scFv-B3 may have a great potential in the treatment of human heart diseases induced by ROS.

Keywords Human single-chain Fv; Selenium; Glutathione peroxidase; H_2O_2; Cardiac myocyte

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1 Introduction

Reactive oxygen species(ROS) such as superoxide radicals, hydroxyl radicals, or hydrogen peroxide are generated by the incomplete reduction of molecular oxygen to water during the exposure to environmental factors or during several metabolic processes. Although ROS is biologically important signaling molecules[1—3], their accumulation can cause several chronic human diseases[4], including heart diseases. Reactive oxygen species plays a key role in myocardial hypertrophy, contractile dysfunction, and fibrosis[5—8]. With the increase of oxidative stress, a cellular imbalance between the production and elimination of ROS may contribute to ventricular remodeling and dysfunction in heart failure[9—11]. Therefore, it is important to develop effective antioxidants to protect cardiac myocytes against oxidative injure in some heart diseases.

Selenium-containing glutathione peroxidase (GPX) is one of the most important antioxidant enzymes, which protects cells and tissues against oxidative damage as it catalyzes the reduction of hydroperoxides with glutathione(GSH). Selenium, in the form of selenocysteine(Sec), consists in the active center of GPX, which functions as a redox center[12,13].

However, therapeutic usage of native GPX is limited because of its instability and limited availability. Many artificial mimics of GPX have been made, but most of them have shown low GPX activities for lack of GSH-substrate binding site. Use of abzyme is a new strategy for preparing GPX mimics with GSH binding site. The mice monoclonal antibody and scFv antibody with GPX activity have been synthesized in our laboratory[14—17]. ScFv is the smallest antibody fragment of retaining the ability to bind antigen. A previous study shows that the mice scFv antibody(Se-scFv2F3) can protect epidermal cells against UVB injury[18]. However, mice scFv antibody may cause potential immunoreaction because of antigenicity to humans. The generation of abzyme with less antigenicity to humans is more important to pharmaceutical application.

Recently, we have generated a new GPX mimic, human selenium-containing single-chain Fv(se-scFv-B3) by screening phage display antibody library, expression, purification, and chemical mutation. The aim of this study is to investigate antioxidant effect of the se-scFv-B3 in rat cardiac myocytes.

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2 Materials and Methods

Se-scFv-B3 was generated by our laboratory. Fetal bovine serum and trypsin were obtained from Gibco(Rockville, MD, USA). Malondialdehyde(MDA) detection reagent kit and LDH colorimetry kit were purchased from Nanjing Jiancheng Research Institute of Biotechnology(Nanjing, China). 2′,7′-Dichlorofluorescin diacetate(DCFH-DA) was from Molecular Probes(Eugene, OR, USA). Culture medium(Iscove's modified Dulbecco's medium, IMEM) and all other chemicals were purchased from Sigma(St. Louis, MO, USA).

2.1 Isolation and Culture of Rat Cardiac Myocytes

Primary neonatal rat heart cell cultures were prepared from ventricular myocardium by previously described method[19] with some modification. Briefly, the rats were killed with ethyl ether. The ventricular myocardium was surgically removed, minced into 1—3 mm^3 pieces, and then washed twice with phosphate-buffered saline(PBS). The minced tissues were dissociated by 4—6 repeated exposures to 0.25% g/mL trypsin solution at 37 °C under gentle agitation. Supernatants were collected and centrifuged at 500*g* for 5 min. The cell pellets were resuspended in IMEM with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin and plated on a culture dish. The cells were incubated for 2 h at 37 °C in 5% CO_2 to promote early adherence of fibroblasts. Subsequently, the floating cardiac myocytes were collected, counted, plated in the 96-well plates or culture dishes and cultured for 3—4 d at 37 ºC in 5% CO_2. Further experiments were performed after the culture had been completely carried out.

2.2 Measurement of Cardiac Myocytes Viability

Rat cardiac myocytes (5×10^5 cells/mL) were seeded in the 96-well plates and cultured for 72 h. The cardiac myocytes were treated with various concentrations of H_2O_2(0—1000 μmol/L) for 4 h or preincubated with Se-scFv-B3(0.2—0.8 U/mL) for 1 h and then incubated with additional 500 μmol/L H_2O_2 for 4 h, respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) test[20] was performed to measure cardiac myocytes viability on an enzyme-linked immuno-sorbent assay plate reader at 490 nm(TECAN, Austria).

2.3 Determination of Malondialdehyde(MDA)

Rat cardiac myocytes(5×10^5 cells/mL) were seeded in the 96-well plates and cultured for 72 h. The cells were preincubated with or without various concentrations of Se-scFv-B3(0.2—0.8 U/mL) for 1 h. Afterward, additional 500 μmol/L H_2O_2 was added to the cells that were incubated for 4 h. Lipid peroxidation product MDA was determined by means of MDA detection reagent kit as described in manufacturer's instructions. The level of MDA was estimated by the absorbance value at 532 nm.

2.4 Assay of Lactate Dehydrogenase(LDH) Release

Rat cardiac myocytes were treated as described in the determination of MDA. The LDH content of the medium was assayed *via* LDH colorimetry kit as described in manufacturer's instructions. The LDH release was estimated by the absorbance value of the medium at 510 nm.

2.5 Assay of Intracellular H_2O_2 Levels

The level of intracellular H_2O_2 in cardiac myocytes after addition of H_2O_2 was assayed after 2′,7′-dichlorofluorescin diacetate(DCFH-DA) was added, which was rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescin(DCF) in response to ROS production within the cells[21]. Rat cardiac myocytes were plated in culture dishes and cultured for 96 h. The cells were preincubated with or without various concentrations of Se-scFv-B3(0.2—0.8 U/mL) for 1 h. Afterward, additional 500 μmol/L H_2O_2 was added to the cells that were incubated for 4 h. After treatment, the medium was removed and the cardiac myocytes were washed twice with PBS. The cells were incubated with 5 μmol/L DCFH-DA for 20 min in a fresh culture medium. After that the cells were washed twice again with PBS. Fluorescence was measured on a fluorescence spectrophotometer with excitation and emission spectra set at 488 and 525 nm, respectively. Fluorescence intensity reflects the intracellular H_2O_2 level.

2.6 Statistical Analysis

Two-tailed unpaired *t* tests were performed for comparison between the groups. $P<0.05$ or $P<0.01$

was accepted as statistical significance. All the results were expressed as means±SD of three independent experiments.

3 Results and Discussion

3.1 Effect of Se-scFv-B3 on Cardiac Myocytes Viability

Since ROS contributes to the pathologic process of many heart diseases, we examined antioxidant effect of Se-scFv-B3 in rat cardiac myocytes, which were damaged by H_2O_2. To obtain a working concentration of H_2O_2 that causes approximately 50%—60% cardiac myocytes death, we performed concentration-response experiments. Fig.1(A) shows that 500 μmol/L H_2O_2 caused approximately 55% cardiac myocytes death. Therefore, all subsequent experiments were performed with 500 μmol/L H_2O_2. Fig.1(B) shows that pretreatment with Se-sFv-B3 increased the viability of H_2O_2-treated cardiac myocytes in a concentration-dependent manner. A total of 0.8 U/mL Se-scFv-B3 exhibited a maximal protective effect, which increased cell viability from 43.3% to 86.1%. In addition, there was no statistically significant difference between cardiac myocytes viabilities of Se-scFv-B3(0.2—0.8 U/mL) alone and normal control group(data not shown).

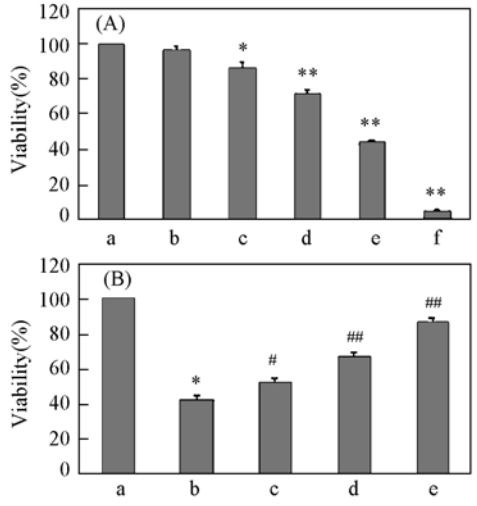

Fig.1 Effects of Se-scFv-B3 on rat cardiac myocytes viability by MTT assay

(A) Cardiac myocytes were incubated with indicated concentrations of H_2O_2 for 4 h. * P<0.05 or ** P<0.01, compared with 0 μmol/L H_2O_2. $c(H_2O_2)$/(μmol·L^{-1}): a. 0; b. 50; c. 100; d. 200; e. 500; f. 1000. (B) Cardiac myocytes were preincubated with or without different concentrations of Se-scFv-B3 for 1 h, then treated with 500 μmol/L H_2O_2 for 4 h. * P<0.01, compared with control. # P<0.05 or ## P<0.01, compared with 500 μmol/L H_2O_2 group. a. Control; b. 500 μmol/L H_2O_2; c. 0.2 U/mL Se-scFv-B3+500 μmol/L H_2O_2; d. 0.4 U/mL Se-scFv-B3+500 μmol/L H_2O_2; e. 0.8 U/mL Se-scFv-B3+500 μmol/L H_2O_2.

3.2 Effect of Se-scFv-B3 on MDA Production

Lipid peroxidation is an important mechanism of ROS-mediated cellular injury. Malondialdehyde is the main lipid peroxidation product. It is usually used to estimate the level of lipid peroxidation[22]. Table 1 shows that 500 μmol/L H_2O_2 significantly increased the content of MDA in cardiac myocytes, and MDA production was partly inhibited with the increase in concentration of Se-scFv-B3. These data indicate that Se-scFv2F3 has a strong ability to inhibit lipid peroxidation.

Table 1 Effect of Se-scFv-B3 on the level of MDA in rat cardiac myocytes exposed to H_2O_2*

Group	MDA($A_{532\ nm}$)
Normal control	0.020±0.005
500 μmol/L H_2O_2	0.084±0.006**
0.2 U/mL Se-scFv-B3+500 μmol/L H_2O_2	0.072±0.006#
0.4 U/mL Se-scFv-B3+500 μmol/L H_2O_2	0.051±0.006##
0.8 U/mL Se-scFv-B3+500 μmol/L H_2O_2	0.034±0.005##

* Cardiac myocytes were treated as indicated. The level of MDA was estimated by the absorbance value at 532 nm on a spectrophotometer. ** P<0.01, compared with normal control. # P<0.05 or ## P<0.01, compared with 500 μmol/L H_2O_2 group.

3.3 Effect of Se-scFv-B3 on LDH Release

With the occurrence of lipid peroxidation, the cardiac myocyte membrane was damaged, and the cellular components began to leak into the culture medium. Lactate dehydrogenase is a sensitive indicator of cell membrane integrity[23]. Table 2 shows that 500 μmol/L H_2O_2 significantly increased the LDH release, which reached 5.17 times that of the normal control in the medium. Se-scFv-B3 prevented cardiac myocytes from leaking LDH induced by H_2O_2. A total of 0.8 U/mL Se-scFv-B3 furthest reduced LDH release to 1.51 times that of the control.

Table 2 Effect of Se-scFv-B3 on LDH release from rat cardiac myocytes exposed to H_2O_2*

Group	LDH($A_{510\ nm}$)
Normal control	0.029±0.007
500 μmol/L H_2O_2	0.150±0.013**
0.2 U/mL Se-scFv-B3 + 500 μmol/L H_2O_2	0.121±0.007#
0.4 U/mL Se-scFv-B3 + 500 μmol/L H_2O_2	0.081±0.011##
0.8 U/mL Se-scFv-B3 + 500 μmol/L H_2O_2	0.044±0.003##

* Cardiac myocytes were treated as indicated. The LDH release was estimated by the absorbance value of medium at 510 nm using spectrophotometer. ** P< 0.01, compared with normal control; # P< 0.05 or ## P< 0.01, compared with 500 μmol/L H_2O_2 group.

3.4 Effect of Se-scFv-B3 on Intracellular H_2O_2 Levels

H_2O_2 is one of several kinds of ROS. Glutathione

peroxidase can reduce H_2O_2 by converting H_2O_2 to H_2O. So the analysis of intracellular H_2O_2 levels is helpful to explaining the protection mechanism of Se-scFv-B3. As shown in Table 3, the levels of intracellular H_2O_2 were significantly increased after treatment with 500 μmol/L H_2O_2 compared with that of control. However, the levels of intracellular H_2O_2 gradually decreased after pretreatment of the cells with different concentrations of Se-scFv-B3. This demonstrates that Se-scFv-B3 exerts an antioxidant mechanism similar to that of the natural GPX.

Table 3 Effect of Se-scFv-B3 on intracellular H_2O_2 level*

Group	H_2O_2 levels($A_{525\ nm}$)
Normal control	245±11
500 μmol/L H_2O_2	1012±12**
0.2 U/mL Se-scFv-B3+500 μmol/L H_2O_2	922±9##
0.4 U/mL Se-scFv-B3+500 μmol/L H_2O_2	500±10##
0.8 U/mL Se-scFv-B3+500 μmol/L H_2O_2	379±9##

* Cardiac myocytes were treated as indicated. The levels of intracellular H_2O_2 were assayed by fluorescence emission from DCF on a fluorescence spectrophotometer with excitation and emission spectra set at 488 and 525 nm, respectively. ***P*<0.01, compared with control. ##*P*<0.01, compared with 500 μmol/L H_2O_2 group.

In this study, we have demonstrated that a range of Se-scFv-B3(0.2—0.8 U/mL) exerts an antioxidant effect similar to that of the natural GPX in rat cardiac myocytes. Previous study reported that the GPX mimic(Se-scFv2F3) from mice monoclonal antibody protected rat epidermal cells against UVB injury. When 0.2—0.4 U/mL of Se-scFv2F3 was administered to the cells before UVB radiation, the MDA production, LDH leakage, and intracellular H_2O_2 content reached the levels of those in the normal control group. The antioxidant effect of Se-scFv-B3 is lower than that of Se-scFv2F3 in different cells[18]. However, mice scFv may cause potential immunoreaction because of antigenicity to humans. In addition, many small-molecule GPX mimics, such as ebselen, 2-SeCD, and 2-TeCD, exhibited antioxidant abilities in different cells[24]. But their GPX activities are lower than that of Se-scFv-B3 because of the lack of substrate binding sites. The human Se-scFv-B3 may have a great potential in the treatment of human diseases induced by ROS because of its higher GPX activity, lower antigenicity, and smaller size.

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