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The effects of glucose on proteasome activities in neuronal cells under in vitro ischemia conditions

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Medical Student Research Project Report

The effects of glucose on proteasome activities in neuronal cells under *in vitro* ischemia conditions

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Abstract

Reducing the volume of brain damage after stroke has been a focus of recent research due to its effect on recovery time and quality of life. Changes in proteasomal activities are related to ischemic damage of brain. Proteasome inhibition has been suggested as a potential treatment option for stroke. However, the mechanism of alteration of proteasomal activities in neurons under ischemic conditions is not known. In study, we investigated the role of glucose in regulating proteasomal activities in neuronal cells under ischemic condition. We found that glucose concentration had remarkable differential effect on both 20S and 26S proteasomal activities in SH-SY5Y cells under hypoxic exposures. Further investigation revealed that reactive oxygen species caused increase or decrease of 20S and 26S activities, dependent on the level of ROS. Finally, antioxidant treatment confirmed that ROS was responsible, at least in part, for the changes of proteasomal activities in ischemic neurons.

Introduction

According to American Stroke Association, there are 700,000 Americans suffering new or recurrent stroke each year, and stroke is the 3rd leading cause of death after heart disease and cancer in the U.S. In 2006, fifty eight billion dollars were spent on stroke related medical care and disability. A significant amount of recent research on stroke focuses on reducing the volume of brain damage that is directly related the recovery time and quality of life. Changes in proteasomal activities are related to ischemic damage of brain. It has been reported that treatment with proteasome inhibitor reduces effectively neuronal and astrocytic degeneration, cortical infarct volume, infarct neutrophil infiltration (Wojcik and Di Napoli, 2004). And thus, proteasome inhibition has been suggested as a potential treatment option for stroke (Phillips et al., 2000). However, the mechanism of alteration of proteasomal activities in neurons under ischemic conditions is not known. Results from non-neuronal cells have shown that reactive oxygen species (ROS) plays a critical role in regulating proteasomal activities. Ischemia causes abnormal ROS metabolism, due to the interruption of nutrients and oxygen supplies and their abnormal metabolisms. Glucose is one of the major nutrients in blood. It is not only a major energy supplier, but also a major reducing agent provider through the pentose phosphate pathways in cells. It has been shown that glucose quenches ROS in neurons under ischemic conditions (Shi and Liu, 2006). Based on these findings, we hypothesize that glucose plays a critical role in regulating proteasomal pathways through reducing ROS levels in neuronal cells under ischemic conditions. This study will provide experimental evidence to shed new light on the mechanism of proteasomal

activity changes in neurons under ischemic conditions, and thus provide assistance in designing potential safe and effective stroke treatments.

Material and Method

Cell culture and treatments: SH-SY5Y Cells was chosen for its similar property to neuronal cells. SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin 1:100) at 37 °C in a humidified incubator gassed with 95% air and 5% CO_2. Medium was changed to DMEM without FBS and without antibiotics at 80% confluence. For glucose and hypoxic treatments, cells were gently washed twice with pre-warmed phosphate buffered saline (PBS, pH 7.4), and then placed in DMEM with a range of glucose concentrations (0, 2.5, 5.5,10, and 25 mM), which had been gassed with nitrogen for 10 min. The range of glucose concentration is chosen according to Shi et al (Shi and Liu, 2006). Cells were incubated at 37 °C for 3 hrs in a humidified hypoxia chamber (Billups-Rothenberg Inc., Del Mar, CA) with 1% O_2, 5% CO_2 and balance nitrogen. For ROS or antioxidants treatment, cells are handled as same method as glucose treatment.

Proteasome activity assay: 26S proteasome functions were measured by their abilities to cleave a substrate (SucLLVY-AMC) as described by Fekete et al (Fekete et al., 2005). Briefly, one million cells were washed twice with phosphate buffer (pH 7.4) and then lysed by repeated freeze–thaw cycles in 0.25 M sucrose, 25 mM HEPES (pH 7.8), 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol. Lysates were centrifuged at 14,000*g* for 30 min. Protein levels were determined using a Bio-Rad reagent. Cell lysate (10 μg protein) was diluted with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2 mM

ATP) to a final volume of 100 µl and the fluorogenic proteasome substrate SucLLVY-AMC (80 µM in 1% DMSO, chymotrypsin-like, Sigma) was added. The mixture was incubated at 37°C for 1 h. The reaction was stopped by adding an equal volume of ice-cold ethanol and 10 vol of 0.125 M sodium borate (pH 9.0). All the procedure described above was carried out under a low O_2 condition by using a hypoxia chamber. Free AMC was detected with a fluorescence plate reader (Wallac 1420 victor^2) at ex 380 nm and em 440 nm using free AMC as a standard. To access 20S function, buffer I was replaced by an ATP-free buffer containing SDS (20 mM HEPES, pH 7.8; 0.5 mM EDTA, 0.03% SDS).

Data analysis

Each datum point was repeated 4-8 times. Results were expressed as means ± SE. The statistical analysis will be evaluated by unpaired Student's t-test, with the level of significance chosen at $p<0.05$.

Results

To investigate the effects of glucose on proteasomal activities, we studied 20S and 26S proteasomal activities in SH-SY5Y cells after *in vitro* ischemic treatments. The cells were exposed to different glucose levels (0, 2.5, 5.5,10, and 25 mM) and low oxygen (1%) 3 hours (hrs). After the treatments, cells were collected, and their proteasomal activities were analyzed. As shown in Fig. 1, the 20S proteasomal activity was glucose concentration dependent in the hypoxic cells. In range of 0-10 mM, glucose increased the proteasomal activity. When glucose concentration was greater than 10 mM, the

proteasomal activity decreased. The activity at 25 mM glucose was significant lower than these at 0, 5.5, and 10 mM. In addition, it seemed that the activity increased again when the glucose concentration was greater than 25 mM. Fig. 2. shows the effect of glucose on the 26S proteasomal activities in the hypoxic cells. Glucose at the range of 0-25 mM primarily decreased the 26S activities concentration dependently. In the presence of glucose concentration greater than 25 mM, the activity increased. The results revealed that glucose had great effect on both 20S and 26S activities in the hypoxic cells and that cells treated with glucose at 25 mM had the lowest activities of 20S and 26S proteasomes.

To confirm that reactive oxygen species (ROS) was involved in the glucose-mediated proteasomal activity changes, we carried out experiments to study the effect of ROS on the activity of proteasomes. ROS refers to a group of oxygen centered reactive species. Superoxide anion radical and H_2O_2 are two very important ROS in causing oxidative damage and altering signal transduction under oxidative stress such as pathological condition of ischemia. We tested the effects of superoxide and H_2O_2 on the activity of 20S and 26S proteasomes in SH-SY5Y cells under hypoxic conditions. Fig. 3 shows that superoxide decreased 26S activity under hypoxic conditions concentration-dependently. In the presence of 50 μM KO_2, the 26S activity decreased about 50%. In contrast, the effect of superoxide on 20S activity was different from that of 26S activity. KO_2 increased 20S activity in a concentration up to 20 μM. When KO_2 concentration was greater than 20 μM, it reduced 20S activity. KO_2 at 50 μM only slightly reduced 20S activity without significance, compared to the control group (in the absence of KO_2).

H_2O_2 also showed ability to affect 20S and 26S activities concentration dependently (Fig. 4). In a smaller dose (smaller than 50 μM), H_2O_2 elevated 26S activity. As the concentration of H_2O_2 increases (greater than 50 μM), it seemed to inhibit the activity. On the other hand, H_2O_2 elevated 20S activity during the range of 0-200 μM. These results demonstrated that superoxide and H_2O_2 had great effect on 20S and 26S activities in hypoxic SH-SY5Y cells although their effects were concentration dependent and differential dependent on the species of proteasomes.

The above results indicated that ROS could increase or inhibit 20S and 26S activities in hypoxic SH-SY5Y cells. However, there was no direct link that ROS was responsible for glucose-mediated proteasomal activity changes in hypoxic cells. To further confirm that ROS was involved in proteasomal activity changes, we used specific antioxidants as ROS scavenger to reduce the levels ROS in hypoxic cells. A membrane permeable SOD mimic MnTmPyp was used to suppress $O_2^{\bullet-}$. Catalase was used to suppress the generation of H_2O_2. In these experiments, cells were pre-incubated with these antioxidants 30 min before *in vitro* hypoxic treatments. Fig. 5 shows the effect of MnTmPyp on 26S and 20S activities. In the concentration range of 0-20 μM , MnTmPyp decreased both 26S and 20S activities. Catalase also decreased the activity of 26S (Fig. 6). However, the effect of catalase on 20S was a different from that on 26S. It increased the activity at 250 units/ml but decreased the activity when its concentration was greater than 250 units/ml. These results confirmed that ROS were involved in the alteration of proteasomal activities in ischemic SH-SY5Y cells.

Discussion

Proteasomes are large multicatalytic proteinase complexes in cytosol and nucleus of eukaryotic cells. The proteasome complexes exist in two forms, 26S, which mainly mediates the ubiquitin-dependent degradation of cellular proteins, and 20S, which is ATP- and ubiquitin-independent. The two different complexes usually have different protein substrate specificities. The 26S pathway is greatly dependent on the covalent attachment of ubiquitin to proteins, which is known as ubiquitination, to form conjugates. The ubiquitin-proteasome system has a central role in the selective degradation of intracellular proteins, which are critical in regulating critical cell processes including differentiation, cytokine-induced gene expression, apoptosis, and the stress response (Brahimi-Horn and Pouyssegur, 2005; Ciechanover, 2006; Ding et al., 2006; Wilkinson, 1995). In addition, the ubiquitin-proteasome system component are among the most abundant proteins of central nervous system. Cellular oxidized proteins are degraded primarily through ubiquitin-independent 20S proteasome pathway (Ding et al., 2006). Proteasome activities under ischemic condition have been related to activation of proinflammatory protein through nuclear factor-kappa B (NF-kB) (Palombella et al., 1994). The involvement of proteasome in the degradation of hypoxia-inducible factor 1 has been proved to be critical to the progression and extension of cerebral injuries during stroke. More significantly, it has been reported that treatment with proteasome inhibitor reduces effectively neuronal and astrocytic degeneration, cortical infarct volume, infarct neutrophil infiltration (Wojcik and Di Napoli, 2004). Thus, proteasome inhibition has been suggested as a potential treatment option for stroke (Phillips et al., 2000).

Glucose, an essential nutrient for brain function has been proved to play an important role in ischemic injury. Both glucose deprivation and exposure to elevated glucose levels have been shown to be pathogenic to cells. Besides as an energy source, glucose is also a source providing reductants in cells and maintains cellular redox status. Glucose is a main substrate to produce the principal intracellular reductant NADPH by the pentose phosphate pathways (PPP) (Averill-Bates and Przybytkowski, 1994; Przybytkowski and Averill-Bates, 1996). Glutathione (GSH) can be regenerated from glutathione disulfide (GSSG) by glutathione reductase, which uses NADPH as an electron donor. The pathways have been suggested as the major source of NADPH production for the maintenance of GSH level, which maintains a cellular reduced milieu, in the brain (Ben-Yoseph et al., 1994; Hotta, 1962; Hotta and Seventko, 1968). Consequently, it would be expected that lack of glucose results in a condition of metabolic oxidative stress characterized by increased pro-oxidant production and increased levels of GSSG because of interfering with NADPH production by the PPP pathway. In fact, it has been demonstrated that glucose greatly affect the ROS levels in hypoxic neurons (Shi, 2006). The published results showed that the presence of glucose decreased the level of ROS and cells incubated with glucose at 25 mM had the lowest levels of ROS.

ROS have been suggested to be able to affect both 26S and 20S proteasome pathways. For example, $O_2^{\bullet-}$ may play a role like O_2 in activating prolyl hydroxylase under hypoxia, thus increasing the activity of ubiquitin-proteasomal pathways as a whole (Callapina et al., 2005; Haddad, 2002), although the 26S may be vulnerable to ROS exposure. On the other hand, ROS may facilitate protein degradation through 20S pathway because

oxidized proteins are preferred substrates of 20S proteasome (Grune et al., 1996, 1997; Grune et al., 1995; Levine et al., 1996) and ROS rapidly upregulate 20S proteasome activity (Ullrich et al., 1999). However, it is not completely known whether ROS affect the proteasomal activities in cells under hypoxic conditions, especially in neuronal cell. The results in this report clearly demonstrate that ROS affect the activity of proteasomal activities in SH-SY5Y neuronal cells under hypoxic exposures. As shown in Fig. 1 and 2, cells treated hypoxia and glucose at 25 mM had the lowest 20S and 26S proteasomal activities. As discussed previously, glucose at 25 mM induced the lowest level of ROS in hypoxic neurons. This indicates that ROS may be was responsible for the elevation of proteasomal activities in ischemic neurons (in the absence or low levels of glucose). Indeed, our results from experiments with ROS and antioxidant treatments confirmed that both superoxide and hydrogen peroxide could increase 20S and 26S activities in hypoxic neurons although there was a concentration range for this specific effect.

Cellular injury induced by both low and high glucose has been linked to the generation of reactive oxygen species (ROS) and increased oxidative stress. It has been reported that glucose deprivation causes perturbations in cellular sensitivity to oxidative stress, which is mediated by increased generation of pro-oxidants and decreased scavenging of free radicals. Formation of hydrogen peroxide (Ho et al., 2000) as well as more general measurements of ROS generation (Sharpe et al., 1998) have been observed under high glucose conditions and are suggested to be responsible for high glucose-induced cell injury and/or death in non-neuronal cells. The previous study in Dr. Shi's lab has proven that the optimal glucose concentration increase cellular GSH/GSSG ratio which is the

main criteria of cellular redox status in neuronal cell. The study of the effect of glucose on proteasome activities will not only help us to understand the mechanism of ROS on ischemic injury but also illustrate the interaction of nutrient and oxygen under ischemia condition.

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Figure Legend

Fig. 1. Effect of glucose on 20S proteasomal activity under hypoxic conditions. SH-SY5Y cells were exposed to hypoxia (1% O_2) 3 hrs. The proteasomal activities were expressed as the percentage of the value obtained in the presence of 0 mM glucose. Data are shown as means ± SE, n=8. $^{*}p<0.05$, compared to glucose at 25 mM.

Fig. 2. Effect of glucose on 26S proteasomal activity under hypoxic conditions. SH-SY5Y cells were exposed to hypoxia (1% O_2) 3 hrs. The proteasomal activities were expressed as the percentage of the value obtained in the presence of 0 mM glucose. Data are shown as means ± SE, n=8. $^{*}p<0.05$, compared to glucose at 25 mM.

Fig. 3. Effects of superoxide anion radical on 20S and 26S proteasomal activities under hypoxic conditions. KO_2 was used as a source of superoxide anion radicals. SH-SY5Y cells were treated with different concentrations of KO_2 and hypoxia for 3 hrs. Data are shown as means ± SE, n=3-4. $^{*}p<0.05$, compared to the group without KO_2 treatment.

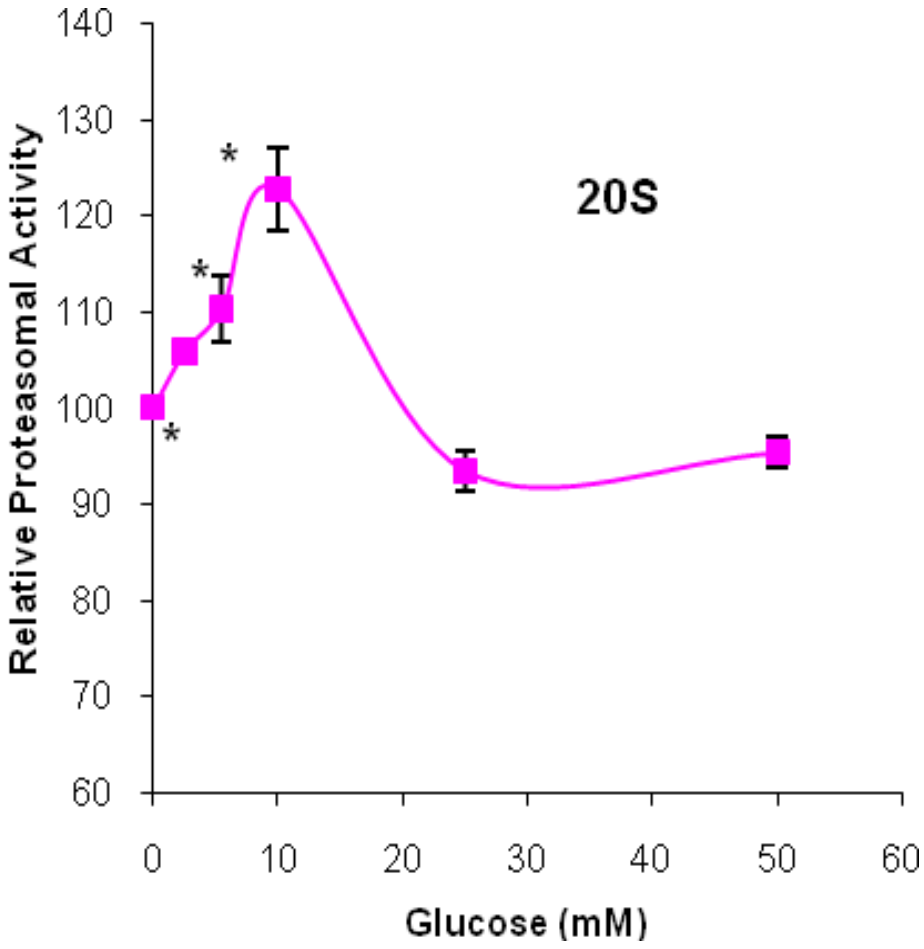
Fig. 4. Effects of hydrogen peroxide on 20S and 26S proteasomal activities under hypoxic conditions. SH-SY5Y cells were treated with different concentrations of H_2O_2 and hypoxia for 3 hrs. Data are shown as means ± SE, n=3-4. $^{*}p<0.05$, compared to the group without H_2O_2 treatment.

Fig. 5. Effects of antioxidant MnTMPyP on 20S and 26S proteasomal activities under hypoxic conditions. SH-SY5Y cells were treated with different concentrations of

MnTMPyP and hypoxia for 3 hrs. Data are shown as means ± SE, n=3-4. $^{*}p<0.05$, compared to the group without MnTMPyP treatment.3

Fig. 6. Effects of antioxidant catalase on 20S and 26S proteasomal activities under hypoxic conditions. SH-SY5Y cells were treated with different concentrations of catalase and hypoxia for 3 hrs. Data are shown as means ± SE, n=3-4. $^{*}p<0.05$, compared to the group without catalase treatment.

Fig.1


20S
Relative Proteasomal Activity
Glucose (mM)
140
130
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110
100
90
80
70
60
0
10
20
30
40
50
60
*
*
*

Fig. 2

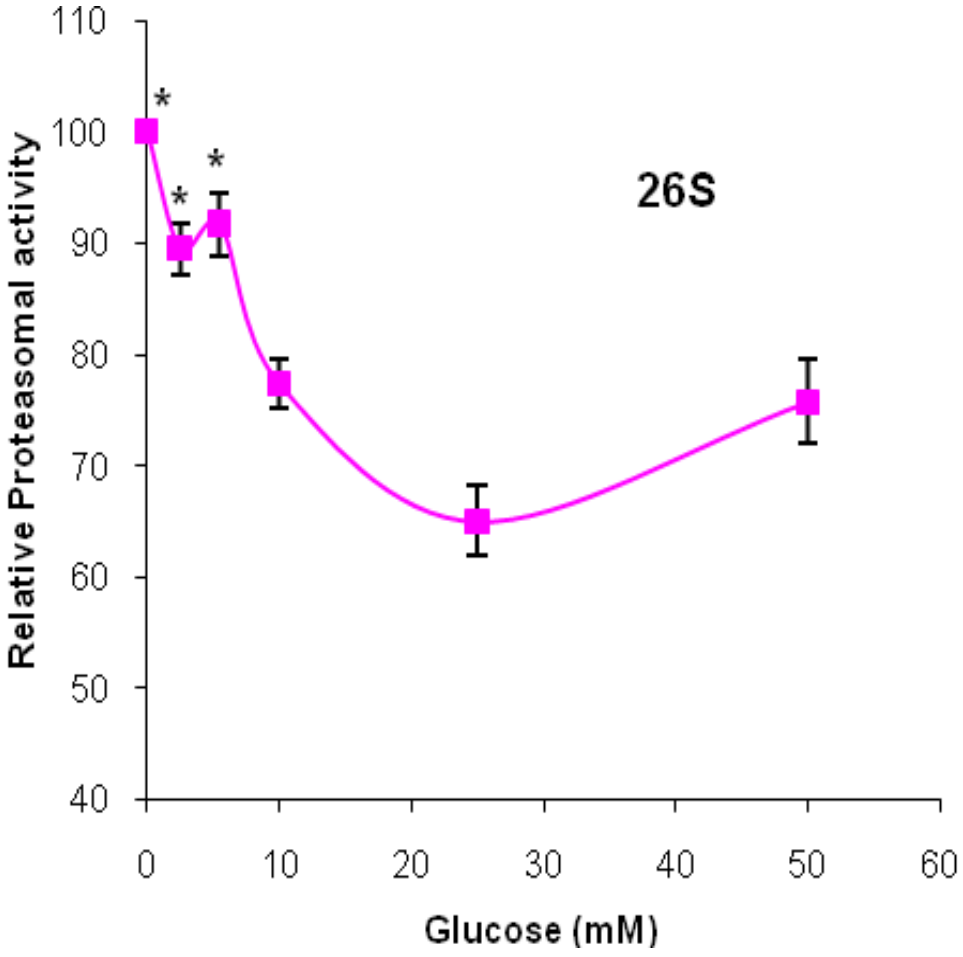

110
100
90
80
70
60
50
40
Relative Proteasomal activity
*
*
*
26S
0
10
20
30
40
50
60
Glucose (mM)

Fig. 3

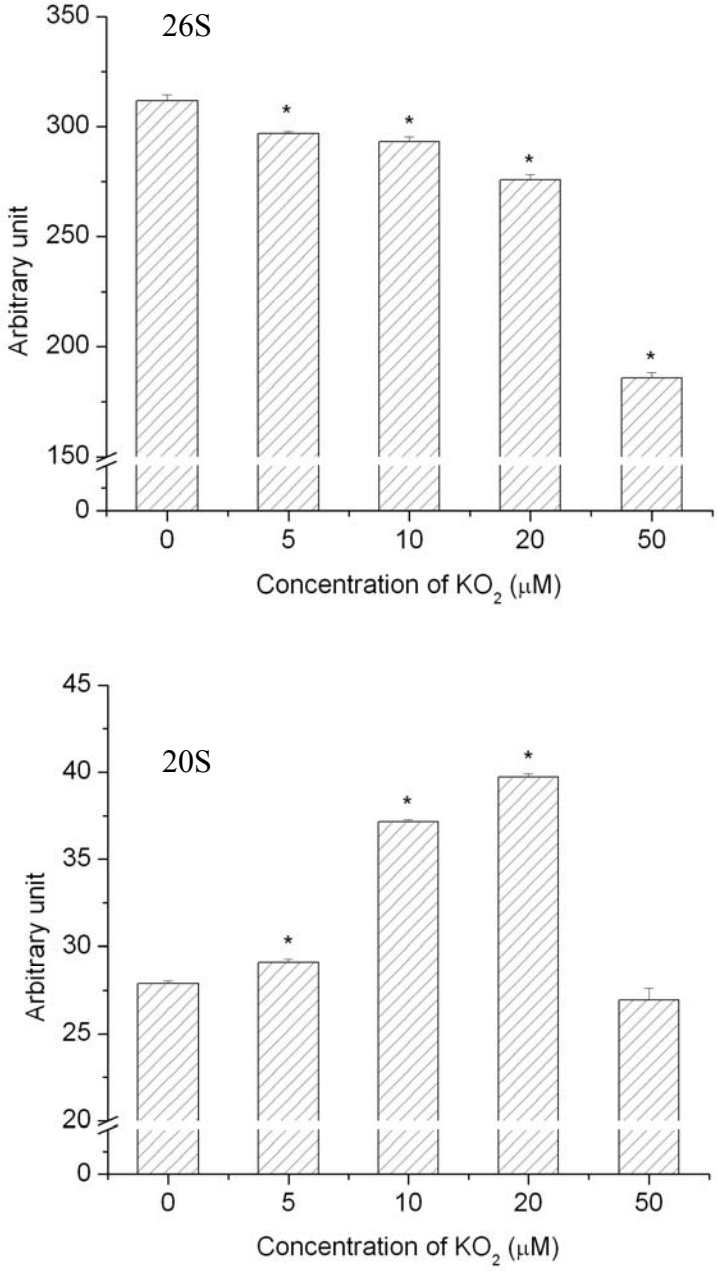
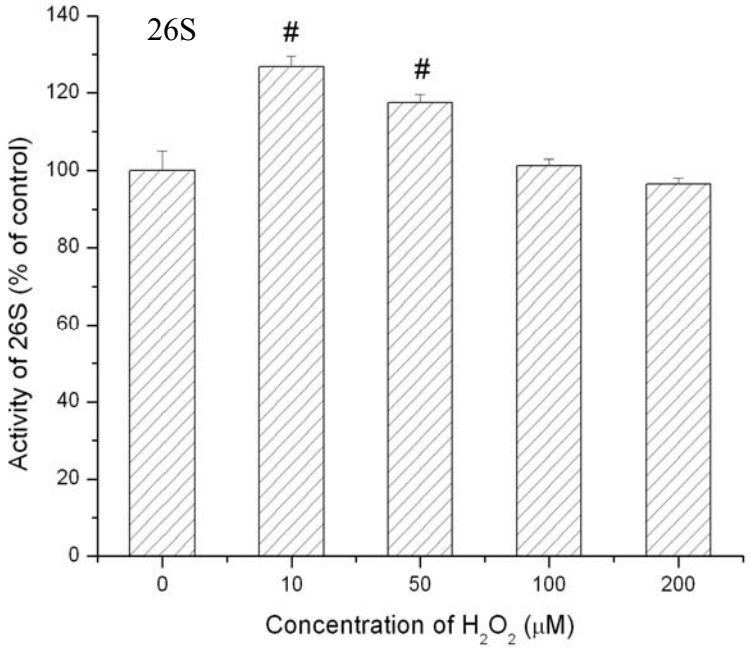

26S
350
300
250
200
150
0
Arbitrary unit
*
*
*
*
0
5
10
20
50
Concentration of KO2 (μM)
20S
45
40
35
30
25
20
0
Arbitrary unit
*
*
*
0
5
10
20
50
Concentration of KO2 (μM)

Fig. 4


26S
#
#
Activity of 26S (% of control)
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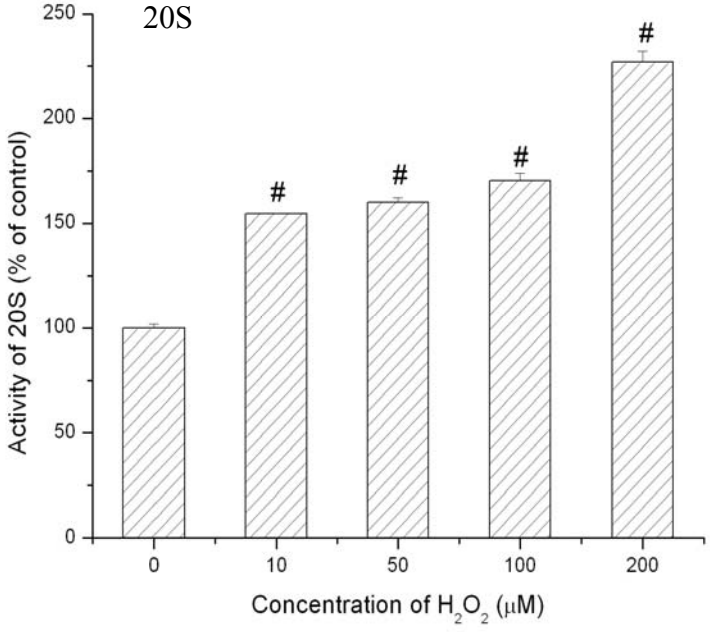
120
100
80
60
40
20
0
0
10
50
100
200
Concentration of H_2O_2 (μM)


20S
#
#
#
#
Activity of 20S (% of control)
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200
150
100
50
0
0
10
50
100
200
Concentration of H_2O_2 (μM)

Fig. 5

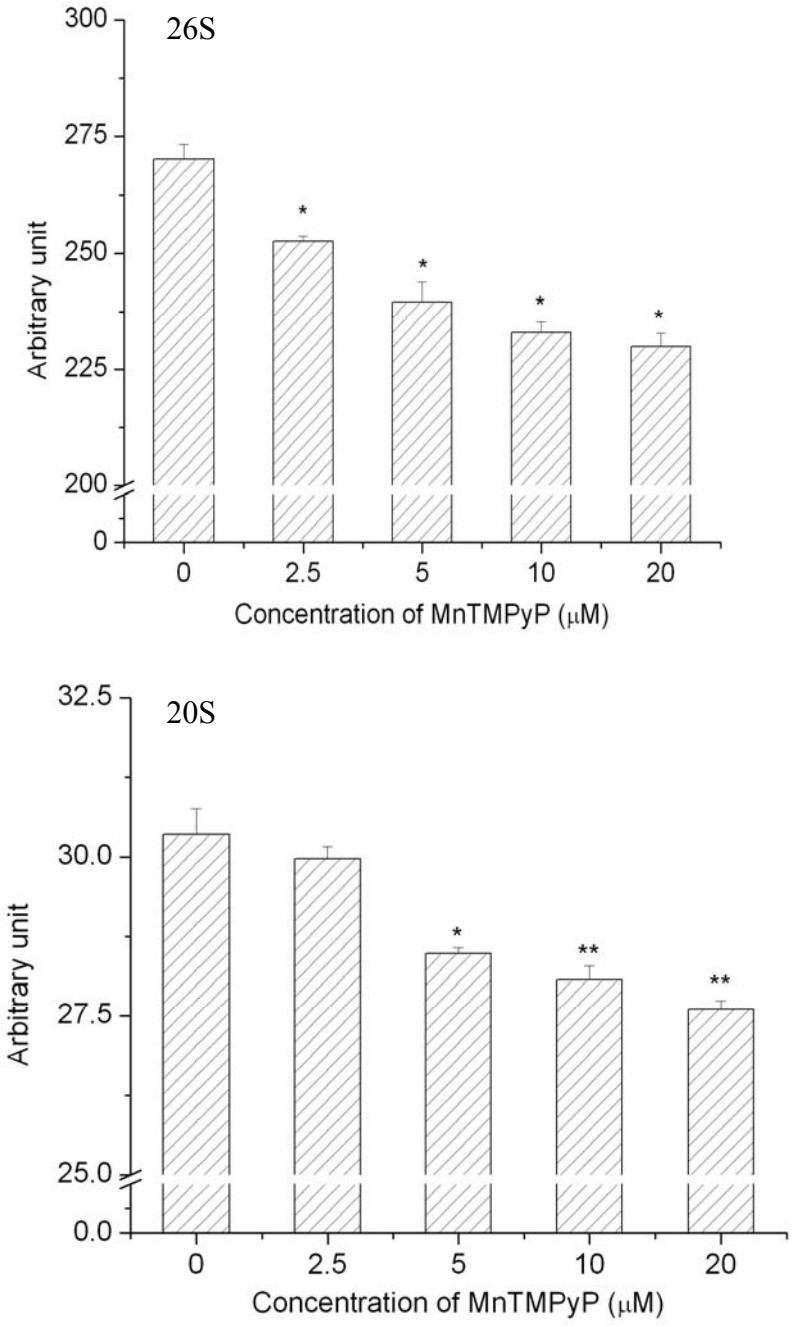
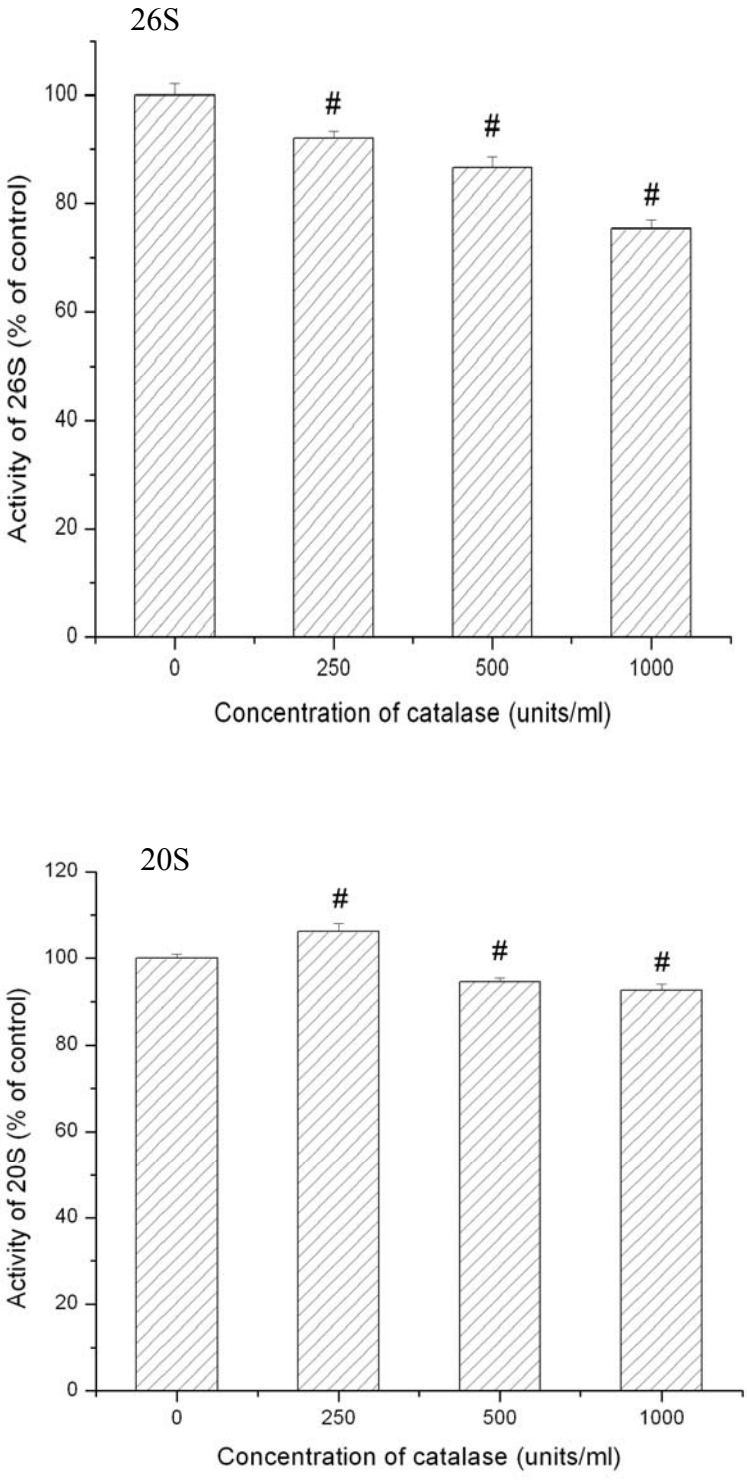

26S
300
275
250
225
200
0
Arbitrary unit
*
*
*
*
0
2.5
5
10
20
Concentration of MnTMPyP (μM)
20S
32.5
30.0
27.5
25.0
0.0
Arbitrary unit
*
**
**
0
2.5
5
10
20
Concentration of MnTMPyP (μM)

Fig. 6


26S
Activity of 26S (% of control)
100
80
60
40
20
0
#
#
#
0
250
500
1000
Concentration of catalase (units/ml)
20S
Activity of 20S (% of control)
120
100
80
60
40
20
0
#
#
#
0
250
500
1000
Concentration of catalase (units/ml)