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Antioxidative action of *N*-α-tosyl-L-lysine chloromethyl ketone prevents death of glutathione-depleted cardiomyocytes induced by hydrogen peroxide

Kiyoshi Takahashi, Kyohei Oyama, Hironori Motoshige, Koichi Sakurai*

Division of Biochemistry, Department of Life science, Hokkaido Pharmaceutical University School of Pharmacy, Katsuraoka-cho, Otaru, Hokkaido, Japan; *Corresponding Author: ks51@hokuyakudai.ac.jp

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ABSTRACT

Hydrogen peroxide (H_2O_2) induces the hypertrophy in cultured H9c2 cardiomyocytes and cell death in glutathione (GSH)-depleted H9c2 cells. In the present study, we observed that pretreatment with a serine protease inhibitor, *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), significantly prevented the H_2O_2-induced cell damages in GSH-depleted H9c2 cells in a concentration-dependent manner. The phase contrast microscopy revealed that although the exposure of the GSH-depleted H9c2 cells to H_2O_2 resulted in a globular shape of the cells, TLCK prevented the occurrence of H_2O_2-induced morphological changes. TLCK also inhibited the generation of reactive oxygen species in the cells after addition of H_2O_2, suggesting that the antioxidant action of TLCK is involved in the protection against the cell damages by H_2O_2. Application of TLCK after ~30 min of exposure to H_2O_2 could significantly protect the cells from cell damages. The other serine protease inhibitors that were tested could not prevent the cell damages in GSH-depleted H9c2 cells. Pretreatment with an inhibitor of nuclear factor-κB translocation into the nucleus and a proteasome inhibitor did not prevent the cell damages in GSH-depleted H9c2 cells. An inhibitor of p53 significantly prevented the cell damages in GSH-depleted H9c2 cells. These results suggest that antioxidative action of TLCK prevents the death of GSH-depleted H9c2 cardiomyocytes induced by H_2O_2.

Keywords: TLCK; Cardiomyocytes; Oxidative Damage; H_2O_2; p53; ROS; Antioxidant

1. INTRODUCTION

Studies on the reactive oxygen species (ROS), such as superoxide, H_2O_2, and the hydroxyl radical, have indicated their involvement in pathogenesis of several diseases. Although ROS are involved in atherosclerosis, myocardial ischemia/reperfusion injury, and heart failure [1,2], the mechanism by which ROS initiate these diseases is not yet fully understood. In primary cultured neonatal rats cardiomyocytes, H_2O_2 at higher doses causes necrotic cell death, whereas at lower doses, it induces apoptosis of a fraction of cells; the surviving cells undergo hypertrophy [3]. We had previously suggested that intracellular free thiol levels determine the fate of H_2O_2-treated H9c2 cardiomyocytes, *i.e.*, cell death or hypertrophy [4]. In GSH-depleted H9c2 cells, hypertrophy did not occur after treatment with H_2O_2 at any concentrations, but the viability of the cells decreased markedly in comparison with that of normal GSH-containing cells [4]. These findings suggest that the GSH-depleted cells are vulnerable to oxidative stress. It has been reported that GSH functions as an important cellular defense system against oxidative stress and that GSH participates in the detoxification reactions of free radicals and peroxides, either directly or indirectly as a coenzyme of antioxidant enzymes, such as glutathione peroxidases [5,6].

It is well known that the ratio of GSH to GSSG in cells represents the intracellular redox environment. Additionally, the intracellular redox state of GSH is involved in signal transduction through the redox regulation of a number of proteins that have critical thiols, such as receptors, proteins involved in ubiquitinylation, several protein kinases, and some transcription factors [7,8]. The modulation of the activity of several transcription factors, *i.e.*, nuclear factor-κB (NF-κB), activator protein-1, and heat shock factor-1, by thiol redox state has been reviewed [5].

While caspases, a family of cysteine proteases, have been predominantly studied in cell death research, serine proteases may also play a significant role in cell death process [9]. The roles of these serine proteases, *i.e.*, Omi /HtrA2, AP24, and thrombin, have been reviewed [10]. During the study that was conducted to clarify whether serine proteases might be involved in the H_2O_2-induced cell death in cardiomyocytes, we had the opportunity to determine the protective effect of *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), a well-known serine protease inhibitor, against the H_2O_2-induced cell damages in GSH-depleted cardiomyocytes. The serine protease inhibitors inhibited apoptosis-associated proteolysis and DNA fragmentation [9]. TLCK is recognized as an inhibitor of translocation of NF-κB [11] as well as an inhibitor of serine proteases. The translocation of NF-κB is involved in lipopolysaccharide-mediated cytotoxicity in macrophages, and the inhibition of this translocation by TLCK abrogated the cell death in macrophages [12]. Moreover, Gong *et al*. (1999) and Rideout *et al*. (2001) demonstrated that TLCK might interfere with p53 dependent cell death pathway [13,14]. Thus TLCK can exert its protective effect through various sites of action. The goal of the present study is to clarify the mechanism through which TLCK protects the GSH-depleted H9c2 cardiomyocytes against the H_2O_2-induced cell damages.

2. MATERIALS AND METHODS

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-buthionine (S, R) sulfoximine (BSO), TLCK, *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), pifithrin-α, and catalase were obtained from Sigma Chemical Co., St. Louis, MO. SN50 and MG-132 were obtained from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA. Phenylmethanesulfonyl fluoride (PMSF) and H_2O_2 were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. 4-(2-aminoethyl)-benzensulfonyl fluoride hydrochloride (AEBSF) was obtained from Roche Diagnostics GmbH, Mannheim, Germany. Leupeptin was obtained from Peptide Institute, Inc., Osaka, Japan. 2',7'-dichlorofluorescin diacetate ($DCFH_2$-DA) was purchased from CALBIOCHEM, San Diego, CA. The 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay kit was obtained from Promega Co., Madison, WI. All the other chemicals used in this study were of the highest grade available from commercial suppliers.

2.2. Culture and Treatment of Cells

Rat cardiomyocyte H9c2 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in DMEM containing 10% (v/v) heat- inactivated FBS. The cells were incubated in an atmosphere of 5% CO_2 and 95% air at 37°C. The details of the standard treatment of H9c2 cells are as follows: the cells were seeded onto 96-well plates at a concentration of 1 x 10^4 cells/well for performing cell survival assays. After overnight preincubation with 0.1 mM BSO, the cells were incubated with different concentrations of H_2O_2 for various time intervals. TLCK and TPCK were usually added to the cell suspension concurrently with BSO unless otherwise noted, and the other inhibitors were added 30 min (leupeptin, PMSF, SN50) [15] or 1 h (AEBSF, MG-132, pifithrin-α) prior to the addition of H_2O_2 [16-18]. The details regarding the intracellular GSH levels in H9c2 cells pretreated with or without BSO have been described in our previous report [4].

2.3. Measurement of Cytotoxicity

Cell damages were determined using an MTT assay according to the manufacturer's instructions. Briefly, after the incubation with H_2O_2, DMEM containing 10% FBS and a dye solution were added to each of the 96 wells and the assay plates were incubated for 4 h at 37°C. A solubilization/stop solution was added for overnight incubation. The absorbances at 540 nm and 690 nm were recorded using a microplate reader (Labsystems Multiskan Bichromatic, Helsinki, Finland). Other culture conditions were same as those mentioned earlier. Cell morphology was investigated using a phase contrast microscope (Nikon Diaphoto-TMD, Tokyo, Japan).

2.4. Measurement of Antioxidant Effect

Intracellular ROS levels were assessed using $DCFH_2$-DA [19,20]. Briefly, $DCFH_2$-DA was preincubated with cells in culture medium at a final concentration of 10 μM for 15 min. Following incubation with H_2O_2 for 15 min, the cells were trypsinized, collected and washed with PBS by centrifugation at 300 × g for 5 min. The cell pellets were resuspended with PBS (1 ml) and analyzed using a flow cytometer (Beckman Coulter, Fullerton, CA). DCF fluorescence was measured by fluorescence channel 1 of the flow cytometer. Antioxidant effect of TLCK on lipid peroxidation was examined in rat liver microsomes incubated with ascorbic acid and $FeCl_3$ [21]. Briefly, microsomes (0.5 mg protein/mL) prepared from male Wistar strain rats were incubated with ascorbic acid (0.5 mM) and $FeCl_3$ (10 μM) in 50 mM phosphate buffer, pH 7.4 at 37°C for 1 h. The production of lipid peroxidation was assessed using thiobarbituric acid reactive substance (TBARS). TLCK and TPCK were added to reaction system at the beginning of incubation. The TBARS

values represented in the data was corrected by subtracting the value obtained in the reaction system without ascorbic acid and $FeCl_3$ from total value.

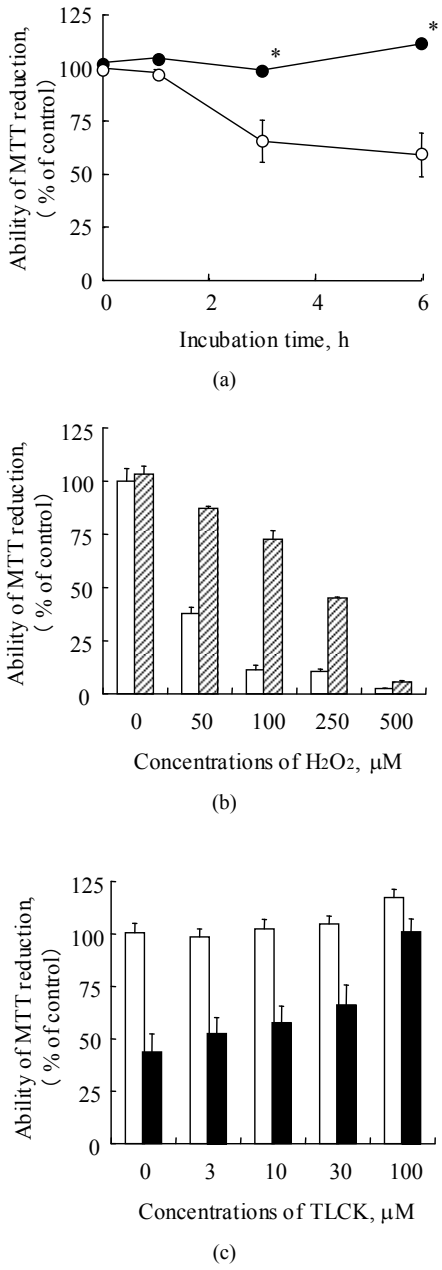
2.5. Data Analysis

The data are expressed as mean ± SD of at least three experiments. Student's *t*-test was used to analyze statistical difference. *P* values less than 0.05 were considered significant.

3. RESULTS

3.1. Protective Effects of TLCK against Cell Oxidative Damages by H_2O_2

We had previously reported that H_2O_2 induces hypertrophy in H9c2 cardiomyocytes and cell death in GSH-depleted H9c2 cells. An exposure to 100 μM H_2O_2 for more than 3 h induced an increase in the damages of the GSH-depleted H9c2 cells (**Figure 1(a)**), as assessed by the function of MTT reduction. The damages were inhibited by the addition of antioxidants such as Trolox and butylated hydroxyanisol (data not shown). H_2O_2 (100 μM) alone did not induce the damages in the cells pretreated without BSO. These results suggest that H_2O_2 induces the oxidative damages in the GSH-depleted H9c2 cells. Pretreatment with TLCK significantly inhibited the cell damages induced by H_2O_2 up to 6 h of test (**Figure 1(a)**). Although data not shown, the loss of MTT reductive activity was observed in the cells treated with H_2O_2 for 24 h in the presence of TLCK, suggesting that TLCK prevented oxidative damages 6 h but dit not the cell death 24 h after the addition of H_2O_2. Therefore, we selected 6 h as the duration of H_2O_2 treatment for the GSH-depleted cells in further experiments unless otherwise stated. Regarding the dose-related cytotoxic effect of H_2O_2, an increase in the concentration of H_2O_2 resulted in an increase in the damages of the cells; however, TLCK significantly prevented H_2O_2-induced cell damages caused by ~250 μM H_2O_2 (**Figure 1(b)**). TLCK at a concentration of more than 30 μM, particularly a concentration of 100 μM, significantly prevented the cell damages induced by H_2O_2 (**Figure 1(c)**). On the other hand, TLCK at a concentration of 300 μM exerted a deleterious effect in a preliminary experiment (data not shown). Therefore, we selected 100 μM TLCK as the dose in further experiments. An analysis using a phase contrast microscope was performed to observe the effect of TLCK on morphological changes in the H_2O_2-treated cells. Exposure to 100 μM H_2O_2 for 6 h resulted in a globular shape of the cells and detachment of the cells from the well in the GSH-depleted H9c2 cells (**Figure 2(b)**) in comparison with the H_2O_2-unexposed control

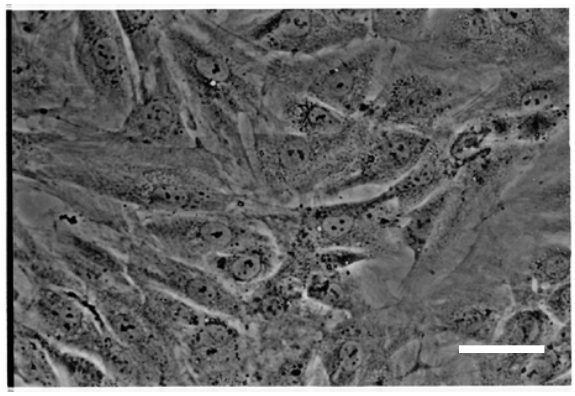


(a) GSH-depleted H9c2 cells pretreated with (solid circles) or without (open circles) TLCK were incubated with 100 μM H_2O_2. *$p < 0.01$ vs. corresponding TLCK-untreated cells. (b) GSH-depleted H9c2 cells pretreated with (shaded bars) or without (open bars) TLCK were incubated with various concentrations of H_2O_2 for 6 h. (c) GSH-depleted H9c2 cells pretreated with various concentrations of TLCK were incubated with (solid bars) or without (open bars) 100 μM H_2O_2 for 6 h.

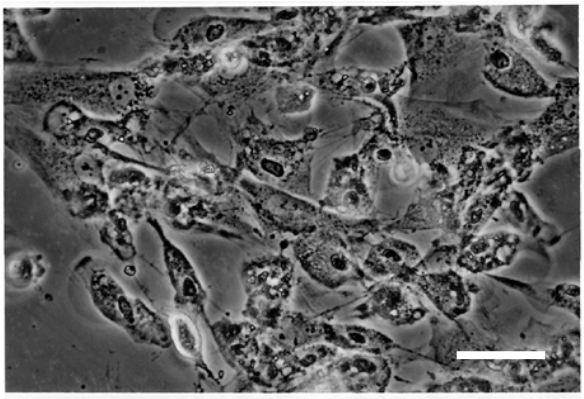
Figure 1. Effects of TLCK on the cytotoxicity of GSH-depleted H9c2 cells exposed to H_2O_2.

cells (**Figure 2(a)**). Moreover, the cells remained attached had shrunken nuclei and cytoplasm (**Figure 2(b)**). When the cells were pretreated with TLCK, none of these morphological changes was observed in the cells

even after incubation with H_2O_2 for 6 h (**Figure 2(c)**), however, marked decrease of cell numbers was observed for 24 h. These results show that TLCK protects the GSH-depleted H9c2 cells against the H_2O_2-induced cell oxidative damages and morphological changes, but not the cell death.



(a)



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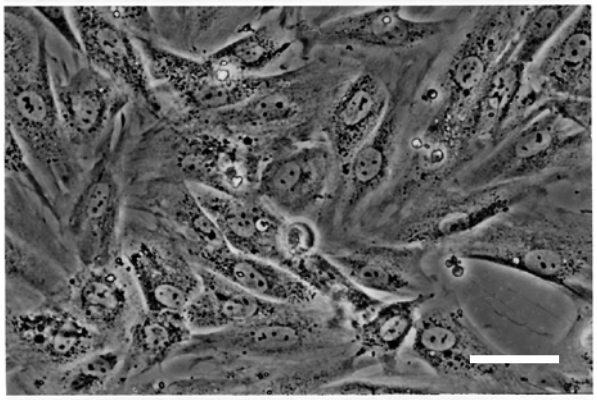


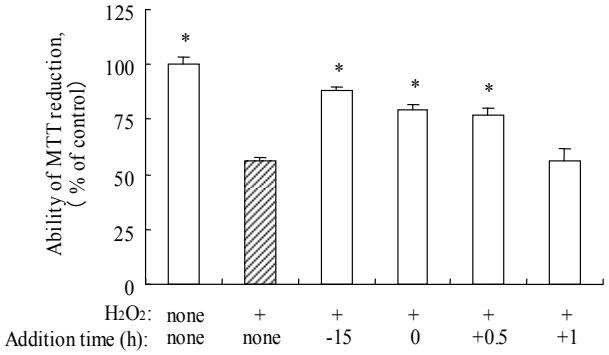
(c)

H9c2 cells pretreated with BSO (a, b) or BSO and TLCK (c) were incubated without (a) or with (b, c) 100 μM H_2O_2 for 6 h. The scale bar represents 50 μm.

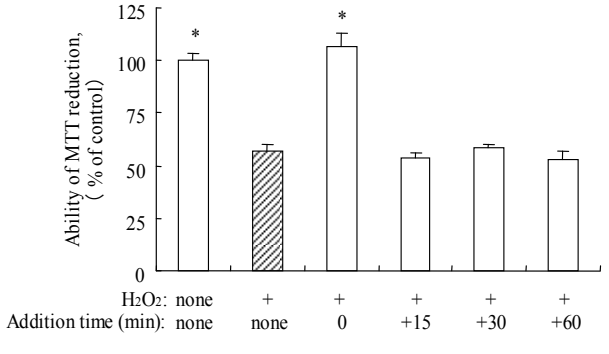
Figure 2. TLCK prevents H_2O_2-induced morphological changes in GSH-depleted H9c2 cells.

3.2. Effect of Addition Time of TLCK and Catalase on the Cell Damages Induced by H_2O_2

TLCK was added to the GSH-depleted H9c2 cells at different time intervals before or after the addition of H_2O_2. TLCK could prevent the H_2O_2-induced cell damages when it was added to the cells at the same time as H_2O_2, 15 h before the addition of H_2O_2, or 0.5 h after the addition of H_2O_2. However, it could not prevent the cell damages when added 1 h after the addition of H_2O_2 (**Figure 3(a)**). On the other hand, catalase completely prevented the cell damages when it was added at the same time as H_2O_2. However, it did not prevent cell damages when it was added 15 min after addition of H_2O_2 (**Figure 3(b)**). These results suggest that H_2O_2 induces the cell damages within 15 min after its addition and that TLCK may prevent the H_2O_2-induced cell damages by interfering with some steps in the signaling pathway that results in H_2O_2-induced cell damages, but not by quenching H_2O_2.



(a)



(b)

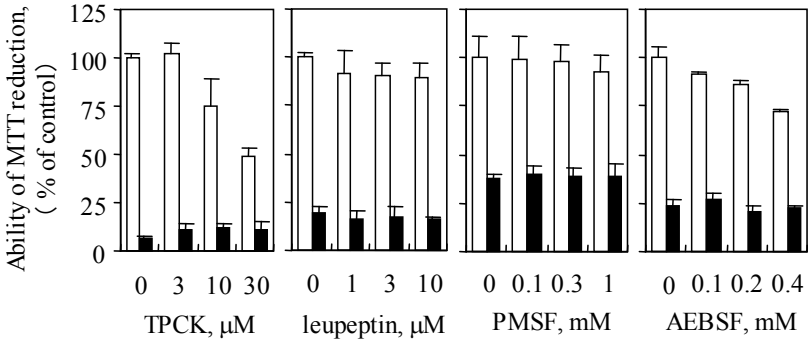
GSH-depleted H9c2 cells were treated with 100 μM TLCK (a) or 100 units of catalase (b) at different time intervals before or after the addition of 100 μM H_2O_2. The cytotoxicity was measured 6 h after the addition of H_2O_2. *, $p < 0.05$ vs. H_2O_2 alone (shaded bars).

Figure 3. Effects of addition time of TLCK and catalase on the cytotoxicity of GSH-depleted H9c2 cells exposed to H_2O_2.

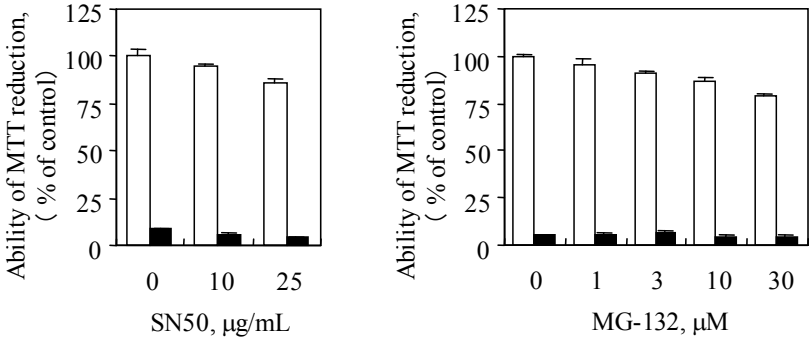
3.3. Effects of Various Inhibitors on H_2O_2-Induced Cell Damages

To clarify the mechanism through which TLCK protects the GSH-depleted H9c2 cells against the H_2O_2-induced cell damages, we first tested whether inhibitors of serine protease other than TLCK could prevent the cell damages as with TLCK. The inhibitors tested in the present study included TPCK, leupeptin, PMSF, and AEBSF; and their test concentrations are shown in the **Figure 4(a)**. None of these four inhibitors could protect the cells against the H_2O_2-induced cell damages; in fact, higher concentrations of TPCK and AEBSF exerted by themselves a deleterious effect on the ability of the cells for MTT reduction (**Figure 4(a)**). These results suggest that the protection of cells by TLCK against the H_2O_2-induced cell damages could not be due to its inhibitory effect on serine protease.

TLCK blocks the activation of NF-κB by inhibiting



(a)



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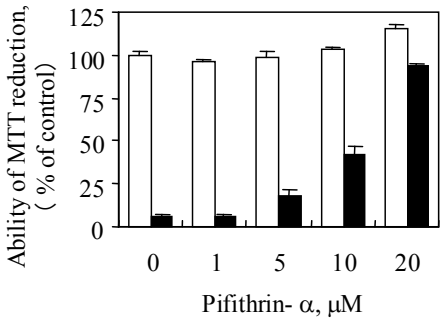


(c)

GSH-depleted H9c2 cells were pretreated with inhibitors of serine protease (a), NF-κB (b), or p53 (c) before the addition of H_2O_2. Then, the cells were incubated with (solid bars) or without (open bars) 100 μM H_2O_2 for 6 h.

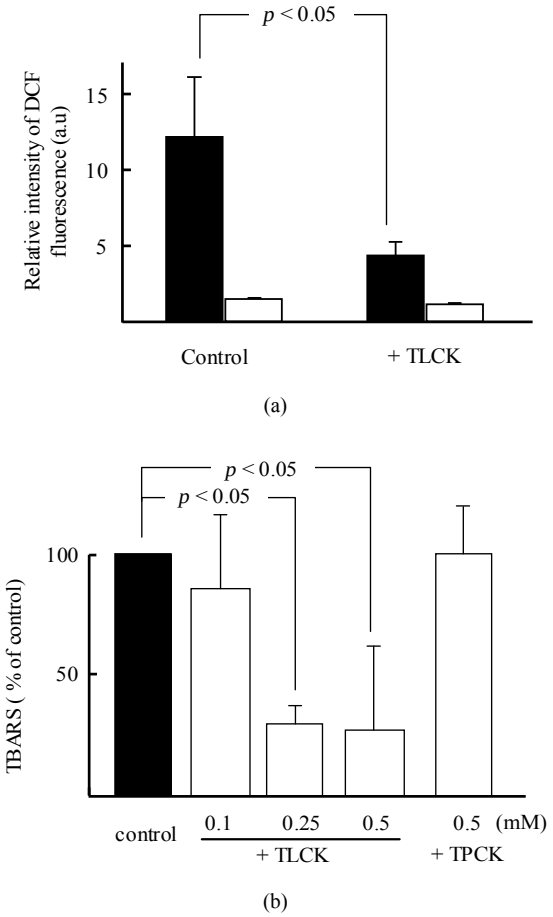
Figure 4. Effects of various inhibitors on H_2O_2-induced cytotoxicity.

the proteolytic cleavage of IκB, a cytosolic inhibitor of NF-κB. Accordingly, we examined the effects of SN50, an inhibitor of NF-κB translocation into the nucleus, and MG-132, a proteasome inhibitor that inhibits NF-κB activation by preventing IκB degradation, on the H_2O_2-induced cell damages. Neither SN50 nor MG-132 could protect the cells against the H_2O_2-induced cell damages; however, at higher concentrations, both the inhibitors had a little tendency to increase the damages of the control cells (**Figure 4(b)**). These results indicate that the protection of cells by TLCK against the H_2O_2-induced cell damages may not be mediated by the inhibition of NF-κB activation.

TLCK prevents neuronal cell death, which is induced by UV irradiation or by treatment with camptothecin, through the inhibition of the induction and nuclear accumulation of p53 [14]. H_2O_2 induces phosphorylation of p53, leading to accumulation of p53 and transcription of its target gene $p21^{Cip1/WAF1}$ in endothelial cells [22]. If p53 participates in the H_2O_2-induced cell damages in our model, suppression of p53 should prevent the cell damages. Therefore, we examined the effect of pifithrin-α, an inhibitor of p53 [18], on the H_2O_2-induced cell damages. **Figure 4(c)** shows that pifithrin-α at a concentration of more than 5 μM protected the GSH-depleted H9c2 cells against H_2O_2-induced cell damages in a concentration-dependent manner, and 20 μM pifithrin-α almost completely blocked the cell damages. Pifithrin-α itself did not exert any deleterious effects on the ability of the cells for MTT reduction in the range of concentrations that were tested.

3.4. Antioxidant Effects of TLCK

To evaluate the effect of TLCK on oxidative stress in the GSH-depleted H9c2 cells after the addition of H_2O_2, the generation of intracellular ROS was measured using a fluorescent probe $DCFH_2$-DA. $DCFH_2$ reacts with ROS to generate a highly fluorescent derivative DCF [23]. As shown in **Figure 5(a)**, an increase in the intensity of DCF fluorescence was observed after the addition of H_2O_2 in the GSH-depleted H9c2 cells, while TLCK significantly inhibited it after addition of H_2O_2. In the cells preincubated with BSO, TLCK does not affect the intensity of DCF fluorescence. TLCK did not induce the increase of GSH levels in the cells pretreated with BSO, or rather TLCK slightly decreased the level (16 ± 8.0%, $p > 0.05$). To characterize the antioxidative action of TLCK, we evaluated the effect of TLCK on lipid peroxidation of rat liver microsomes in the reaction system of ascorbic acid and $FeCl_3$. As shown in **Figure 5(b)**, TLCK significantly inhibited the production of TBARS in a dose-dependent manner. However, TPCK did not inhibit the



A, H9c2 cells were pretreated with BSO or BSO and TLCK overnight. Then, the cells were incubated with (solid bars) or without (open bars) 100 μM H_2O_2. B, Antioxidant effect of TLCK on lipid peroxidation.

Figure 5. TLCK inhibits generation of ROS in GSH-depleted H9c2 cells.

increase in production of TBARS, suggesting that TLCK may have some antioxidant action in the cells.

4. DISCUSSION

In our experiments, TLCK, which is known as an inhibitor of serine proteases, significantly protected GSH-depleted H9c2 cardiomyocytes from oxidative damages (**Figure 1**) and morphological changes (**Figure 2**) induced by H_2O_2 up to 6 h of test, but did not from cell death for 24 h, suggesting that TLCK prevented the oxidative damages and delayed the induction of cell death. The other serine protease inhibitors, *i.e.*, TPCK, leupeptin, PMSF, and AEBSF, did not exert any protective effect on the damages (**Figure 4(a)**); however, pharmacological inhibitors of serine proteases have been shown to prevent cell death under several other conditions [14]. These results suggest that serine proteases may not be involved in H_2O_2-induced cytotoxicity of GSH-depleted cardiomyo-

cytes. Therefore, we also investigated the other effects of TLCK on GSH-depleted H9c2 cardiomyocytes apart from the effect of serine protease inhibition.

The present study showed that pifithrin-α, an inhibitor of p53 function [18], significantly prevented the H_2O_2-induced cell damages (**Figure 4(c)**). The transcription factor p53, originally identified as a tumor suppressor protein, is activated in response to DNA damage, resulting in the induction of the transcription of the regulatory genes involved in cell cycle and DNA repair [24]. When DNA damage is serious and irreparable, p53 causes the cell death by inducing an apoptosis program in order to prevent formation of neoplasia in mammals [25]. It has also been demonstrated that oxidative stress is involved in the activation of p53 [26]. Gong *et al*. reported that TLCK prevented p53 induction and protected leukemic cells against ionizing radiation-induced cell death [13]. Rideout *et al*. also reported that TLCK prevented the induction and accumulation of p53 that was induced by DNA damage in cortical neurons, leading to the increase in the survival of the cells [14]. In the present study, pifithrin-α exerted a protective effect on the damages, suggesting the involvement of the p53-dependent pathway in the cytotoxicity. It has also been reported that TLCK prevents the activation of NF-κB through the inhibition of IκBα-protease [11,27]. We demonstrated that SN50, an inhibitor of translocation of NF-κB into nucleus, and MG-132, an inhibitor of proteasome that is responsible for the proteolysis of IκBα, did not protect the cells against the cytotoxicity. This result suggests that activation of NF-κB is not involved in the cell damage process.

Pretreatment with BSO presumably decreases the ability of cells to scavenge ROS, because BSO inhibits *de novo* synthesis of intracellular GSH [4,28]. The present data showed that an increase in the intensity of DCF fluorescence was observed after the addition of H_2O_2 on BSO-pretreated cells, suggesting that ROS are concerned with the cell damages. TLCK markedly inhibited the generation of ROS in the cells after addition of H_2O_2, and the production of TBARS in rat liver microsomes incubated with ascorbic acid and $FeCl_3$. However, the levels of GSH in the cells pretreated with BSO were not significantly affected by the addition of TLCK. These results suggest that TLCK have the ability to scavenge ROS.

We had previously demonstrated that H_2O_2 induces the hypertrophy in the normal (BSO-nontreated) cells and cell death in the GSH-depleted cells, suggesting that the intracellular free thiol levels determine the signal pathway resulting in the cell death or hypertrophy in H_2O_2-treated cardiomyocytes [4]. We demonstrated that TLCK did not induce the increase of GSH levels in H9c2 cells pretreated with BSO. These results suggest that neither the increase of GSH levels in the cells nor the interfering with the depletion of GSH by the pretreatment with BSO are involved in the protective action of TLCK on the oxidative damages. In addition, TLCK significantly inhibited the microsomal lipid peroxidation in the reaction system of ascorbic acid with $FeCl_3$. We guess that rich electrons on 3- and 5-position of benzene ring and/or the primary amino group can participate in the protective action of TLCK against the production of TBARS and the oxidative damage(s) in the cells.

The H_2O_2-induced cell damages were abolished when catalase was added along with H_2O_2 (**Figure 3(b)**), indicating that the cell damages are caused by the oxidative damages initiated by H_2O_2. After its addition, H_2O_2 initiates the process of the cell damages within 15 min, because no cytoprotection was observed when catalase was added 15 min after the addition of H_2O_2 (**Figure 3(b)**). In contrast, TLCK prevented the cell damages even when it was added 30 min after the addition of H_2O_2; however, it did not prevent cell damages when added 1 h after the addition of H_2O_2 (**Figure 3(a)**). These results suggest a possibility that TLCK also block the some signaling pathway involving p53, that is activated within 1 h after stimulation. Based on these results, we inferred that TLCK abrogated the H_2O_2-induced cell damages via the antioxidant effect accompanied by the inhibitory effect on p53 function under defined experimental conditions. Further investigations are required to clear the target molecules and the protective mechanism of TLCK in cardiomyocyte.

5. ACKNOWLEDGEMENTS

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REFERENCES

[1] Takano, H., Zou, Y., Hasegawa, H., Akazawa, H., Nagai, T. and Komuro, I. (2003) Oxidative stress-induced signal transduction pathways in cardiac myocytes: Involvement of ROS in heart diseases. *Antioxidants & Redox Signaling*, **5**, 789-794.

[2] Trachootham, D., Lu, W., Ogasawara, M.A., Nilsa, R.D. and Huang, P. (2008) Redox regulation of cell survival. *Antioxidants & Redox Signaling*, **10**, 1343-1374.

[3] Chen, Q.M., Tu, V.C., Wu, Y. and Bahl, J.J. (2000) Hydrogen peroxide dose dependent induction of cell death or hypertrophy in cardiomyocytes. *Archives of Biochemistry and Biophysics*, **373**, 242-248.

[4] Tanaka, H., Sakurai, K., Takahashi, K. and Fujimoto, Y. (2003) Requirement of intracellular free thiols for hydrogen peroxide-induced hypertrophy in cardiomyocytes. *Journal of Cellular Biochemistry*, **89**, 944-955.

[5] Arrigo, A.P. (1999) Gene expression and the thiol redox state. *Free Radical Biology & Medicine*, **27**, 936-944.

[6] Carmel-Harel, O. and Storz, G. (2000) Roles of the glu-

tathione- and thioredoxin-dependent reduction systems in the Escherichia coli and Saccharomyces cerevisiae responses to oxidative stress. *Annual Review of Microbiology*, **54**, 439-461.

[7] Filomeni, G., Rotilio, G. and Ciriolo, M.R. (2002) Cell signaling and the glutathione redox system. *Biochemical Pharmacology*, **64**, 1057-1064.

[8] Shukla, G.S., Shukla, A., Potts, R.J., Osier, M., Hart, B.A. and Chiu, J.F. (2000) Cadmium-mediated oxidative stress in alveolar epithelial cells induces the expression of gamma-glutamylcysteine synthetase catalytic subunit and glutathione S-transferase alpha and pi isoforms: potential role of activator protein-1. *Cell Biology and Toxicology*, **16**, 347-362.

[9] Solary, E., Eymin, B., Droin, N. and Haugg, M. (1998) Proteases, proteolysis, and apoptosis. *Cell Biology and Toxicology*, **14**, 121-132.

[10] Stenson-Cox, C., FitzGerald, U. and Samali, A. (2003) In the cut and thrust of apoptosis, serine proteases come of age. *Biochemical Pharmacology*, **66**, 1469-1474.

[11] Kim, H., Lee, H.S., Chang, K.T., Ko, T.H., Beak, K.J. and Kwon, N.S. (1995) Chloromethyl ketones block induction of nitric oxide synthase in murine macrophages by preventing activation of nuclear factor-kappa B. *The Journal of Immunology*, **154**, 4741-4748.

[12] Abate, A. and Schröder, H. (1998) Protease inhibitors protect macrophages from lipopolysaccharide-induced cytotoxicity: Possible role for NF-κB. *Life Science*, **62**, 1081-1088.

[13] Gong, B., Chen, Q., Endlich, B., Mazumder, S. and Almasan, A. (1999) Ionizing radiation-induced, Bax-mediated cell death is dependent on activation of cysteine and serine proteases. *Cell Growth & Differentiation*, **10**, 491-502.

[14] Rideout, H.J., Zang, E., Yeasmin, M., Gordon, R., Jabado, O., Park, D.S. and Stefanis, L. (2001) Inhibitors of trypsin- like serine proteases prevent DNA damage-induced neuronal death by acting upstream of the mitochondrial checkpoint and of p53 induction. *Neuroscience*, **107**, 339-352.

[15] Adams, J. and Stein, R. (1996) Novel inhibitors of the proteasome and their therapeutic use in inflammation. *Annual Reports in Medicinal Chemistry*, **31**, 279-288.

[16] Grisham, M.B., Palombella, V.J., Elliott, P.J., Conner, E.M., Brand, S., Wong, H.L., *et al.* (1999) Inhibition of NF-κB activation *in vitro* and *in vivo*: Role of 26S proteasome. *Methods in Enzymology*, **300**, 345-363.

[17] Chen, K., Albano, A., Ho, A. and Keaney, J.F.Jr. (2003) Activation of p53 by oxidative stress involves platelet-derived growth factor-β receptor-mediated ataxia telangiectasia mutated (ATM) kinase activation. *The Journal of Biological Chemistry*, **278**, 39527-39533.

[18] Setsukinai, K., Urano, Y., Kakinuma, K., Majima, H.J. and Nagano, T. (2003) Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *The Journal of Biological Chemistry*, **278**, 3170-3175.

[19] Gao, D., Sakurai, K., Katoh, M., Chen, J. and Ogiso, T. (1996) Inhibition of microsomal lipid peroxidation by baicalein: A possible formation of an iron-baicalein complex. *Biochemistry and Molecular Biology International*, **39**, 215-225.

[20] Myhre, O., Andersen, J.M., Aarnes, H. and Fonnum, F. (2003) Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. *Biochemical Pharmacology*, **65**, 1575-1582.

[21] Stone, J.R. and Yang, S. (2006) Hydrogen peroxide: A signal messenger. *Antioxidants & Redox Signaling*, **8**, 243-270.

[22] Lin, Y.Z., Yao, S.Y., Veach, R.A., Torgerson, T.R. and Hawiger, J. (1995) Inhibition of nuclear translocation of transcription factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *The Journal of Biological Chemistry*, **270**, 14255-14258.

[23] Komarov, P.G., Komarova, E.A., Kondratov, R.V., Christov-Tselkov, K., Coon, J.S., Chernov, M.V., *et al.* (1999) A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science*, **285**, 1733-1737.

[24] Burns, T.F. and El-Deiry, W.S. (1999) The p53 pathway and apoptosis. *Journal of Cellular Physiology*, **181**, 231-239.

[25] Chen, S.J., Wang, J.L., Chen, J.H. and Huang, R.N. (2002) Possible involvement of glutathione and p53 in trichloroethylene- and perchloroethylene-induced lipid peroxidation and apoptosis in human lung cancer cells. *Free Radical Biology & Medicine*, **33**, 464-472.

[26] Renzing, J., Hansen, S. and Lane, D.P. (1996) Oxidative stress is involved in the UV activation of p53. *Journal of Cell Science*, **109**, 1105-1112.

[27] Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Neriah, Y. and Baeuerle, P.A. (1993) Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. *Nature*, **365**, 182-185.

[28] Bai, J. and Cederbaum, A.I. (2006) Overexpression of CYP2E1 in mitochondria sensitizes HepG2 cells to the toxicity caused by depletion of glutathione. *The Journal of Biological Chemistry*, **281**, 5128-5136.

