Effects of Choto-san and Its Related Constituents on Endogenous Antioxidant Systems

Pramote MAHAKUNAKORN, Michihisa TOHDA,†,‡ Yukihisa MURAKAMI, Hiroshi WATANABE, and Kinzo MATSUMOTO∗,†

∗Division of Medicinal Pharmacology, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University; and †21st Century COE Program, Toyama Medical and Pharmaceutical University; Toyama 930–0194, Japan.

Received June 7, 2004; accepted October 26, 2004; published online October 29, 2004

We previously reported that Choto-san acts as an antioxidant and cytoprotective agents against H2O2-induced oxidative damage in NG108-15 cells, and the effect is due at least partly to the phenolic compounds. To further investigate the detail mechanisms of this cytoprotection effects of Choto-san and related compounds on enzyme activities of antioxidant systems were examined. Choto-san (5—100 μg/ml) and Chotoko (5—100 mg/ml) stimulated the activity of superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX). These also increased the level of glutathione. Although Choto-san without Chotoko (w/o CKO) did not show the effects on SOD and catalase, GPX activity and glutathion content also, but weakly, stimulated by w/o CKO. The effects of phenolic compounds, epicatechin, caffeic acid and quercetin were also investigated. Epicatechin stimulated catalase, GPX and glutathione content, but not SOD. On the other hand, caffeic acid stimulated SOD activity but had no effects on others. Quercetin stimulated all, although intensities were different among. These results suggest that simultaneous induction of cellular antioxidant defense systems by Choto-san and its related constituents may be an important mechanisms underlying the protective effects of Choto-san on ischemia-induced neuronal cells injury, and the characteristics of the stimulative effects of phenolic compounds were depend on enzymes.

Key words Choto-san; superoxide dismutase; catalase; glutathione peroxidase; glutathione; NG108-15 cell

Reactive oxygen species (ROS) have been implicated in neurotoxicity following cerebral ischemia-reperfusion. Accumulation of ROS, particularly superoxide anion (O2•−) and hydrogen peroxide (H2O2) causes damage to membrane phospholipids, proteins and DNA.1) Under normal circumstances, these oxidant species can be effectively scavenged by antioxidant enzymes, namely superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). In mammalian SOD is able to convert O2•− to H2O2.2) The H2O2 formed is subsequently removed by the cytosolic catalase and GPX to form water and molecular oxygen.3) However, the sudden burst of ROS, for example, during cerebral ischemia-reperfusion can overwhelm the antioxidant defense, resulting in lipid peroxidation and hence cerebral infarction. In this regard, transgenic mice over-expressing SOD, catalase, or GPX showed significant reduction of infarct volume and edema formation.4,5) Intravenous administration of polyethylene-conjugated SOD or GPX could also reduce the size of brain infarction.5) In contrast, after transient focal cerebral ischemia, knockout mice without SOD, catalase, or GPX expression showed an increased extent of lipid peroxidation, leading to an exacerbated infarction.6,5)

In our previous report,7) the antioxidant activities of Choto-san and its related constituents were investigated employing various established in vitro systems, such as a protective effect on H2O2-induced oxidative damage in NG108-15 cells, lipid peroxidation in mouse brain homogenate induced by Fe2+/ascorbate system, DPPH, superoxide, nitric oxide and hydroxyl radical scavenging, reducing power, and iron chelation. Choto-san and its related constituents significantly protected NG108-15 cells from injury induced by H2O2 exposure in vitro and also inhibited lipid peroxidation in the brain homogenate. Furthermore, they exhibited a strong concentration-dependent scavenging effect on DPPH radical, superoxide, nitric oxide and hydroxyl radical. The multiple antioxidant activity of Choto-san and its related constituents was evident as it showed strong reducing power, and also ferrous ion chelating potency. The data obtained in the in vitro models clearly establish the antioxidant activity of Choto-san. These in vitro results suggest the possibility that Choto-san could be effectively employed as an antioxidant, to alleviate oxidative stress.

Activation of the function of endogenous antioxidant systems in tissue is the characteristic of antioxidant compounds. Many plant extracts or antioxidants have been reported to exert an indirect scavenging effect on free radical-induced tissue injury by activating the function of endogenous antioxidant systems.8) Thus it is likely that the antioxidant effect of Choto-san is due to not only free radical-scavenging activity but also to the modulation of endogenous antioxidant defense system. Therefore, we investigated the effect of Choto-san and its related constituents on the activities of the antioxidant enzymes such as SOD, catalase and GPX as well as the level of intracellular glutathione in vitro using NG108-15 cell cultures system.

MATERIALS AND METHODS

Materials The following reagents were purchased from the indicated sources. Medicinal herbs comprising Choto-san from Tochimoto (Osaka, Japan) and the extract were prepared as described previously. Aminopterin, epicatechin, caffeic acid, resveratrol, catalase, GPX and Bradford’s reagent were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); quercetin, and EDTA from Nacalai Tesque, Inc. (Kyoto, Japan); FK506 from Fujisawa (Osaka, Japan); glutathione, 5,5T-dithio-bis(2-nitrobenzoic acid), NADPH, glutathione reductase, phenazine methosulfate (PMS), SOD (Cat. No. 196-08773), nitroblue tetrazolium (NBT), NADH, from Wako Pure Chemical (Osaka, Japan); H2O2 (30% w/v) from Santoku Chemical In-
Glutathione Determination Total cellular glutathione was quantified using the glutathione reductase-DTNB recycling assay, modified for microtiter plates. NG108-15 cells were treated with various concentrations of test compounds. After 24 h of incubation, cultures were washed with cold PBS, and cells were then lysed in 2 ml of 0.1% Triton X-100 in phosphate buffered (143 mM NaH₂PO₄, 6.3 mM EDTA, pH 7.4). After protein precipitation with 2.5% (w/v) 5-sulfosalicylic acid and centrifugation (10000 rpm, 15 min, 4°C), the supernatant was collected. To each well of a 96-well plate was then added: 10 µl samples or GSH standards and 200 µl of a solution of 1 mM 5,5‘-dithio-bis(2-nitrobenzoic acid) (DTNB), 0.3 mM NADPH in phosphate buffer. The reaction was started by quickly adding glutathione reductase to give a final concentration of 1 U/ml. Absorbance was read at 30 s intervals for 10 min at 405 nm with a microplate reader, and compared with that of a glutathione standard curve.

Statistical Analysis All results are expressed as mean±S.D. The data for biochemical parameters such as SOD, catalase, GPX, and glutathione were analyzed statistically using one-way ANOVA, followed by Dunnnett’s test for comparison of treated groups with control groups. Differences of p<0.05 were considered statistically significant.

RESULTS

Assay for Antioxidant Enzymes Activity In order to investigate whether the antioxidant activities of Choto-san and its related constituents are mediated by an increase in antioxidant enzymes, we measured SOD, catalase and GPX activities in NG108-15 cells treated with extracts of Choto-san, Chotoko, and Choto-san without Chotoko as well as its phenolic constituents; epicatechin, caffeic acid, and quercetin. In addition, we compared these results with those of a reference drug, resveratrol.

Effects of Choto-san and Its Related Constituents on Catalase Activity in NG108-15 Cells Treatment of NG108-15 cells with a reference drug, resveratrol (20—100 µM) for 3 h resulted in a significant increased of SOD activity compared to that of the untreated control cells. The SOD activity in NG108-15 cells also significantly increased from that of control after treated with Choto-san (5—100 µg/ml), Chotoko, and Choto-san without Chotoko as well as its phenolic constituents; epicatechin, caffeic acid, and quercetin. In addition, we compared these results with those of a reference drug, resveratrol.

Assays for Antioxidant Enzymes NG108-15 cells were treated with 5, 20 and 100 µg/ml of Choto-san, Chotoko, and Choto-san without Choto extracts or 5, 20 and 100 µM of epicatechin, caffeic acid, and quercetin for 3 h, and then homogenized in a 0.1% Triton X-100 in 50 mM Tris–HCl buffer (pH 7.6). Cells homogenate was centrifuged at 10000 rpm for 15 min. The supernatant was removed for the enzyme assays. Results are expressed as relative enzyme activity per mg of protein compared with the corresponding control cultures.

Assays for SOD Activity SOD activity was assayed based on inhibition of the formation of NBT formazan. NBT is reduced by superoxide anion to form a blue formazan which has a strong absorbance at 540 nm and the formation of blue formazan is inhibited in the presence of SOD. The SOD activity was determined at room temperature according to the method of Bartosz et al. The assay mixture contained 1.2 ml sodium phosphate buffer (25 mM, pH 8.3), 0.1 ml PMS (186 µM), 0.3 ml NBT (300 µM), 0.2 ml NADH (780 µM) and 0.1 ml of cell homogenate. A system devoid of enzyme served as control. Samples were incubated for 5 min at room temperature and the absorbance of formazan at 540 nm was then measured using the microplate reader. The enzyme activity was calculated according to the SOD standard curve. Protein content in sample was determined by Bradford method.

Assays for Catalase Activity Catalase activity was measured by the modification of the method of Carrillo et al. The assay was carried out in reaction mixture contained 12 ml of 30 % (v/v) H₂O₂ and 100 µl of cell homogenate in 50 mM phosphate buffer (pH 7.0) to a final volume of 1.0 ml. Samples were incubated for 2 min at 37°C and the absorbance of the samples were monitored for 3 min at 240 nm. Changes in absorbance were taken to be proportional to the breakdown of H₂O₂. The enzyme activity was calculated according to the catalase standard curve.

Assays for GPX Activity GPX activity was determined at room temperature by a slight modification of a method of Paglia and Valentine. The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 1 mM EDTA, 10 mM glutathione, 1 mM NaNO₃, 1 unit of glutathione reductase, 1.5 mM NADPH and 0.1 ml of cell homogenate. After incubating for 10 min at 37°C, H₂O₂ was added to the reaction mixture to a final concentration of 1 mM. GPX activity was measured at 340 nm for 3 min as the rate of NADPH oxidation. The enzyme activity was calculated according to the GPX standard curve.

dustries Co. (Tokyo, Japan); thymidine and hypoxanthine from Kohjin Co. (Tokyo, Japan); minomycin from Lederle (Japan); Dulbecco’s modified Eagle’s medium (DMEM) from Gibco BRL, Life Technologies, Inc. (New York, U.S.A.); and fetal bovine serum (FBS) from Canasera International, Inc. (Canada). All other chemicals were of the highest analytical grade and purchased from common sources.

Cell Cultures NG108-15 cells were continuously cultured using the method described previously, with minor modifications. Briefly, the cells were cultured in DMEM supplemented with 4% FBS, hypoxanthine (100 µM), thymidine (16 µM), aminopterin (1 µM), and minomycin (1 µg/ml). The culture medium was changed every 2—3 d. All cultures were maintained at 37°C under 10% CO₂. For experiments, cells were plated onto 3.5-cm polyomithine (polymerization 50) coated plates and used after 3—4 d of incubation.

Assays for Antioxidant Enzymes NG108-15 cells were treated with 5, 20 and 100 µg/ml of Choto-san, Chotoko, and Choto-san without Choto extracts or 5, 20 and 100 µM of epicatechin, caffeic acid, and quercetin for 3 h, and then homogenized in a 0.1% Triton X-100 in 50 mM Tris–HCl buffer (pH 7.6). Cells homogenate was centrifuged at 10000 rpm for 15 min. The supernatant was removed for the enzyme assays. Results are expressed as relative enzyme activity per mg of protein compared with the corresponding control cultures.

Assays for SOD Activity SOD activity was assayed based on inhibition of the formation of NBT formazan. NBT is reduced by superoxide anion to form a blue formazan which has a strong absorbance at 540 nm and the formation of blue formazan is inhibited in the presence of SOD. The SOD activity was determined at room temperature according to the method of Bartosz et al. The assay mixture contained 1.2 ml sodium phosphate buffer (25 mM, pH 8.3), 0.1 ml PMS (186 µM), 0.3 ml NBT (300 µM), 0.2 ml NADH (780 µM) and 0.1 ml of cell homogenate. A system devoid of enzyme served as control. Samples were incubated for 5 min at room temperature and the absorbance of formazan at 540 nm was then measured using the microplate reader. The enzyme activity was calculated according to the SOD standard curve. Protein content in sample was determined by Bradford method.

Assays for Catalase Activity Catalase activity was measured by the modification of the method of Carrillo et al. The assay was carried out in reaction mixture contained 12 ml of 30 % (v/v) H₂O₂ and 100 µl of cell homogenate in 50 mM phosphate buffer (pH 7.0) to a final volume of 1.0 ml. Samples were incubated for 2 min at 37°C and the absorbance of the samples were monitored for 3 min at 240 nm. Changes in absorbance were taken to be proportional to the breakdown of H₂O₂. The enzyme activity was calculated according to the catalase standard curve.

Assays for GPX Activity GPX activity was determined at room temperature by a slight modification of a method of Paglia and Valentine. The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 1 mM EDTA, 10 mM glutathione, 1 mM NaNO₃, 1 unit of glutathione reductase, 1.5 mM NADPH and 0.1 ml of cell homogenate. After incubating for 10 min at 37°C, H₂O₂ was added to the reaction mixture to a final concentration of 1 mM. GPX activity was measured at 340 nm for 3 min as the rate of NADPH oxidation. The enzyme activity was calculated according to the GPX standard curve.

Statistical Analysis All results are expressed as mean±S.D. The data for biochemical parameters such as SOD, catalase, GPX, and glutathione were analyzed statistically using one-way ANOVA, followed by Dunnnett’s test for comparison of treated groups with control groups. Differences of p<0.05 were considered statistically significant.

RESULTS

Assay for Antioxidant Enzymes Activity In order to investigate whether the antioxidant activities of Choto-san and its related constituents are mediated by an increase in antioxidant enzymes, we measured SOD, catalase and GPX activities in NG108-15 cells treated with extracts of Choto-san, Chotoko, and Choto-san without Chotoko as well as its phenolic constituents; epicatechin, caffeic acid, and quercetin. In addition, we compared these results with those of a reference drug, resveratrol.

Effects of Choto-san and Its Related Constituents on Catalase Activity in NG108-15 Cells Treatment of NG108-15 cells with a reference drug, resveratrol (20—100 µM) for 3 h resulted in a significant increased of SOD activity compared to that of the untreated control cells. The SOD activity in NG108-15 cells also significantly increased from that of control after treated with Choto-san (5—100 µg/ml), Chotoko, and Choto-san without Chotoko as well as its phenolic constituents; epicatechin, caffeic acid, and quercetin. In addition, we compared these results with those of a reference drug, resveratrol.

Effects of Choto-san and Its Related Constituents on Catalase Activity in NG108-15 Cells Treatment of NG108-15 cells with a reference drug, resveratrol (100 µM) for 3 h resulted in a significant enhanced of catalase activity (Fig. 2). Consistent with the induction of SOD, the incubation of cells with Choto-san (5—100 µg/ml), Chotoko (5—100 µg/ml), and Choto-san without Chotoko (100 µg/ml) for 3 h also led to a concentration-dependent increase in CAT activity. Phenolic constituents of Chotoko; epicatechin (5—100 µM) and quercetin (100 µM) also led to the marked increased in the catalase activity. In contrast, no significant changed in the catalase activity was found in
NG108-15 cells treated with Choto-san without Chotoko (5—100 μg/ml)

**Effects of Choto-san and Its Related Constituents on GPX Activity in NG108-15 Cells** Treatment of NG108-15 cells with resveratrol (20—100 μM) for 3 h resulted in a significant activation of GPX activity. Incubation of NG108-15 cells with Choto-san (20—100 μg/ml), Chotoko (20—100 μg/ml), and Choto-san without Chotoko (20—100 μg/ml) resulted in a concentration-dependent induction of GPX activity. Notably, a significant increased in GPX activity was observed in the cells treated with epicatechin (20—100 μM) and quercetin (20—100 μM). However, in this study, treatment of the cells with caffeic acid (5—100 μM) for 3 h did not show any significant effect on GPX activity (Fig. 3).

**Effects of Choto-san and the Constituents on Glutathione Level in NG108-15 Cells** Treatment of NG108-15 cells with FK506 (20—500 nM), a reference drug, for 24 h resulted in a marked increased in glutathione level in a concentration-dependent manner (Fig. 4). Concentrations of glutathione in NG108-15 cells significantly increased from the basal level in that of control after Choto-san (20—100 μg/ml), Chotoko (20—100 μg/ml) and Choto-san without Chotoko (20—100 μg/ml) treatment for 24 h. Also in this test, a significant induction of intracellular glutathione was observed in NG108-15 cells treated with epicatechin and quercetin for 24 h. However, glutathione content was not change in NG108-15 cells treated with caffeic acid (5—100 μM) for 24 h.

**DISCUSSION**

ROS, including superoxide anion and H₂O₂, are generated constantly by a number of cellular sources. Because ROS are highly reactive to biomolecules, including lipid, protein, and nucleic acids, mammalian cells have evolved variety of antioxidant enzymes to counteract the ROS generated during normal cell metabolism and/or various pathophysiological processes. Major antioxidants and oxidative free radical scavenging enzymes are SOD, catalase and GPX. Among the cellular antioxidants, SOD, catalase, GPX, and glutathione have received extensive studies. SOD catalyzes the breakdown of
O2\textsuperscript{-} to H\textsubscript{2}O\textsubscript{2}. Because H\textsubscript{2}O\textsubscript{2} is still harmful to the cells, catalase and GPX further catalyze the decomposition of H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O and O\textsubscript{2}. In the reaction catalyzed by GPX, glutathione is oxidized to glutathione disulfide, which can then be reduced back to glutathione by glutathione reductase. Thus, the coordination actions of various cellular antioxidants in the cells are critical for effectively detoxified ROS. During oxidative stress such as in transient cerebral ischemia, sudden burst of ROS can not be handled efficiently by endogenous system which protects neurons in the normal conditions. As a result the highly reactive ROS damages the components (cellular membranes, proteins, and DNA) of neuron. Accumulation of hydrogen peroxide was reported to impair the mitochondrial function.\textsuperscript{13} Superoxide along with hydroxyl radical produces modification in the primary secondary and tertiary structure and aggregation and/or fragmentation of cellular proteins including SOD, catalase, and GPX.\textsuperscript{14,15} Dysfunction of SOD, catalase, and GPX may result in loss of protective activity exerted by these enzymes. There are no analogous enzymes to counter hydroxyl radicals. Instead, hydroxyl radicals management depends on GSH.\textsuperscript{16} which are the most abundant low molecular weight antioxidants. Glutathione is an important constituent of intracellular protective mechanisms against various noxious stimuli including oxidative stress. However, reduced glutathione as the main component of endogenous non-protein sulphhydryl pool is known to be a major low molecular weight scavenger of free radicals in the cytoplasm.\textsuperscript{17,18} Because of their exposed sulphhydryl groups, non-protein sulphhydryl bind a variety of electrophilic radicals and metabolites that may be damaging to cells.\textsuperscript{19} Recently, the antioxidant resveratrol has been reported to enhance the activity of antioxidant enzymes in a various cell culture systems.\textsuperscript{20} This evidence was supported by our present results that treatment of the cells with resveratrol also enhanced SOD, catalase and GPX activity in NG108-15 cells.

In the present study, I also observed that treating the NG108-15 cells with Choto-san and its related constituents significantly increased the activity of the antioxidant enzymes including SOD, catalase and GPX. In addition, they also increased the glutathione level in the cells in a concentration-dependent manner. The simultaneously increase of...
antioxidant enzyme activity and cellular glutathione content by Choto-san and its related constituents may contribute to the increase resistance of the cells to oxidative stress. It was found that the upregulation of enzyme activities started as early as 3 h after treatment of Choto-san and its related constituents. In this regard, the increased SOD activity may play a protective role in development of ischemic tolerance and the survival of neurons after ischemia.21,22) In the same way, the treatment with Choto-san and its related constituents associated with the increase in catalase and GPX activity was observed in the present study. The increase in catalase and GPX activity may reveal a compensatory response to the increase in H$_2$O$_2$ production arising from the SOD-catalyzed reaction.21 It has been reported that a rise in SOD activity, without a concomitant rise in the activity of catalase and/or GPX might be detrimental since SOD generates hydrogen peroxide as a metabolite, which is more cytotoxic than oxygen radicals and must be scavenged by catalase or GPX. Thus a simultaneous increase in catalase and/or GPX activity are essential for an overall beneficial effect of an increase in SOD activity. In this regard, Choto-san and its related constituents treatment likely provides an appropriate antioxidant balance by increasing all of SOD, catalase and GPX activities thereby reducing the extent of oxidative damage in ischemic-reperfusion brain tissues. In the glutathione assay, FK506, an immunosuppressive immunophilin ligand, was included as a reference compound. FK506 was previously reported to increase glutathione level in NG108-15 cells.23) In this study, the ability of FK506 to increase intracellular glutathione level was also observed. Our present results clearly demonstrated that Choto-san and some of its related constituents were able to increase the intracellular concentration of glutathione in NG108-15 cells. Glutathione is the most important nonprotein thiol source used to maintain cells in a reduced environment. It has been recently found that glutathione is an efficient scavenger of superoxide radicals (O$_2^-$) and hydroxyl radicals, as well as single oxygen.24) Decreased tissue GSH levels have been associated with several diseases, including stroke, ischemic reperfusion injury.25,26) Deficient glutathione status contributes to a weakened antioxidant defense system.27—30) Increased cellular glutathione levels may be beneficial in conditions where glutathione levels are decreased. A key question which remains unclear in our study is the mechanism of Choto-san induced augmentation of endogenous antioxidants. Although we have not isolated the compounds responsible for the enhancement of antioxidant defense systems by Choto-san, we speculated that it may be related to the phenolic constituents in the extract. This possibility was supported by the present result that phenolic compounds especially quercetin significantly enhance activity of all antioxidant enzymes and glutathione. Furthermore, the treatment of the cells with Choto-san significantly enhanced the activity of catalase. Whereas the increased of catalase activity was not observed in NG108-15 cells treated with Choto-san without Chotoko. These results suggest that Chotoko and its constituents may play an important role in the induction of catalase in NG108-15 cells. The induction of antioxidants defense systems by Choto-san and its related constituents has not been previously reported. The molecular mechanism underlying Choto-san-mediated elevation of antioxidant enzymes and glutathione level remains unclear. A number of studies have demonstrated that induction of antioxidant enzymes by some phenolic antioxidants in mammalian tissue occur via Nrf2-mediated antioxidant response element (ARE)-driven transcription mechanism.31) However, further studies are needed to clarify this mechanism.

In summary, simultaneous induction of cellular antioxidant defense systems by Choto-san and its related constituents may be an important mechanisms underlying the protective effects of Choto-san on ischemia-induced neuronal cells injury.

REFERENCES