Neuroprotective Effects of Curcumin and Highly Bioavailable Curcumin on Oxidative Stress Induced by Sodium Nitroprusside in Rat Striatal Cell Culture

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Curcumin, a polyphenolic compound extracted from Curcuma longa, has several pharmacological activities such as anticancer, anti-inflammatory, and antioxidant effects. The purpose of this study was to investigate the protective effects of curcumin and THERACURMIN, a highly bioavailable curcumin, against sodium nitroprusside (SNP)-induced oxidative damage in primary striatal cell culture. THERACURMIN as well as curcumin significantly prevented SNP-induced cytotoxicity. To elucidate the cytoprotective effects of curcumin and THERACURMIN, we measured the intracellular glutathione level in striatal cells. Curcumin and THERACURMIN significantly elevated the glutathione level, which was decreased by treatment with SNP. Moreover, curcumin showed potent 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging ability. Finally, a ferrozine assay showed that curcumin (10–100 µg/mL) has potent Fe2+-chelating ability. These results suggest that curcumin and THERACURMIN exert potent protective effects against SNP-induced cytotoxicity by free radical-scavenging and iron-chelating activities.

Key words curcumin; THERACURMIN; sodium nitroprusside; oxidative stress

Oxidative stress has been implicated in the progression of neurodegenerative disorders including Parkinson’s, Alzheimer’s, and Huntington’s diseases as well as stroke and trauma. 1–6) Oxidative stress is caused when the balance between production of reactive oxygen species (ROS) and level of antioxidants is considerably disturbed and results in damage to cells by excessive ROS production. The brain is more vulnerable to oxidative stress than other tissues because of its high oxygen consumption and consequent generation of large amounts of ROS and limited antioxidant capacity.7,8) However, cells normally employ several defense mechanisms against ROS such as antioxidant enzymes, vitamins E and C, and glutathione. When the antioxidant defense mechanisms fail to protect cells from ROS, lipids, proteins, and DNA are damaged and this subsequently results in cell death.9) Because many degenerative disorders are closely related to free radical overloading and intracellular oxidative stress, antioxidant supplements that can reduce ROS in cells are useful for treatment of neurodegenerative diseases.10) Thus, development of natural antioxidants and drugs from natural products will be a useful strategy in the prevention of oxidative stress and associated neurological disorders.

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a yellow polyphenolic compound derived from Curcuma longa. It is widely used in South and Southeast Asia as well as in other parts of the world.11) In recent years, there has been a growing interest in studying curcumin because of its beneficial health properties. Several reports have suggested that curcumin can be used as a potential drug for the treatment of cancer, cardiovascular diseases, inflammatory conditions, and neurodegenerative disorders.12–15) Curcumin has also been suggested to be effective against oxidative insults.16–18) Recently, a new highly bioavailable curcumin THERACURMIN has been developed for oral applications against systemic diseases.19,20) Therefore, it is important to evaluate the effects of curcumin and THERACURMIN against oxidative stress in vivo experiments. Results from these experiments may provide insights on the potential of these compounds for treatment of systemic diseases, particularly central nervous system (CNS) disorders.

Therefore, the aim of our study was to evaluate the protective effects of curcumin and THERACURMIN against oxidative stress damage induced by sodium nitroprusside (SNP) toxicity in primary striatal cell culture and to understand the main mechanisms involved in the protective effects of curcumin against SNP-induced oxidative stress.

MATERIALS AND METHODS

Materials Wistar rats were obtained from Nihon SLC (Shizuoka, Japan). SNP; 2,3,5-triphenyltetrazolium chloride (TTT); ferrous chloride (FeCl2) tetrahydrate; ethylenediaminetetraacetic acid (EDTA); l-ascorbic acid; methanol; and Triton X-100 were purchased from Nacalai Tesque (Kyoto, Japan). Curcumin; 1,1-diphenyl-2-picrylhydrazyl (DPPH); 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine); and l-buthionine-[S,R]-sulfoximine (BSO) were obtained from Sigma (St. Louis, MO, U.S.A.). Reduced glutathione (GSH) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Eagle’s minimum essential medium (Eagle’s MEM) was purchased from Nissui Pharmaceutical Co. (Tokyo). Fetal bovine serum was obtained from JRH Biosciences (Lenexa, KS, U.S.A.). The cytotoxicity detection lactate dehydrogenase (LDH) kit was purchased...
from Kyokuto Pharmaceutical Industrial Corp. (Tokyo, Japan). THERACURMIN was obtained from Theravalex Corporation (Tokyo, Japan).

**Cell Culture** Primary cell cultures were prepared from the striatum of fetal Wistar rats on gestational days 17–19 according to a previously described method.\(^2\) In brief, the striatum of the fetal rats was removed, minced using scalpel blades, and filtered through a stainless steel mesh to obtain a single-cell suspension. Striatal cells were then cultured in 96-well tissue culture plates coated with 0.1% polyethyleneimine. Cultures were maintained in Eagle’s MEM supplemented with 10% heat-inactivated fetal bovine serum, glucose (2 mM), NaHCO\(_3\) (24 mM), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (10 mM) at 37°C in a humidified atmosphere of 95% O\(_2\) and 5% CO\(_2\). Curcumin and THERACURMIN were dissolved in dimethyl sulfoxide. Cells were treated with curcumin or THERACURMIN for 24 h prior to addition of SNP (300 µM) and then treated with curcumin or THERACURMIN for the next 24 h. The reproducibility of the results was confirmed by at least three separate experiments. The experiments were conducted in accordance with ethical guidelines of the Kyoto University Animal Experimentation Committee and the guidelines of the Japanese Pharmacological Society.

**LDH Release Assay** Cell viability was assessed by measuring the amount of LDH released into the culture medium using the cytotoxicity detection LDH kit according to the method described previously.\(^2\) Briefly, culture supernatant was added to the LDH substrate mixture in a 96-well plate and incubated at 37°C. After incubation, the reaction was stopped by adding 1 N HCl and the absorbance was read at 570 nm using a microplate reader (Bio-Rad). The percentage chelation activity of the Fe\(^{2+}\)-ferrozine complex was calculated as follows:

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\text{Chelating activity (\%)} = \left(\frac{A_0 - A_t}{A_0}\right) \times 100
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where \(A_0\) is the absorbance of the control reaction (FeCl\(_2\) + ferrozine) and \(A_t\) is the absorbance in the presence of the test compound.

**Statistical Analyses** The results were expressed as mean±S.E.M. One way analysis of variance (ANOVA) was used, followed by Tukey’s post test to determine statistical significant among three or more groups. Student’s \(t\)-test was performed to determine statistical significance between two groups. All statistical analyses were conducted using GraphPad InStat (GraphPad Software Inc., San Diego, CA, U.S.A.).

**RESULTS**

**Effects of SNP, Curcumin, and THERACURMIN on the Viability of Rat Striatal Cell Culture** To evaluate the effect of SNP-induced cytotoxicity and the protective effects of curcumin and THERACURMIN in striatal cell culture, we first determined the sublethal concentration of SNP and the nontoxic concentrations of curcumin and THERACURMIN. SNP (300 µM) induced considerable striatal cell death (Fig. 1A). To determine the nontoxic concentrations of curcumin and THERACURMIN, cells were treated with various concentrations of the drugs for 48 h. Curcumin and THERACURMIN did not show the toxicity at 10 µM (Figs. 1B, C).

**Effects of Curcumin and THERACURMIN against SNP-Induced Cytotoxicity in Rat Striatal Cell Culture** We examined the effects of curcumin and THERACURMIN against SNP-induced cytotoxicity. As shown in Fig. 2, treatment with curcumin (10 µM) or THERACURMIN (10 µM) for 48 h significantly attenuated SNP-induced cytotoxicity. These results suggest that curcumin and THERACURMIN have the potential to protect striatal cells from SNP-induced oxidative stress.

**Effect of Curcumin on the Intracellular GSH Level of Striatal Cell Culture** GSH has been reported to play an important role against oxidative stress in cells.\(^2\) To determine whether cellular GSH is involved in the protective effect of curcumin against SNP-induced cell death, we measured the GSH level in striatal cell culture. Curcumin (10 µM) significantly increased the intracellular GSH level in a concentration-dependent manner (Fig. 3A). Curcumin or THERACURMIN (10 µM) also increased the intracellular GSH level in a time-dependent manner (Figs. 3B, C). Furthermore, treatment with SNP (300 µM) for 24 h significantly reduced the intracellular GSH level, although a 24-h pretreatment with curcumin (10 µM) significantly inhibited this SNP-induced decrease in GSH level (Fig. 3D). In addition, treatment with BSO (10 µM), an inhibitor of \(\gamma\)-glutamylcysteine synthase, which is the rate-limiting enzyme in GSH synthesis, significantly decreased the intracellular GSH level in striatal cell culture (Fig. 3E). These results suggest that the protective
Fig. 1. Effects of Curcumin and THERACURMIN on the Viability of Rat Striatal Cell Cultures

Cell cultures were treated with SNP for 24 h (A) and curcumin (B) or THERACURMIN (C) for 48 h. ***p<0.001, compared with vehicle. Values represent mean±S.E.M.

Fig. 2. Effects of Curcumin and THERACURMIN on SNP-Induced Cytotoxicity in Rat Striatal Cell Cultures

Cultures were pretreated with curcumin or THERACURMIN for 24 h, followed by treatment with SNP (300 µM) for 24 h. SNP significantly reduced cell viability (B, D), but 10 µM curcumin (C, D) and 10 µM THERACURMIN (E) attenuated SNP-induced cytotoxicity. ***p<0.001, compared with vehicle; ##p<0.01, ###p<0.001, compared with SNP treatment. Values represent mean±S.E.M. Scale bar, 50 µm.
Fig. 3. Effect of Curcumin on the Intracellular GSH Level in Rat Striatal Cell Culture

Cultures were treated with curcumin for 48 h (A) or curcumin and THERACURMIN (10 µM) for 1–48 h (B and C), followed by measurement of the intracellular GSH level. Cultures were pretreated with or without 10 μM curcumin, followed by treatment with either 300 μM SNP (D) or 10 μM BSO (E) for 24 h. ** p<0.01, *** p<0.001, compared with vehicle; ### p<0.001, compared with SNP treatment; +++ p<0.001, compared with curcumin treatment. Values represent mean±S.E.M.

Fig. 4. DPPH Radical-Scavenging Activity of Curcumin, Ascorbic Acid, and Glutathione

Values represent mean±S.E.M. of triplicate samples.

Fig. 5. Iron-Chelating Activity of Curcumin and EDTA

Values represent mean±S.E.M. of triplicate samples.
effect of curcumin is mediated in part by an elevation of intracellular GSH levels.

**DPPH Radical-Scavenging Activity** We previously reported that iron-related radical reactions have crucial roles in SNP-induced brain damage. Thus, to elucidate the protective mechanism of curcumin, we determined the free radical-scavenging activity of curcumin using DPPH. As shown in Fig. 4, curcumin potently scavenged the DPPH radicals. However, the scavenging activity of curcumin was almost comparable with that of ascorbic acid at higher concentrations (30–100 µg/mL). These results indicate that curcumin has potent free radical-scavenging activity, showing that this is a part of the mechanism by which curcumin exerts protective effects against SNP-induced cytotoxicity.

**Fe²⁺-Chelating Activity** Production of ROS such as hydroxyl radicals is catalyzed by free iron via the Fenton reaction. To investigate the iron-chelating activity of curcumin, the ferrozine assay was performed. As shown in Fig. 5, curcumin potently chelated free iron ions (10–100 µg/mL) in a concentration-dependent manner. These results indicate that curcumin has chelating activity, showing that this is also a part of the mechanism by which curcumin exerts protective effects against SNP-induced cytotoxicity.

**DISCUSSION**

In this study, we evaluated the protective effects of curcumin and THERACURMIN, a highly bioavailable curcumin, against SNP-induced oxidative stress. Curcumin and THERACURMIN demonstrated significant protective effects against SNP-induced oxidative stress in vitro.

Curcumin, a naturally occurring polyphenolic compound, has been reported to exhibit several biological and pharmacological activities, including potent antioxidant, anticancer and anti-inflammatory effects. It was previously demonstrated that curcumin effectively prevented amyloid β-induced toxicity in rat cortical cells. Curcumin has also been reported to be effective in the treatment of focal cerebral ischemia.

However, there is no report on the effect of THERACURMIN against SNP-induced neurotoxicity in vitro. Thus, we performed experiments to evaluate SNP-induced oxidative stress in striatal cell culture as well as the protective effect of curcumin or THERACURMIN. We observed that SNP induced significant cytotoxicity in primary striatal cell culture, as determined by the LDH release assay (Fig. 1). Treatment with curcumin or THERACURMIN significantly inhibited SNP-induced cytotoxicity. These results suggest that curcumin and THERACURMIN are protective against SNP-induced oxidative stress in primary striatal cell culture.

The mechanism of action of curcumin is complex and multifaceted. Curcumin has been suggested to exert its effects by inducing the expression of antioxidant enzymes and molecules or by directly scavenging free radicals and chelating metal ions. Thus, to investigate whether the intracellular GSH level was increased as part of the protective effect of curcumin against SNP-induced cytotoxicity, we treated rat striatal cell culture with SNP and observed that incubation of cells with SNP for 24 h significantly decreased the intracellular GSH level, but curcumin compensated the GSH level. Moreover, 1-h incubation with SNP did not alter cell viability in the LDH release assay, but it decreased the intracellular GSH level, indicating that cell death was not involved in SNP-induced GSH depletion (data not shown). Under normal conditions, the intracellular GSH level is properly maintained, but it is decreased under oxidative stress conditions. GSH is important for cellular defense against ROS in the brain, acting directly in detoxification of free radicals in nonenzymatic reactions and functioning as a substrate for various peroxidases. Moreover, several reports have suggested that elevated GSH levels in brain cells are associated with a protective effect against oxidative stress. However, the effects of SNP or curcumin on the intracellular modulation of GSH level in striatal cell culture have not been reported till date. Our results clearly illustrate that SNP causes intracellular GSH depletion and curcumin compensates the GSH level in rat striatal cell culture. However, curcumin did not affect GSH reduction induced by BSO (Fig. 3). Previous report showed that curcumin increased the expression of γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme of the synthesis of glutathione. Thus, curcumin compensates GSH level after treatment of SNP. On the other hand, curcumin did not affect the GSH depletion by BSO because increased γ-GCS could not synthesize glutathione in the presence of BSO. Our results suggest that minor part of the protective effect of curcumin against oxidative damage induced by SNP cytotoxicity involves increase in the intracellular GSH level.

In an attempt to elucidate the main mechanisms through which curcumin protected cells from SNP-induced cytotoxicity, the radical-scavenging activity of curcumin was assessed using the DPPH assay, which is based on the hydrogen-donating ability of antioxidants. The assay showed that curcumin reduced the DPPH free radical more potently than the well-known endogenous antioxidant GSH in a concentration-dependent manner (Fig. 4). After release of NO, the iron moiety of SNP may contribute to the formation of ROS such as OH radicals via the Fenton reaction. The two phenolic OH groups and the methylene group of the β-diketone moiety in curcumin are important functional groups responsible for its potent antioxidant and free radical-scavenging properties. To investigate the metal-chelating activity of curcumin, we performed the ferrozine assay, which showed that curcumin can potently bind iron. These results indicate that curcumin may prevent the redox cycling of iron and reduce oxidative stress (Fig. 5). It has been proven that the hydroxyl groups and β-diketone moiety in curcumin are involved in metal chelation. In terms of the permeability of curcumin into cells, it was reported that curcumin is able to diffuse through the cell membrane and to suppress intracellular ROS production. Minear et al. reported that curcumin significantly decreased intracellular iron levels. Thus, our results suggested that curcumin entered the cells and protected against SNP-induced cytotoxicity by its free radical-scavenging and iron-chelating abilities.

In conclusion, curcumin and THERACURMIN prevented cell death resulting from exposure to SNP-induced oxidative stress in vitro. These protective effects are possibly caused by radical-scavenging and iron-chelating mechanisms. Moreover, our results suggest that curcumin and THERACURMIN have potent antioxidant effects. Thus, these compounds may be used as potent neuroprotective agents against neurological disorders associated with oxidative stress.

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REFERENCES


