Evaluation of the Nitric Oxide Radical Scavenging Activity of Manganese Complexes of Curcumin and Its Derivative

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Curcumin manganese complex (CpCpx) and diacetylcurcumin manganese complex (AcylCpCpx) were determined as to their effect on the nitric oxide (NO) radical scavenging *in vitro* method using a sodium nitroprusside generating NO system compared with their parent compound and astaxanthin, an extreme antioxidant. All compounds effectively reduced the generation of NO radicals in a dose dependent manner. They exhibited strong NO radical scavenging activity with low IC_{50} values. The IC_{50} values of curcumin, diacetylcurcumin, CpCpx and AcylCpCpx obtained are 20.39±4.10 μM, 28.76±1.48 μM, 9.79±1.50 μM and 8.09±0.99 μM, respectively. CpCpx and AcylCpCpx show greater NO radical scavenging than their parent compounds, curcumin and acetylcurcumin, respectively. However, the IC_{50} values of curcumin and related compounds were found to be less than astaxanthin, an extreme antioxidant, with the lower IC_{50} value of 3.42±0.50 μM.

Key words curcumin; diacetylcurcumin; nitric oxide; manganese complex

Nitric oxide (NO), a short-lived free radical generated endogenously, exerts influence on a number of functions including vasodilation, neurotransmission, synaptic plasticity and memory in the central nervous system.[1,2] Besides mediating normal function, NO has been implicated in pathophysiologic states. Overproduction of NO can mediate toxic effects, *e.g.* DNA fragmentation, cell damage and neuronal cell death.[3] NO also shows neurotoxicity and acts as a pathological mediator in pathophysiological processes such as cerebral ischemia, epilepsy, Alzheimer's disease, Parkinson's disease and certain neurodegenerative disease.[4]

Curcumin (diferuloylmethane) is a major active component of the food flavour tumeric. It is extracted from the powdered dry rhizome of *Curcuma longa* LINN (Zingiberaceae), a perennial herb widely cultivated in tropical regions of Asia. It has been used for centuries in indigenous medicine for the treatment of a variety of inflammatory, and infectious diseases, cancer and other diseases.[5,6] Several studies have shown that a trigger mechanism that allows for the treatment of many diseases is due to its antioxidant properties. Curcumin is a powerful scavenger of many free radicals such as superoxide anion,[7] hydroxyl radical[8] and nitric oxide.[9]

In a previous study, Vajragupta *et al.* synthesized the manganese complexes of the curcumin and diacetylcurcumin as superoxide dismutase mimics. The structures of curcumin and its derivatives are shown in Fig. 1. Superoxide dismutase (SOD) enzyme is the metalloenzyme that catalyzes the dismutation of superoxide anion to hydrogen peroxide and dioxygen. Curcumin manganese complex (CpCpx) and diacetylcurcumin manganese complex (AcylCpCpx) are low molecular weight synthetic compounds that showed much greater SOD activity and an inhibitory effect on lipid peroxidation.[10] AcylCpCpx also gave the highest inhibitory activity to H_2O_2-induced cell damage (oxidative stress) in NG108-15 cells, which were more potent than curcumin and acetylcurcumin. In addition, the manganese complexes also potentiated neuroprotective effects against learning and memory impairment in transient cerebral ischemic mice.[11]

The aim of this study is to investigate the effect of CpCpx and AcylCpCpx on NO radical scavenging *in vitro* using a

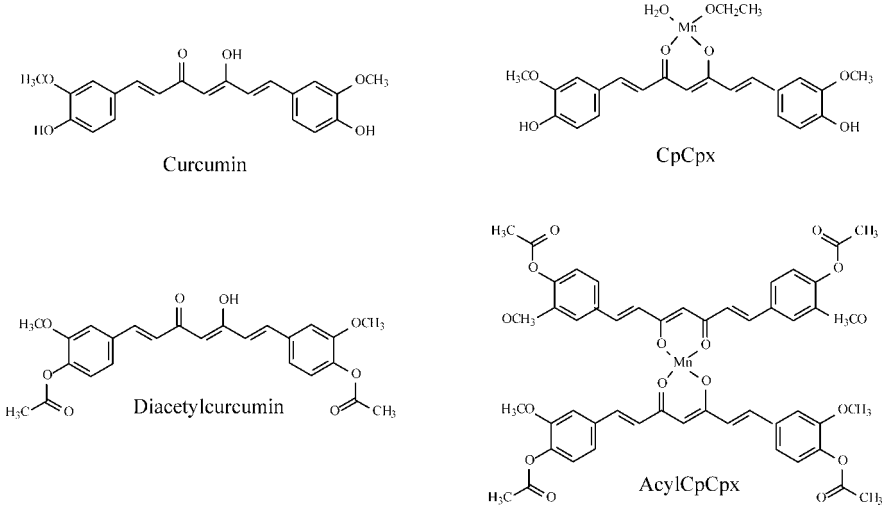

Fig. 1. Structure of Curcumin, Diacetylcurcumin and Their Manganese Complexes

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sodium nitroprusside (SNP) generating NO system.

MATERIALS AND METHODS

Materials Curcumin, diacetylcurcumin, CpCpx and AcylCpCpx were obtained from Boonchoong P. and coworkers. CpCpx and AcylCpCpx were prepared by the reaction of manganese acetate with the curcumin and diacetylcurcumin, respectively, in ethanolic solution. The reactions were run under nitrogen gas and an appropriately controlled temperature (60—70 °C). The manganese complexes were then precipitated.[11] SNP was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium nitrite, sulphanilamide and *N*-naphthylethylenediamine dihydrochloride were purchased from Wako Pure Chemicals Industries Co., Ltd. (Osaka Japan).

Assay of NO Radical Scavenging Activity. Incubation Condition NO generated from SNP was measured by the Griess reagent, as described previously.[12,13] 100 mM of SNP was prepared by dissolving the powder in phosphate buffered saline (PBS) pH 7.4. The reaction mixture (2 ml) containing 100 mM SNP (0.2 ml, final concentration 10 mM) and PBS (1.8 ml) was incubated at 25 °C for 180 min. At 30 min intervals, samples (1 ml) of the incubation were removed and diluted with 1 ml of Griess reagent (1% sulphanilamide and 0.1% naphthyletylenediamine dihydrochloride in 2% H_3PO_4). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylene-diamine was read at 540 nm, and referred to the absorbance of standard solutions of sodium nitrite treated in the same way with Griess reagent. The plot between the concentration of nitrite and incubation time exhibited the best incubation time for nitrite production from SNP.

Effect of Tested Compounds on NO Radical Scavenging Activity Various concentrations of the tested compounds and SNP (10 mM, final concentration) in PBS in a final volume of 2 ml were incubated at 25 °C for 150 min. A control experiment without tested compounds but with the equivalent amount of vehicles was conducted in an identical manner of control. After incubation, 1.0 ml samples of reaction mixtures containing nitrite were removed and diluted with 1.0 ml of Griess reagent. Astaxanthin was used as a reference standard. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. The IC_{50} value is the concentration of sample required to inhibit 50% of the NO radicals. Curcumin and their complexes were the spectrophotometrically detectable compounds. The absorbances of their chromophores at measured wavelengths were in a concentration-dependent manner. Addition of varied concentrations of these compounds into the reaction mixture affected an increase in total absorbance upon treatment with Griess reagent. It was indicated that the tested compounds interfered with the absorbance value of nitrite detection. Therefore, in these experiments we had to exclude this interference by subtracting their absorbance at each concentration.

RESULTS

SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce a nitrite ion which can be estimated using Griess reagent. The incubation time for SNP to generate a maximum concentration of nitrite ion is 150 min. Incubation of solutions of SNP in PBS at 25 °C for 150 min resulted in linear time-dependent nitrite production (Fig. 2).

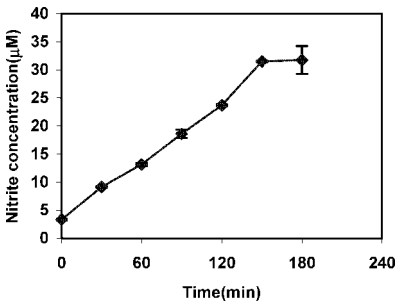

Fig. 2. Time-Dependent Nitric Oxide Production from a Solution of 10 mM Sodium Nitroprusside

At 30 min intervals, 1 ml of incubation solution was removed and reacted with 1 ml of Griess reagent. The absorbance was read at 540 nm, and referred to the absorbance of standard solutions of sodium nitrite treated in the same way with Griess reagent. Data are means±S.D. (n=3).

In this study, astaxanthin and quercetin were used as the reference NO radical scavengers. The compound that possesses NO scavenging activity inhibited nitrite formation by competing with oxygen to react with NO. This lead to the reduction of nitrite concentration in the assay media. Astaxanthin, an extreme antioxidant, exhibited potent NO radical scavenging activity with an IC_{50} value of 3.42±0.50 μM, whereas quercetin appeared much less active than astaxanthin with an IC_{50} value of 100.61±7.99 μM (Fig. 3).

Curcumin, diacetylcurcumin, CpCpx and AcylCpCpx effectively reduced the generation of NO radicals. All tested compounds scavenged NO radicals in a dose dependent manner (Fig. 4).

All tested compounds exhibited strong NO radical scavenging with low IC_{50} values. The IC_{50} values of curcumin, diacetylcurcumin, CpCpx and AcylCpCpx obtained were 20.39±4.10 μM, 28.76±1.48 μM, 9.79±1.50 μM and 8.09±0.99 μM, respectively. CpCpx and AcylCpCpx showed greater NO radical scavenging than their parent compounds, curcumin and diacetylcurcumin, respectively.

DISCUSSION

The present study demonstrates that curcumin, diacetylcurcumin, CpCpx and AcylCpCpx are potent NO radical scavengers. NO generated from SNP in aqueous solution at physiological pH reacts with oxygen to form nitrite ions. All tested compounds inhibited nitrite formation by competing with the oxygen atom to react with NO. This is consistent with the previous report on the effect of curcumin and its derivatives on NO scavenging activity by Sreejayan and Rao. Curcumin, demethoxycurcumin, bisdemethoxycurcumin and diacetylcurcumin have the ability to scavenge oxide directly.[9] Besides, it has previously been reported that curcumin inhibits the generation of NO from activated macrophages, as measured by the nitrite method.[14] This inhibition might also be a result of direct scavenging of NO by curcumin. In addition, mechanisms of anticarcinogenic properties of curcumin

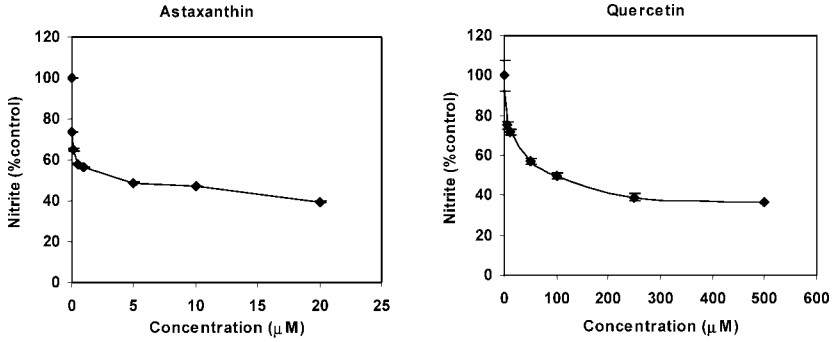

Fig. 3. The Effect of Astaxanthin and Quercetin, the Reference Nitric Oxide Scavenger, on the Accumulation of Nitrite upon Decomposition of Sodium Nitroprusside (10 mM)

Incubation time 150 min; temperature 25 °C. Data are means±S.D. (n=3).

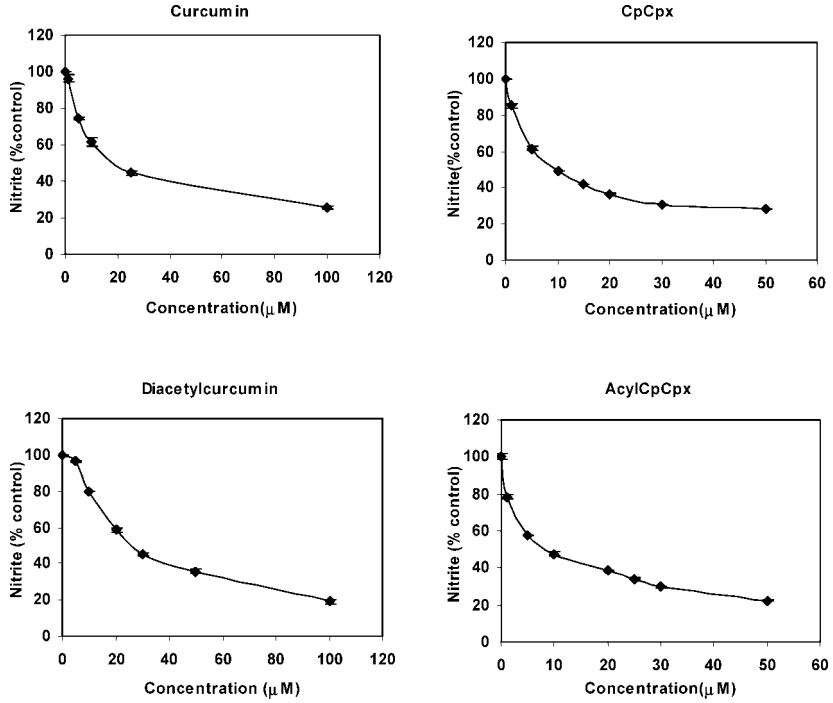

Fig. 4. The Effect of Curcumin, Diacetylcurcumin and Their Manganese Complexes on the Accumulation of Nitrite upon Decomposition of Sodium Nitroprusside (10 mM)

Incubation time 150 min; incubation temperature 25 °C. Data are means±S.D. (n=3).

were linked to the antioxidant activity.[15] According to extensive studies on the structure–activity relationship of curcumin, the antioxidant properties of curcumin are thought to be imparted by its β-diketone moiety, cleavage of the C–C bond at the methylene carbon between two carboxyls in the diketone moiety, and the formation of relatively stable free radicals due to its extended conjugated double bond structure.[16] In addition, the phenolic moiety is an important part that is also involved in the antioxidative mechanism of curcumin compound and it derivatives.[17] The mechanism of the antioxidant activity of phenols is widely known to involve their ability to act as free radical scavengers, leading to the formation of phenoxyl radicals. There are two possible mechanisms for the production of phenoxyls from the reaction of $^{\bullet}$NO with a phenol moiety. First, H-atom abstraction produces HNO and phenoxyls. Second, it is also possible that phenols reduce $^{\bullet}$NO by single electron transfer to produce the phenol radical cation, with subsequent loss of a proton to form phenoxyl radicals. Reaction of $^{\bullet}$NO with phenolic groups may serve to attenuate the concentration of $^{\bullet}$NO in the experiment.[18,19] This finding supports our results that acetylation at two hydroxyl groups of the phenolic part of curcumin results in the decreased NO scavenging activity of diacetylcurcumin relative to curcumin. The results are in the line with those reported.[20—22] Acetylation of phenolic groups resulted in significant reduction of the inhibitory effect of nitrite induced oxidation of haemoglobin in both erythrocytes and purified human haemoglobin.[20] According to the results of a previous study, there is a high correlation between the total phenolic content and the NO scavenging ability and antioxidant activity of plant extracts. Since total phenolic compounds in the extracts were higher, it might be inferred that the antioxidant activity of those extracts were increased.[21,22] On the other hand, Sreejayan and Rao reported that the phenolic groups were not essential for NO scavenging activity, because diacetylcurcumin was almost as active as cur-

cumin.[9] These inconsistent results may be due to the different resources of tested compounds and experiment conditions. For manganese complexes, CpCpx and AcylCpCpx were synthesized by incorporating the manganese atom into the structure of their parent ligands. CpCpx, manganese atom, was bonded at the β-diketone part of the curcumin ligand and was solvated with ethanolic and one molecule of water (Fig. 1). The phenolic and methoxy groups on the benzene ring remained and contributed to their antioxidant activity. Besides the phenolic and methoxy group, manganese atoms at the active site of CpCpx may be involved in the antioxidant activity. The proposed mechanism might be the changing of the oxidation state of the manganese atom from a redox reaction between manganese atoms and free radicals. In the case of AcylCpCpx, the manganese atom was coordinated with the β-diketone moiety of two molecules of diacetylcurcumins (Fig. 1). The high oxidation state manganese complex is stabilized by strong π electron-donor ligands of the β-diketone system. In addition to electron density provided by σ-coordination, the π electrons of such ligands donate additional electron density to the electron deficient metal center.[23] The antioxidant property was obtained from two parts of the methoxy group and manganese atom at the active site of AcylCpCpx due to the phenolic group being blocked by acetylation. CpCpx and AcylCpCpx exhibited greater NO radical scavenging activity than their parent compounds, corresponding with the previous study that CpCpx and AcylCpCpx had a much better effect on superoxide ($O_2^{-\bullet}$) than curcumin and diacetylcurcumin, respectively, in both nitroblue tetrazolium and electron paramagnetic resonance spin-trapping assays[10]; also, the manganese-centered atom at the active site of manganese complexes is important since it possesses SOD activity. Therefore, the higher NO radical scavenging activity of CpCpx and AcylCpCpx than their parent ligands may be mainly from the redox reaction of the manganese-centered atom.

CONCLUSION

Curcumin, diacetylcurcumin and their manganese complexes show potent NO radical scavenger activity *in vitro* using a SNP generating NO system method. However, the exact pathway of electron abstraction from the manganese atom is, as yet, not fully understood. The complete antioxidant mechanism of curcumin manganese complex and its derivative also needs to be clarified. In addition to reactive oxygen species, NO is also implicated in various pathophysiological processes. Therefore, it is interesting to further investigate the therapeutic efficacy of manganese complexes of curcumin and its derivative in the other model of pathophysiologic states mediated by NO, at least partly, according to their ability to scavenge NO radicals.

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