Inhibitory Action of Oren-gedoku-to Extract on Enzymatic Lipid Peroxidation in Rat Liver Microsomes

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We examined the inhibitory action of the extract of Oren-gedoku-to, a traditional herbal medicine known to act as an antioxidant, on enzymatic lipid peroxidation in rat liver microsomes. Simultaneous addition of a spray-dried preparation of Oren-gedoku-to extract (Tsumura TJ-15) inhibited enzymatic lipid peroxidation induced by reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) and ADP/Fe^{3+} complex in liver microsomes in a dose-dependent manner. When the inhibition by TJ-15 of enzymatic lipid peroxidation in liver microsomes was kinetically analyzed, this medicine showed a competitive inhibition against NADPH or ADP/Fe^{3+} complex. TJ-15 inhibited the NADPH-driven enzymatic reduction of ADP/Fe^{3+} complex or cytochrome c in liver microsomes competitively. TJ-15 enhanced NADPH consumption by liver microsomes with ADP/Fe^{3+} complex. Treatment with TJ-15 after the onset of enzymatic lipid peroxidation in liver microsomes inhibited the progression of lipid peroxidation in a dose-dependent manner. The present results indicate that Oren-gedoku-to extract inhibits enzymatic lipid peroxidation in rat liver microsomes not only through its antioxidant action but also through reduction of the supply of electrons derived from NADPH to ADP/Fe^{3+} complex in liver microsomes both in a competitive manner and through stimulation of NADPH oxidation.

Key words Oren-gedoku-to; enzymatic lipid peroxidation; liver microsome (rat); electron transport system

Oren-gedoku-to (Huanglian-Jie-Du-Tang), a traditional Chinese herbal medicine, is the boiled extract of the four herbs Coptidis Rhizoma, Scutellariae Radix, Phellodendri Cortex, and Gardeniae Fructus, and has been used for the treatment of gastric diseases, brain diseases, and liver diseases in Japan. A spray-dried preparation of Oren-gedoku-to extract (Tsumura TJ-15) has often been used to treat these diseases. There are several reports showing that TJ-15 exerts a protective or therapeutic effect on gastric mucosal lesions induced by compound 48/80, a mast cell degranulator, or water immersion-restraint stress in rats, possibly through its antioxidant action. It is known that TJ-15 exerts a therapeutic effect on carbon tetrachloride-induced acute liver injury in rats, possibly through its antioxidant action. It is also known that TJ-15 prevents a decrease in serum superoxide dismutase activity in rats with inflammation.

TJ-15 has been shown to scavenge superoxide radical and hydroxyl radical in vitro. TJ-15 has been reported to inhibit lipid peroxidation induced by Fe^{2+}/H_{2}O_{2} in phosphatidylcholine liposomes and lipid peroxidation induced by Fe^{2+} or Fe^{2+}/H_{2}O_{2} in rat liver homogenates. TJ-15 has also been reported to inhibit lipid peroxidation induced by reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), cumene hydroperoxide, or iron/ascorbate in rat liver microsomes. We have shown that TJ-15 inhibits lipid peroxidation induced by ADP/Fe^{3+}-NADPH, carbon tetrachloride, cumene hydroperoxide, or the water-soluble radical initiator 2,2′-azobisis(2-aminopropane) in rat liver microsomes. However, the detailed inhibitory actions of Oren-gedoku-to extract on these lipid peroxidation reactions in rat liver microsomes are still unclear.

In the present study therefore, we attempted to elucidate the inhibitory action of Oren-gedoku-to extract on enzymatic lipid peroxidation, i.e., NADPH-induced lipid peroxidation, in rat liver microsomes with ADP/Fe^{3+} complex using TJ-15.

MATERIALS AND METHODS

Materials TJ-15, a spray-dried preparation using boiled water extracts of Coptidis Rhizoma 2.0 g, Scutellariae Radix 3.0 g, Phellodendri Cortex 1.5 g, and Gardeniae Fructus 2.0 g, was kindly provided by Tsumura & Co. (Tokyo, Japan). The extraction percentage of TJ-15 was 17.6%. Glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-PDH) were purchased from Boehringer Mannheim (Tokyo, Japan), and bovine serum albumin (fraction V), cytochrome c (from horse heart), ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), 2,2′-dipyridyl, NADPH, 2-thiobarbituric acid (TBA), and other chemicals from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). All chemicals were used without further purification.

Preparation of Liver Microsomes Liver microsomes were prepared from 7-week-old male Wistar rats, which were purchased from Japan SLC Inc. (Hamamatsu, Japan) and fasted overnight, as described previously. In brief, the liver of rats was removed after ether anesthesia under substantial perfusion with ice-cold KCl 0.15 M to remove the residual blood in the organ. The removed liver was rinsed, weighed, and then homogenized in 9 volumes of ice-cold KCl 0.15 M–EDTA 1 mM using a glass homogenizer with a Teflon pestle. The preparation of liver microsomes was conducted at 4 °C as follows: the prepared homogenate was centrifuged at 105,000 g for 20 min to obtain the postmitochondrial fraction which was then centrifuged at 105,000 g for 60 min to obtain the microsomal fraction. The microsomes were suspended in ice-cold KCl 0.15 M–EDTA 1 mM using a glass homogenizer with a Teflon pestle. The preparation of liver microsomes was conducted at 4 °C as follows: the prepared homogenate was centrifuged at 10,000 g for 20 min to obtain the postmitochondrial fraction which was then centrifuged at 105,000 g for 60 min to obtain the microsomal fraction. The microsomes were suspended in ice-cold KCl 0.15 M–EDTA 1 mM and then centrifuged at 105,000 g for 30 min for washing. The washed microsomes were suspended in Tris–HCl buffer 0.15 M (pH
7.4) containing 20% glycerol and stored at −80°C until use. The stored microsomes were diluted with Tris–HCl 0.05 M (pH 7.4) to obtain an appropriate protein concentration when used for the measurement of lipid peroxidation. Microsomal protein was determined by the method of Lowry et al.(15) using bovine serum albumin as a standard.

**Lipid Peroxidation Assay** The reaction mixture consisted of NADPH 0.3 mM, ADP/Fe<sup>3+</sup> (ADP 4 mM–FeCl<sub>3</sub> 12 µM), Tris–HCl buffer 0.1 M (pH 7.4), and microsomes (0.25 mg protein/ml) with and without an appropriate concentration of TJ-15, which was suspended in distilled water, in a final volume of 1.0 ml. After preincubation at 37°C for 3 min, NADPH was added to the reaction mixture to initiate lipid peroxidation. The complete reaction mixture was incubated at 37°C for an appropriate time. The activity of ADP/Fe<sup>3+</sup>-NADPH-induced lipid peroxidation in microsomes was determined by the method of Buege and Aust(16) using the TBA reaction. The formation of lipid peroxide (LPO), the TBA-reactive products, in the presence of BHT, an antioxidant, was monitored at 535 nm. The amount of malondialdehyde (MDA) formed was estimated using the molar extinction coefficient of a red pigment produced by the reaction of Tris–HCl buffer 0.1 M (pH 7.4), NADPH 0.1 mM, ADP/Fe<sup>3+</sup> (ADP 4 mM–FeCl<sub>3</sub> 12 µM), and liver microsomes (0.5 mg protein/ml) with and without TJ-15 10 or 50 µg/ml by monitoring the reduction of absorbance at 340 nm for 5 min after the start of the reaction by addition of liver microsomes. The amount of oxidized NADPH was estimated using the molar extinction coefficient of NADPH at 340 nm, i.e., ε=6.27×10<sup>3</sup> M<sup>−1</sup> cm<sup>−1</sup>.

**Statistical Analysis** The results obtained from all experiments except kinetics experiments are expressed as the mean±S.E. of at least three independent experiments. The results obtained from kinetics experiments are expressed as the mean of two independent experiments. If necessary, the statistical analysis was carried out by ANOVA (StatView). Values of p<0.05 were considered to be statistically significant.

**RESULTS**

**Dose Dependence of the Inhibitory Effect of TJ-15 on Enzymatic Lipid Peroxidation** When enzymatic lipid peroxidation in liver microsomes with NADPH and ADP/Fe<sup>3+</sup> complex was examined in the presence of TJ-15 (5, 10, 50, or 100 µg/ml) over a 30-min period, lipid peroxidation was inhibited in proportion to the dose of TJ-15 and an apparent time lag was not observed in lipid peroxidation with TJ-15 50 or 100 µg/ml (Fig. 1A). Enzymatic lipid peroxidation with and without TJ-15 proceeded in a linear fashion at least between 4 and 10 min after the onset of reaction (Fig. 1A). When the dose dependence of the inhibitory effect of TJ-15 on enzymatic lipid peroxidation in liver microsomes was examined in the presence of TJ-15 1.0 to 100 µg/ml at 20 min after the onset of the reaction, TJ-15 showed a dose-dependent inhibition of the lipid peroxidation; TJ-15 1.0 µg/ml caused no significant inhibition but TJ-15 10 and 100 µg/ml resulted in 50% and 95% inhibition, respectively (Fig. 1B).

**Kinetic Analysis of the Inhibitory Effect of TJ-15 on Enzymatic Lipid Peroxidation** When the inhibitory effect of TJ-15 5 or 10 µg/ml on enzymatic lipid peroxidation in liver microsomes was examined in the presence of a certain concentration of ADP/Fe<sup>3+</sup> complex and various concentrations of NADPH and between 4 and 10 min after the onset of reaction, during which time the reaction proceeded in a linear fashion, TJ-15 showed competitive inhibition of the lipid peroxidation as a function of NADPH (Fig. 2A). When the inhibitory effect of TJ-15 5 or 10 µg/ml on enzymatic lipid peroxidation was determined in liver microsomes with a certain concentration of NADPH and various concentrations of ADP/Fe<sup>3+</sup> complex NADPH and between 4 and 10 min after the onset of reaction, during which time the reaction proceeded in a linear fashion, TJ-15 also showed competitive inhibition of lipid peroxidation as a function of the ADP/Fe<sup>3+</sup> complex concentration (Fig. 2B).

**Effect of TJ-15 on the NADPH-dependent Reduction of ADP/Fe<sup>3+</sup> Complex or Cytochrome c** When the effect of TJ-15 50 or 100 µg/ml on NADPH-dependent ADP/Fe<sup>3+</sup>...
complex reduction by liver microsomes was examined in the presence of a certain concentration of NADPH and various concentrations of ADP/Fe$^{3+}$ complex, TJ-15 inhibited the NADPH-dependent ADP/Fe$^{3+}$ complex reduction in a competitive manner, as shown in Fig. 3A. When the effect of TJ-15 50 or 100 µg/ml on NADPH-dependent cytochrome c reduction by liver microsomes was examined in the presence of a certain concentration of NADPH and various concentrations of cytochrome c, it also inhibited the NADPH-dependent cytochrome c reduction in a competitive manner, as shown in Fig. 3B.

**Effect of TJ-15 on NADPH Oxidation** When the effect of TJ-15 10 or 50 µg/ml on the oxidation of NADPH in liver microsomes with the ADP/Fe$^{3+}$ complex was examined for 5 min after addition of NADPH, TJ-15 10 or 50 µg/ml stimulated the NADPH oxidation, as shown in Fig. 4. The stimulatory effect of TJ-15 on the NADPH oxidation was much larger at 50 µg/ml than at 10 µg/ml (Fig. 4).

**Effect of Late Addition of TJ-15 on Enzymatic Lipid Peroxidation** When added to the reaction mixture containing liver microsomes, NADPH, and ADP/Fe$^{3+}$ at 10 min after the onset of reaction, TJ-15 5, 10, 50, or 100 µg/ml inhibited the progression of lipid peroxidation dose dependently, as shown in Fig. 5. The addition of 100 µg/ml of TJ-15 caused an almost complete inhibition of the progression of lipid peroxidation (Fig. 5).

**DISCUSSION**

In the present study, simultaneous addition of TJ-15 at concentrations of 1.0 to 100 µg/ml inhibited enzymatic lipid peroxidation, NADPH-dependent lipid peroxidation in rat liver microsomes and the inhibitory effect of TJ-15 was dose-dependent, as reported previously.$^{13,14}$ In addition, simultaneous addition of TJ-15 inhibited this enzymatic lipid peroxidation without an apparent time lag at higher concentrations (50 µg/ml or greater). It has been suggested that enzymatic lipid peroxidation in rat liver microsomes is initiated by re-
NADPH 0.3 mM, G-6-P 4 mM, G-6-PDH 0.4 U/ml, conducted in a reaction medium containing liver microsomes (0.15 mg protein/ml), each value is the mean of two independent experiments. ADP/Fe3+ by affecting the transport of electrons from NADPH to the hibits enzymatic lipid peroxidation in rat liver microsomes don't against ADP/Fe3+. These results suggest the possibility that TJ-15 in reduced form or against NADPH which acts as an electron donor. These results support the above-mentioned possibility that TJ-15 inhibits enzymatic lipid peroxidation in rat liver microsomes by affecting the transport of electrons from NADPH to the ADP/Fe3+ complex.

When the inhibitory action of simultaneously added TJ-15 on enzymatic lipid peroxidation in rat liver microsomes was analyzed kinetically, the Chinese medicine was found to competitively inhibit enzymatic lipid peroxidation in rat liver microsomes against ADP/Fe3+ complex which acts as a catalyst in reduced form or against NADPH which acts as an electron donor. These results suggest the possibility that TJ-15 inhibits enzymatic lipid peroxidation in rat liver microsomes by affecting the transport of electrons from NADPH to the ADP/Fe3+ complex.

It is known that NADPH-cytochrome P-450 reductase reduces cytochrome c and ADP/Fe3+ complex in the presence of NADPH17,49 Therefore, we attempted to clarify whether simultaneous addition of TJ-15 affects the transport of electrons from NADPH to NADPH-cytochrome P-450 reductase in rat liver microsomes by examining the effect of the Chinese medicine on the reduction of cytochrome c and ADP/Fe3+ complex by liver microsomes with NADPH. Simultaneously adding TJ-15 competitively inhibited the reduction of cytochrome c and ADP/Fe3+ complex by rat liver microsomes with NADPH. These results support the above-mentioned possibility that TJ-15 inhibits enzymatic lipid peroxidation in rat liver microsomes by affecting the transport of electrons from NADPH to the ADP/Fe3+ complex in a competitive manner.

We further examined whether TJ-15 affects the oxidation of NADPH in rat liver microsomes with ADP/Fe3+ complex. Simultaneous addition of TJ-15 was found to stimulate the oxidation of NADPH in rat liver microsomes with ADP/Fe3+ complex in a dose-dependent manner. This result indicates that TJ-15 reduces the flow of electrons from NADPH to the ADP/Fe3+ complex via NADPH-cytochrome P-450 reductase by stimulating NADPH oxidation in rat liver microsomes, although it is unknown at present how TJ-15 stimulates NADPH oxidation with the ADP/Fe3+ complex. TJ-15
may exert an inhibitory action on enzymatic lipid peroxidation in rat liver microsomes by reducing the flow of electrons from NADPH to the ADP/Fe$^{3+}$ complex via NADPH-cytochrome P-450 reductase both in a competitive manner and through stimulation of NADPH oxidation.

Furthermore, it was found that when added to the reaction medium for enzymatic lipid peroxidation in rat liver microsomes after the onset of lipid peroxidation, TJ-15 inhibited enzymatic lipid peroxidation in rat liver microsomes not only in the initiation step but also in the propagation step.

It is known that TJ-15 scavenges oxygen free radicals such as superoxide radical and hydroxyl radical in vitro.\(^8\)\(^{10}\) It has been indicated that oxygen free radicals such as superoxide radical, hydroxyl radical, and singlet oxygen participate in enzymatic lipid peroxidation in rat liver microsomes.\(^27\)\(^{34}\) We have observed that when the effects of superoxide dismutase, catalase, hydroxyl radical scavengers such as mannitol, sodium formate, and dimethylthiourea, and singlet oxygen quenchers such as histidine, diazabicyclooctane, and diphenyluran, on enzymatic lipid peroxidation in rat liver microsomes used in the present study are examined, singlet oxygen quenchers inhibited peroxidation by more than 90%, while superoxide dismutase, catalase, and hydroxyl radical scavengers inhibit it by less than 20% (unpublished data).

These results are consistent with those reported by Wright et al.\(^35\)\(^36\) who have proposed the involvement of singlet oxygen in the initiation of enzymatic lipid peroxidation in rat liver microsomes. Therefore the possibility that TJ-15 exerts an inhibitory action on enzymatic lipid peroxidation in rat liver microsomes by scavenging superoxide radical and/or hydroxyl radical appears slight. It has been reported that baikaline, which is present in Scutellaria Radix, one of the constituent herbs of TJ-15, and berberine, jatrohrrizine, magnoflorine, and palmatine, which are present in Coptidis Rhizoma, another constituent of TJ-15, function as efficient quenchers of singlet oxygen in vitro.\(^35\)\(^36\) Accordingly, the possibility cannot be ruled out that TJ-15 inhibits enzymatic lipid peroxidation in rat liver microsomes by scavenging singlet oxygen generated in the process of lipid peroxidation.

It has been shown that cytochrome P-450 participates in enzymatic lipid peroxidation in rat liver microsomes,\(^22\)\(^{24}\)\(^{26}\)\(^{37}\) although there is a report indicating no such involvement.\(^38\) It has also been shown that a heat-labile component, which is probably a protein distinct from cytochrome P-450, is associated with the iron reduction responsible for enzymatic lipid peroxidation in rat liver microsomes.\(^39\) Furthermore, it has been reported that flavonoids such as baikaline inhibit nonenzymatic, iron-induced lipid peroxidation in rat liver microsomes by forming an inert complex of iron or by enhancing iron oxidation.\(^40\)\(^41\) Therefore further investigation is required to elucidate the detailed mechanism of the inhibitory action of Oren-gedoku-to extract on enzymatic lipid peroxidation in rat liver microsomes.

In conclusion, the results of the present study indicate that Oren-gedoku-to extract inhibits enzymatic lipid peroxidation in rat liver microsomes in the initiation and propagation steps in a dose-dependent manner. These results also suggest that this inhibition occurs not only through its antioxidant action but also by reducing the supply of electrons derived from NADPH to the ADP/Fe$^{3+}$ complex in liver microsomes in both a competitive manner and through stimulation of NADPH oxidation.

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