Antioxidant Effect of Wen-Pi-Tang and Its Component Crude Drugs on Oxidative Stress

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Abstract: Oxidative stress plays a key role in the pathophysiologic process of acute and chronic renal diseases. Intracellular component such as lipids, proteins and nucleic acids are easily and rapidly oxidized by excessive reactive oxygen species (ROS), and such reactions lead to increased levels of lipid peroxide. The present study examined the antioxidant effects of Wen-Pi-Tang and its component crude drugs on 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)- or 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN)-induced ROS generation and lipid peroxidation of linoleic acid. As a result, Wen-Pi-Tang significantly decreased AAPH or AMVN-induced ROS in renal mitochondrial particles. For the components in Wen-Pi-Tang’s prescription, Rhei Rhizoma and Glycyrrhizae Radix extracts strongly inhibited peroxide levels, but Ginseng Radix, Aconiti Tuber and Zingiberis Rhizoma extracts were comparably low. Rhei Rhizoma extract showed the strongest inhibitory activity on oxidative injury, and two of its tannin compounds, (-)-epicatechin 3-O-gallate and procyanidin B-2 3,3′-di-O-gallate, inhibited AAPH or AMVN-induced ROS significantly. Thus, the present data suggest that Wen-Pi-Tang and its component crude drugs effectively prevent biological toxicity on oxidative stress through potent antioxidant and anti-lipid peroxidation activities.

Keywords: Wen-Pi-Tang; Rhei Rhizoma; Glycyrrhizae Radix; (-)-Epicatechin 3-O-gallate; Procyanidin B-2 3,3′-di-O-gallate; Oxidative Stress; Lipid Peroxidation.

Introduction

Great efforts have been made to search for safe and effective therapeutic agents for oxidative stress-induced diseases. Reactive oxygen species (ROS) or free radical-mediated...
lipid peroxidation has a harmful effect on biological systems and may lead to disease or its severe progression (Halliwell and Gutteridge, 1999; Mylonas and Kouretas, 1999; Wood et al., 2003). When cellular accumulation of ROS occurs in biological tissue, these oxygen radicals initiate excessive peroxidation in polyunsaturated fatty acids of membranes, which leads to inactivation of membrane-bound receptors and enzymes, and disruption of membrane structure causing cellular injury, thereby leading to so-called oxidative damage (Mlakar and Spiteller, 1994; Kubo et al., 1997; Haraguchi et al., 2000). In addition, mitochondria, a major source of ROS or free radicals, is considered to be the most important site of electron transfer action on peroxidative process (Sohal, 1997; Landar et al., 2006). Therefore, antioxidant action against oxidative damage could be estimated by measuring ROS generation in mitochondria and ROS-induced lipid peroxidation.

In our previous researches on the pharmacological effects and mechanisms of traditional Chinese medicines on antioxidant activity, Wen-Pi-Tang extract showed prominent antioxidant and free radical-scavenging activities on oxidants such as nitric oxide (NO), superoxide (O$_2^-$) and peroxynitrite (ONOO$^-$) (Yokozawa et al., 1998; 2001). Moreover, broad pharmacological effects of this prescription have been reported, such as increased glomerular filtration rate, suppressed the proliferation of mesangial cells and reduced accumulation of uremic toxins in renal failure (Yokozawa et al., 1986; 1989; 1994; 2006). Wen-Pi-Tang is prepared with five crude drugs, and among the component crude drugs, Rhei Rhizoma, Zingiberis Rhizoma and Glycyrrhizae Radix played important roles in the antioxidant action of Wen-Pi-Tang (Yokozawa et al., 2000; Rhyu et al., 2002). Especially Rhei Rhizoma and its two tannin compounds, (-)-epicatechin 3-O-gallate (ECg) and procyanidin B-2 3,3′-di-O-gallate (PCg), have been reported to have the most protective effect in the thiobarbituric acid-reactive substance and 1,1-diphenyl-2-picrylhydrazyl radical generating systems (Yokozawa et al., 1991; 1998).

Therefore, we have tried to study the antioxidant effects of Wen-Pi-Tang and its component crude drugs on the oxidative stress in renal mitochondrial particles when azo compounds, hydrophilic 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) or lipophilic 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), are intraperitoneally administrated to mice. In addition, anti-lipid peroxidation activity was investigated in a linoleic acid system by the thiocyanate method.

Materials and Methods

Chemicals

AAPH, AMVN, butylhydroxytoluene (BHT) and L-ascorbic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). 2′,7′-Dichlorodihydrofluorescein diacetate and acetyl ester (H$_2$DCFDA) were purchased from Molecular Probes (Eugene, Oregon, USA). Esterase (EC 3.1.1.1) was from Sigma Chemical Co. (St. Louis, MO, USA). Linoleic acid was from ICN Pharmaceuticals, Inc. (Costa Mesa, CA, USA).
Preparation of Wen-Pi-Tang Extract

The Wen-Pi-Tang prescription was prepared with 15 g Rhei Rhizoma (*Rheum officinale* BAILLON), 3 g Ginseng Radix (*Panax ginseng* C.A. MEYER), 9 g Aconiti Tuber (*Aconitum japonicum* THUNBERG), 3 g Zingiberis Rhizoma (*Zingiber officinale* ROSCOE) and 5 g Glycyrrhizae Radix (*Glycyrrhiza glabra* LINN. var. *glandulifera* REGEL et HERDER). Aconiti Tuber was obtained from Japan, Ginseng Radix was produced in Korea and the other elements were from China. These plants were identified by the botanist Prof. I. Nishioka, and a voucher specimen is deposited in the Herbarium of Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan. As previously described (Oura *et al.*, 1984), the extract was prepared by boiling the above crude drugs gently in 1,000 ml of water for 60 min and the mixture was concentrated under reduced pressure, resulting in a yield of about 30% by weight of the original preparation.

Preparation of Crude Drug Extracts

One hundred grams of each crude drug component of Wen-Pi-Tang was boiled gently in 1,000 ml water for 5~60 min, according to the Wen-Pi-Tang preparation procedure described previously (Oura *et al.*, 1984), and each extract was concentrated under reduced pressure to leave a residue. The yields of Rhei Rhizoma, Ginseng Radix, Aconiti Tuber, Zingiberis Rhizoma and Glycyrrhizae Radix were 21%, 32%, 37%, 11% and 20%, respectively, by weight, of the original preparation.

Animals and Treatments

The “Guidelines for Animal Experimentation” approved by the University of Toyama were followed during these experiments. Male ddY mice weighing 25 to 30 g (5 weeks) were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were kept in an air-conditioned room with an alternating 12 hours light/dark cycle, and provided with commercial pellet chow (CLEA Japan Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid and 60.5% carbohydrate) and water *ad libitum*. After several days of adaptation, mice were injected intraperitoneally with 80 mg/kg body weight of AAPH or AMVN dissolved in physiological saline. The single dose and time of injection of AAPH or AMVN were conducted as previously described method of Terao and Niki (1986). After 16 hours of the administration of AAPH or AMVN, the mice were sacrificed, and subsequently, the kidney was retrogradely perfused using a syringe with ice-cold physiological saline. The renal tissues were removed and kept at −80°C until analysis.

Preparation of Mitochondrial Particles

Renal mitochondria were isolated by modifying a previously described method (Paraidathathu *et al.*, 1992). The renal tissue was suspended in 50 mM cold potassium
phosphate buffer (pH 7.4) and homogenized using a glass Heidon homogenizer. The homogenate was centrifuged at 900 g for 15 min to discard nuclei and cell debris. The supernatant was centrifuged at 12,000 g for 15 min at 4°C, and the pellet was resuspended in fresh phosphate buffer for use in all studies as mitochondrial particles (1 mg protein/ml). Protein was determined by the micro-biuret method (Itzhaki and Gill, 1964) with bovine serum albumin as a standard.

Measurement of Mitochondrial ROS

ROS generation in renal mitochondria by azo compound administration (AAPH or AMVN) was measured by using H$_2$DCFDA. It is converted into a non-fluorescent derivative DCFH by esterase after incorporation into cells, and DCFH is rapidly oxidized to the highly fluorescent DCF in the presence of ROS (Valkonen and Kuusi, 1997; Landar et al., 2006). In this study, the solution of 10 µM H$_2$DCFDA and 6 U esterase in 1 ml phosphate buffer was incubated at 22°C for 20 min. Mitochondrial ROS was determined in 96-well plates containing mitochondrial suspension, DCFH and the sample solution (total 250 µl). Following incubation for 1 hour, the fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a microplate fluorescence reader, Tecan SPECTRAFluor (Tecan UK, Goring-on-Thames, UK).

Assay of Lipid Peroxidation in a Linoleic Acid System by the Thiocyanate Method

Autoxidation of linoleic acid was determined using the methods of Kikuzaki and Nakatani (1993) and Park et al. (1999). Different concentrations of samples dissolved in EtOH and phosphate buffer were added to the reaction mixture [2 ml of 2.53% linoleic acid in EtOH, 4 ml of 50 mM phosphate buffer (pH 7.0)] in screwcapped vials. At different intervals during incubation, a 100 µl aliquot of the reaction mixture was taken and diluted with 4.7 ml of 75% EtOH, followed by the addition of 100 µl of 30% ammonium thiocyanate. Precisely 3 min after adding 100 µl of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance at 500 nm was measured.

Statistics

The results were expressed as mean ± SE of four values. The effect on each parameter was examined using the one way analysis of variance. Individual differences between groups were evaluated using Dunnett’s test and those at p < 0.05 were considered significant.

Results

Toxic Effects of AAPH or AMVN Administration

Almost all mice, received an intraperitoneal injection of 80 mg/kg body weight of AAPH, showed symptoms of tachypnea and dyspnea in a few minutes. In addition, two
mice (seven mice total) died within 15 min, but survivors showed no abnormal behavior or symptoms. The mice received AMVN exhibited no obvious symptoms, but two mice (eight mice total) died within 10 min. The toxic effects of AAPH and AMVN were well documented previously by Terao and Niki (1986), on observations of liver, kidney, and thymus damage.

Effects of Wen-Pi-Tang and Its Active Compounds on the Renal Mitochondrial ROS Levels

Figure 1 shows the effect of Wen-Pi-Tang and its component crude drugs on ROS levels in AAPH- or AMVN-induced oxidative stress in renal mitochondria. ROS levels in the control group were significantly increased by AAPH or AMVN administration compared to the normal group. However, this elevated ROS level was effectively decreased to 67.5%, 67.4% and 80.1% by the addition of Wen-Pi-Tang, Rhei Rhizoma, and Glycyrrhizae Radix
extracts (100 µg/ml), respectively (Fig. 1A). Ginseng Radix, Aconiti Tuber and Zingiberis Rhizoma extracts showed comparably lower activities than that of Wen-Pi-Tang. Similarly, in AMVN-administrated group, Wen-Pi-Tang, Rhei Rhizoma, and Glycyrrhizae Radix extracts (100 µg/ml) lowered the increased ROS-generations to 53.3%, 54.8% and 65.9%, respectively (Fig. 1B).

Figure 2 shows the ROS-scavenging activity of ECg and PCg on AAPH- or AMVN-induced ROS generation in renal mitochondria. Mitochondrial ROS levels were significantly increased in both AAPH- and AMVN-administrated groups to 142.6% and 160.3%, respectively, but ROS were effectively inhibited by ECg and PCg. When AAPH was administrated to the mice, ECg and PCg (1 and 10 µg/ml) showed a distinctive decrease of ROS generation from 90.6% to 50.6% and from 82.7% to 69.6%, respectively. Also, these compounds (10 µg/ml) in AMVN-administrated mice had showed lowered ROS-levels of 63.8% and 88.2%, respectively.

Figure 2. Antioxidant effects of (-)-epicatechin 3-O-gallate (ECg) and procyanidin B-2 3,3′-di-O-gallate (PCg) on AAPH- or AMVN-induced ROS generation. (A) AAPH-treated; (B) AMVN-treated; N, non-treated; C, AAPH- or AMVN-treated control. Significance: +p < 0.05, †p < 0.01, ‡p < 0.001 vs. non-treated values, ‡p < 0.001 vs. AAPH- or AMVN-treated control values. ■, 1 µg/ml; □, 5 µg/ml; △, 10 µg/ml.
Measurement of Anti-lipid Peroxidation Activity

Anti-lipid peroxidation activity of Wen-Pi-Tang on the decomposition product of linoleic acid was compared to well-known antioxidants such as BHT and L-ascorbic acid. As shown in Fig. 3, the progression of lipid peroxidation of linoleic acid was shown over three days and it was decreased to about 45% in the presence of Wen-Pi-Tang extract (10 µg/ml), whereas L-ascorbic acid, a natural antioxidant, did not reduce the peroxidation of linoleic acid. However, the anti-lipid peroxidation activity of Wen-Pi-Tang was lower than that of BHT, a synthetic antioxidant.

Figure 4 shows the protective activities of Wen-Pi-Tang and its component crude drugs on lipid peroxidation at the concentrations of 5, 10 and 50 µg/ml. Wen-Pi-Tang extracts, 10 and 50 µg/ml, showed a significant decrease in lipid peroxidation to less than 50%, compared to the control. Rhei Rhizoma showed the strongest activity even in the low
concentration of 5 µg/ml. In addition, Zingiberis Rhizoma and Glycyrrhizae Radix extracts also decreased the progression of lipid peroxidation in a dose-dependent manner, but these extracts exhibited relatively low inhibitory effects compared to Wen-Pi-Tang and Rhei Rhizoma. Ginseng Radix and Aconiti Tuber extracts have nearly no protection against lipid peroxidation even in the highest concentration.

Discussion

Traditional Chinese medicines consist of various crude drugs. Their antioxidant activities to scavenge ROS based on their active constituents. Our previous researches have shown that the Chinese prescription, Wen-Pi-Tang and its component crude drugs strongly ameliorated renal damage and lesions via antioxidant or radical scavenging activity in ROS-induced renal injury (Yokozawa et al., 1989; 2000; 2001). In particular, Wen-Pi-Tang extract attenuated the renal damage induced by ONOO− through scavenging ONOO− and boosting the antioxidative defense systems in an animal model and renal tubular cell cultured system, respectively (Rhyu et al., 2002; Yokozawa et al., 2003). ONOO−, as a strong oxidant formed via the reaction of NO and O2−, has been suggested to be cytotoxic itself and to decompose into other toxic species, such as the hydroxyl radical (Beckman and Koppenol, 1996). However, it has not been established whether Wen-Pi-Tang and its component crude drugs are directly involved in ROS generation in renal mitochondria and ROS-induced lipid peroxidation.

Immoderate ROS level and ROS-induced lipid peroxidation plays a key role in chronic degenerative diseases including cardiovascular disease, some forms of cancers, age-related degeneration, rheumatoid arthritis, neurodegenerative disease, diabetes and renal failure (Sies, 1991; Diplock, 1994; Halliwell and Gutteridge, 1999). Lipid peroxidation of the mitochondrial membrane induces especially strong structural and functional alternations of proteins, nucleic acids and small molecules, and the inhibition of this peroxidative process in mitochondria has received much attention for preventing oxidative stress-related pathological conditions. Therefore, functional and morphological defects in the mitochondrial population have been associated with severe tissue damage (Richter et al., 1995; Scarpulla, 1997; Lee et al., 2000; Landar et al., 2006).

In the present study, we evaluated the antioxidant effect of Wen-Pi-Tang and its component crude drugs on oxidative damage stimulated in AAPH- or AMVN-administrated mice, and the amount of peroxides which are formed in emulsion during incubation of linoleic acid, the target of lipid peroxidation. These azo compounds (AAPH and AMVN), well-known as radical initiators, are useful tools for studying free-radical mediated damage in biological systems, since they generate free radicals by thermal decomposition without biotransformation (Niki, 1990). Hydrophilic AAPH added to the aqueous phase generates radicals in the aqueous region, whereas lipophilic AMVN located in the lipid region of micelles or the membrane initially generates radicals within the lipid region (Yamamoto et al., 1984; Niki et al., 1988). The carbon radicals derived from AAPH or AMVN react with oxygen rapidly to give peroxy radicals, which attack the mitochondrial membrane. On the other hand, AAPH and AMVN compounds have been reported to cause lipid peroxidation
WEN-PI-TANG ON OXIDATIVE STRESS

(Barclay et al., 1984), plasma oxidation (Frei et al., 1988), oxidative modification of low density lipoprotein and enzyme inactivation (Sato et al., 1990). Terao and Niki (1986) also reported that the intraperitoneal administration of AAPH into mice induced swelling and disruption in various organs and fatty degeneration of the liver and kidney. Therefore, we chose AAPH and AMVN, and administered intraperitoneally to mice to study their antioxidant effects on renal mitochondria damage induced.

As shown in the above results, we confirmed that ROS levels of renal mitochondria significantly increased in the intraperitoneal AAPH or AMVN administrated-mice in comparison to normal ones, and the ROS level generated by AMVN was higher than that of AAPH. We also found that Wen-Pi-Tang, Rhei Rhizoma and Glycyrrhizae Radix extracts strongly inhibited generation of AAPH- or AMVN-induced mitochondrial ROS in renal tissue, and reduced peroxide formation of the linoleic acid emulsion. In addition, Wen-Pi-Tang extract showed a higher anti-lipid peroxidation activity than L-ascorbic acid, a natural antioxidant. On the other hand, Ginseng Radix, Aconiti Tuber and Zingiberis Rhizoma extracts showed a reducing response of ROS induced by AAPH or AMVN, but it was not as strong as the other components. Therefore, these findings may suggest that Wen-Pi-Tang decreases ROS generation by the increase of mitochondrial antioxidant defense system and prevents the ROS-mediated damage. In addition, Rhei Rhizoma and Glycyrrhizae Radix were more responsible for the antioxidant effect of Wen-Pi-Tang. Rhei Rhizoma was the most effective component drug.

Furthermore, two active tannin compounds of Rhei Rhizoma, ECg and PCg, were tested. They significantly inhibited ROS generation in renal mitochondria of AAPH- or AMVN-administred mice. In addition, they also reduce activated oxygen in adenine-induced renal failure by the decrease of methylguanidine, a strong uremic toxin in our previous study (Yokozawa et al., 1991). The present observations together with previous findings suggest that these two tannin compounds are more responsible for the antioxidant effects of Wen-Pi-Tang and Rhei Rhizoma on the oxidative damage of renal mitochondria. The inhibition of ROS generation by ECg and PCg in this study is in good agreement with Niki et al. (2005) suggesting that the free radical-mediated lipid peroxidation may be inhibited by the inhibition of free radical formation, chain initiation, chain propagation and/or acceleration of chain termination.

In conclusion, we demonstrated that Wen-Pi-Tang and its component crude drugs possess antioxidant and anti-lipid peroxidation activities through their direct suppression of mitochondrial ROS generation. Therefore, these drugs may improve renal injury through their antioxidant effects and inhibition of functional alterations of renal mitochondria by oxidative stress.

References


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