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Antiperoxidative Activity of Neolignans from *Magnolia obovata*

HIROYUKI HARAGUCHI, HARUMI ISHIKAWA, NORITOSHI SHIRATAKI AND AYUMI FUKUDA

Faculty of Engineering, Fukuyama University, Gakuen-cho, Fukuyama 729-02, Japan

Abstract

Honokiol and magnolol, neolignans in *Magnolia obovata*, have been evaluated as antioxidants.

Microsomal lipid peroxidation induced by Fe(III)-ADP/NADPH and mitochondrial lipid peroxidation induced by Fe(III)-ADP/NADH were inhibited by these compounds. These neolignans protected mitochondrial respiratory chain enzyme activity against NADPH-induced peroxidative stress and protected red cells against oxidative haemolysis. The anti-oxidative activity of honokiol was more potent than that of magnolol.

Neolignans in *M. obovata* were shown to be effective in protecting biological systems and functions against oxidative stress.

Membrane lipids are susceptible to oxidation not only because of their high polyunsaturated fatty-acid content but also because of their association in the cell membrane with enzymic and non-enzymic systems capable of generating free-radical species. The oxidation of unsaturated fatty acids in biological membranes leads to a reduction in membrane fluidity and disruption of membrane structure and function (Slater & Cheeseman 1987). Cellular damage as a result of lipid peroxidation causes serious derangement, such as ischemia-reperfusion injury, diabetes mellitus and coronary arteriosclerosis (Kok et al 1991; Sugawara et al 1992), as well as being linked with ageing and carcinogenesis (Yagi 1987). Some antioxidants have been reported to prevent cancer and coronary heart disease (Gay 1992), to protect the myocardium from experimental myocardial infarction (Menon et al 1992), and to be a prophylactic agent against some neuronal symptoms of ageing (Hiramatsu et al 1992).

The dried bark of *Magnolia obovata* (Magnoliaceae) has been used for the treatment of acute pain, diarrhoea, coughs and urinary problems. Honokiol and magnolol (Fig. 1) are constituents of the bark of this medicinal plant. These neolignans have a variety of pharmacological activity, including anti-platelet activity (Teng et al 1988), anti-inflammatory effects (Wang et al 1995), muscle relaxation activity (Teng et al 1990), central nervous inhibition (Watanabe et al 1983) and anti-microbial activity (Clark et al 1981). These phenolic compounds have, furthermore, been found to be effective scavengers of hydroxyl radicals (Taira et al 1993) and to inhibit UV-induced mutation (Fujita & Taira 1994). In our continuous search for anti-oxidative compounds from botanical sources, honokiol and magnolol showed potent inhibition of lipid peroxidation. This report describes the anti-oxidative activity of these compounds in liver mitochondria and microsomes and in red blood cells.

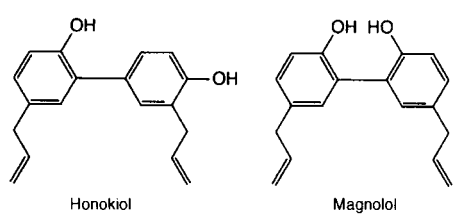

FIG. 1. Structure of antioxidative neolignans from *Magnolia obovata*.

Correspondence: H. Haraguchi, Faculty of Engineering, Fukuyama University, Gakuen-cho, Fukuyama 729-02, Japan.

Materials and Methods

Chemicals

Honokiol and magnolol, isolated from *M. obovata*, were provided by Wako Pure Chemical Industries (Osaka, Japan). Butylated hydroxytoluene, thiobarbituric acid, ADP and cytochrome c were purchased from Sigma (St Louis, MO, USA). NADH and NADPH were obtained from Oriental Yeast Co. (Tokyo, Japan). Other chemical reagents were of commercial grade.

Preparation of mitochondria and microsomes

The livers were quickly removed from male Wistar rats, 100–150 g, and dropped into ice-cold 3 mM Tris-HCl buffer (pH 7·4) containing 0·25 M sucrose and 0·1 mM EDTA. Mitochondria were obtained by centrifugation at 15 000 *g*. Submitochondrial particles were prepared by sonication for 1 min at 4°C by use of a Branson Sonifier 450. Microsomes were obtained after centrifugation at 105 000 *g* for 60 min (Haraguchi et al 1995). Protein concentrations in the suspensions were determined by the method of Lowry et al (1951).

Measurement of lipid peroxidation

The NADPH-dependent peroxidation of microsomal lipid was assayed by a modification of the method described by Pederson et al (1973). Rat-liver microsomes (equivalent to 0·2 mg protein) were incubated at 37°C in reaction mixture (1 mL) containing 0·05 M Tris-HCl (pH 7·5), 2 mM ADP, 0·12 mM $Fe(NO_3)_3$, and 0·1 mM NADPH. The reaction was initiated by addition of NADPH. After 5 min, TCA-TBA-HCl reagent (15% w/v trichloroacetic acid and 0·375% thiobarbituric acid in 0·25 M HCl; 2 mL) and 2% butylated hydroxytoluene (90 μL) were added to the reaction mixture. The solution was heated for 15 min on a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 *g* for 10 min. The absorbance of the supernatant was determined at 535 nm (Buege & Aust 1978).

Mitochondrial lipid peroxidation was assayed by a modification of the method described by Takayanagi et al (1980). Rat-liver submitochondrial particles (equivalent to 0·3 mg protein) were incubated at 37°C in reaction mixture (1 mL) containing 50 mM HEPES-NaOH (pH 7·0), 2 mM ADP, 0·1 mM $FeCl_3$, 10 μM rotenone and 0·1 mM NADH. The reaction was initiated by addition of NADH. After 5 min the reaction was terminated and lipid peroxidation was determined as above.

Mitochondrial peroxidation and assay for enzyme activity

NADPH-dependent peroxidation of rat-liver submitochondrial particles was achieved at 25°C in a medium containing 0·1 M mannitol, 5 mM potassium phosphate (pH 7·4), 10 mM Tris-HCl (pH 7·4), 0·1 mM EDTA, 1 mM ADP and 0·3 mM $FeCl_3$ (Nishida et al 1987). The reaction was started by addition of 0·5 mM NADPH. At intervals during incubation, mitochondrial suspensions were removed from the mixture and NADH-cytochrome c reductase and succinate-cytochrome c reductase activity were measured.

The reductase activity was assayed by measuring the increase in the absorbance at 550 nm resulting from the reduction of cytochrome c. The reaction mixtures contained 50 mM potassium phosphate buffer (pH 7·4), 5 mM NaN_3, 2·1 mg oxidized cytochrome c, and 200 μM NADH or 20 mM sodium succinate in a total volume of 3 mL (Ulrich & Mathre 1972).

Preparation of erythrocyte and assay for haemolysis

Blood from healthy donors was collected in heparinized tubes. Erythrocytes were separated, by centrifugation, from plasma and buffy coat and were washed three times with saline. During the last washing, the cells were centrifuged at 2000 *g* for 10 min to obtain a uniformly packed cell preparation. A 10% suspension of erythrocytes in a solution containing 152 mM NaCl and 10 mM sodium phosphate buffer (pH 7·4) was preincubated at 37°C for 5 min before addition of same volume of 100 mM 2,2′-azo-bis(2-amidinopropane) dihydrochloride in the same buffered saline. The reaction mixture was gently shaken at 37°C. At intervals during the incubation two samples were removed from the mixture; one was diluted with 20 vol. 0·15 M NaCl, the other with distilled water, to effect complete haemolysis. Both samples were centrifuged at 1000 *g* for 10 min. The absorbance of the supernatant was determined at 540 nm. The percent haemolysis was calculated according to the equation described by Miki et al (1986).

Results and Discussion

Microsomes, especially smooth-surfaced endoplasmic reticulum, easily produce lipid peroxides and are thought to supply the peroxidation products to other tissues (Roders 1978). NADPH-cytochrome P450 reductase is involved in NADPH-induced microsomal lipid peroxidation. Lipid peroxidation, which can be measured by the TBA method, occurs when rat-liver microsomes are incubated with Fe(III)-ADP/NADPH (Pederson et al 1973). A common synthetic antioxidant, butylated hydroxytoluene at 30 μM, completely inhibited this peroxidation (data not shown). Honokiol showed potent inhibition of rat-liver microsomal lipid peroxidation; almost complete inhibition was observed at 37·5 μM. The anti-oxidative activity of magnolol in microsomes was, on the other hand, moderate. The 50% inhibitory concentrations are shown in Table 1.

Table 1. Antioxidative activity of neolignans from *Magnolia obovata*.

	IC50 (μM) Microsomal lipid peroxidation	Mitochondrial lipid peroxidation
Honokiol	8·7	7·0
Magnolol	53·4	11·3

Inhibitory activity was expressed as the mean 50% inhibitory concentration of triplicate determinations, obtained by interpolation of concentration–inhibition curves.

Redox reactions frequently occur in mitochondria, which are constantly susceptible to oxidative stress. Electrons, at least, at two sites of the mitochondrial electron transport system, leak and react with oxygen to generate superoxide anions, and subsequently hydrogen peroxide (Nohl 1986). Lipid peroxides produced by the hydroxy radical (OH·), itself derived from H_2O_2 and O_2^-, affect mitochondrial function. Lipid peroxidation by submitochondrial particles is supported by NADH or NADPH in the presence of ADP and Fe(III) (Takayanagi et al 1980). Complete inhibition was obtained with 30 μM butylated hydroxytoluene (data not shown). Neolignans from *M. obovata* showed more potent inhibition of mitochondrial lipid peroxidation than of microsomal peroxidation. Honokiol at 11·2 μM resulted in complete inhibition; magnolol at 37·5 μM also caused 90% inhibition. The 50% inhibitory concentrations of the compounds are shown in Table 1.

Various oxidative stresses affect mitochondrial enzyme activity. NADH-cytochrome c reductase and succinate-cytochrome c reductase are the most sensitive sites of mitochondrial peroxidative injury. NADPH-dependent lipid peroxidation in submitochondrial particles results in a remarkable loss of these enzyme activities (Narabayashi et al 1982). When rat-liver mitochondria were incubated with Fe(III)-ADP/NADPH, membrane lipids were peroxidized and NADH- and succinate-cytochrome c reductase activities were reduced; 80% loss of activity of NADH-cytochrome c reductase and almost complete loss of activity of succinate-cytochrome c reductase were observed for 1·5- and 1-h incubation, respectively. Honokiol and magnolol, anti-oxidative phenolic compounds from *M. obovata*, at 11·2 μM, protected the activity of both enzymes against NADPH-induced peroxidation. Even after 2-h incubation 90–95% of the activity of NADH-cytochrome c reductase and 80–90% of the activity of succinate-cytochrome c reductase were maintained (Fig. 2).

The lipids in the red cell membrane are highly unsaturated and the red cells are exposed to a higher oxygen tension than are all other tissues. The red cells are, furthermore, filled with haemoglobin, one of the most powerful catalysts capable of initiating lipid peroxidation. When erythrocytes were incubated in air at 37°C, they were stable and little haemolysis occurred within 5 h. The peroxy radicals generated by thermal decomposition of the azo-initiator 2,2′-azo-bis(2-amidinopropane) dihydrochloride induce free-radical chain oxidation in erythrocyte membranes (Miki et al 1986). During 5-h incubation with the azo-initiator, 95% of the erythrocytes were damaged. Honokiol and magnolol inhibited the lysis of red

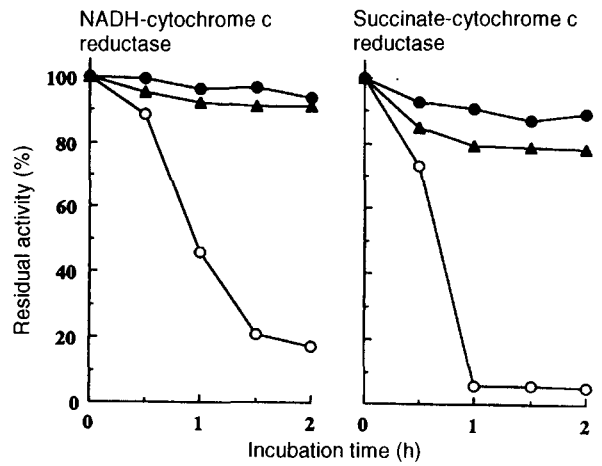

FIG. 2. Effects of neolignans from *Magnolia obovata* on NADPH-dependent oxidative injury of the mitochondrial respiratory chain. Results are the means of triplicate determinations. ●, Honokiol at 11·2 μM; ▲, magnolol at 11·2 μM; ○, control.

cells by peroxy radical attack. Almost 70 and 55% inhibition, respectively, were observed for 37·5 μM concentrations of honokiol and magnolol (Fig. 3).

The neolignans honokiol and magnolol are the oxidative coupling products of phenylpropanoids. Phenylpropanoids have been reported to be potent antioxidants (Kumaravelu et al 1995). Eugenol, a typical hydrophobic phenylpropanoid, has been suggested as interfering with lipid peroxidation by propagation radicals (Nagababu & Lakshmaiah 1992). In the case of anti-oxidative neolignans from *M. obovata*, electron-donating groups are present in the *ortho* position of a phenolic hydroxy function. Phenoxy radicals which would be generated

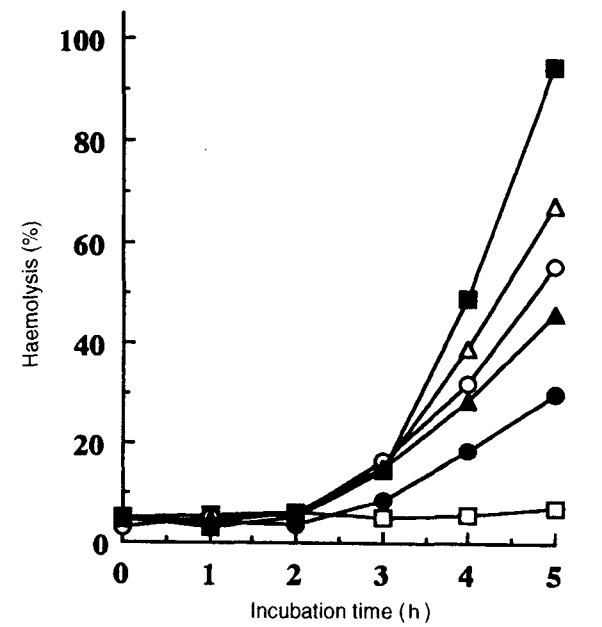

FIG. 3. Inhibition of oxidative haemolysis in human erythrocytes by neolignans from *Magnolia obovata*. Results are the means of triplicate determinations. ●, Honokiol at 37·5 μM; ○, honokiol at 11·2 μM; ▲, magnolol at 37.5 μM; △, magnolol at 11·2 μM; ■, control; □ without 2,2′-azo-bis(2-amidinopropane)dihydrochloride.

in the process of anti-peroxidation might be stabilized not only by their aromatic character, but also by the presence of electron-donating groups (Cuvelier et al 1992). These phenolic antioxidants might act as hydrogen atom donors to peroxy radicals (LOO·), resulting in termination of the chain radical reaction.

These results on biological systems showed that neolignans in *M. obovata* were effective at protecting tissues and cells against a variety of oxidative stress.

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