J. Pharm. Pharmacol. 1996, 48: 441–443
Received July 5, 1995
Accepted December 5, 1995

Inhibition of Lipid Peroxidation by Sesquiterpenoid in *Heterotheca inuloides*

HIROYUKI HARAGUCHI, TAKASHI SAITO, HARUMI ISHIKAWA, YOLANDA SANCHEZ*, TETSUYA OGURA* AND ISAO KUBO†

*Faculty of Engineering, Fukuyama University, Gakuen-cho, Fukuyama 729-02, Japan, *Departamento de Quimica, Universidad Autonoma de Guadalajara, Guadalajara, Mexico, and †Department of Environmental Science, Policy and Management, University of California, Berkeley, CA 94720-3112, USA*

Abstract

A sesquiterpenoid, 7-hydroxy-3,4-dihydrocadalin, isolated from a Mexican medicinal plant *Heterotheca inuloides* was evaluated as an antioxidant.

This sesquiterpenoid inhibited mitochondrial and microsomal lipid peroxidation induced by Fe(III)-ADP/NADH or Fe(III)-ADP/NADPH. Furthermore, 7-hydroxy-3,4-dihydrocadalin protected red cells against oxidative haemolysis.

This sesquiterpene was thus shown to be effective in protecting biological systems against oxidative stresses.

The oxidation of unsaturated fatty acids in biological membranes leads to a decrease in the membrane fluidity and disruption of membrane structure and function (Machlin & Bendich 1987; Slater & Cheeseman 1987). Cellular damage due to lipid peroxidation causes serious damage, such as ischaemia-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). Inhibition of membrane peroxidation has been shown to have a protective effect in the initiation and promotion of certain cancers and in side effects of several cytostatic agents (Lin et al 1992; Rousseau et al 1992).

Sesquiterpenoids and flavonoids were isolated from the dried flower of *Heterotheca inuloides* (Fig. 1), a Mexican medicinal plant, as antimicrobial or tyrosinase inhibitory substances (Kubo et al 1994a, b). In our continuous search for bioactive substances from *H. inuloides*, 7-hydroxy-3,4-dihydrocadalin was found to exhibit potent inhibitory activity against animal tissue lipid peroxidation. This report describes the antioxidative activity of 7-hydroxy-3,4-dihydrocadalin in liver mitochondria and microsome, and red blood cells.

Materials and Methods

Chemicals

7-Hydroxy-3,4-dihydrocadalin was isolated from the dried flower of *H. inuloides* (Kubo et al 1994b). Butylated hydroxytoluene (BHT), thiobarbituric acid and ADP were purchased from Sigma Chemical Co. (St Louis, MO, USA). NADH and NADPH were obtained from Oriental Yeast Co. (Tokyo, Japan). Other chemical reagents were of commercial grade.

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

Preparation of mitochondria and microsomes

Livers of Wistar male rats, 100–150 g, were removed quickly 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 using a Branson Sonifier 450. Microsomes were obtained after centrifugation at 105 000 *g* for 60 min. Protein concentrations of 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 the modified method described by Pederson et al (1973). Rat liver microsomes (equivalent 0·2 mg protein) were incubated at 37°C in 1 mL reaction mixture 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 the addition of NADPH. After 5 min, 2 mL of TCA-TBA-HCl reagent (15% w/v trichloroacetic acid; 0·375% thiobarbituric acid; 0·25 M HCl) and 90 μL of 2% BHT were added to the reaction mixture. The solution was heated for 15 min in 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 the modified method described by Takayanagi et al (1980). Rat liver submitochondrial particles (equivalent 0·3 mg protein) were incubated at 37°C in 1 mL of reaction mixture 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 the addition of NADH. After 5 min, the reaction was terminated and lipid peroxidation was determined as above.

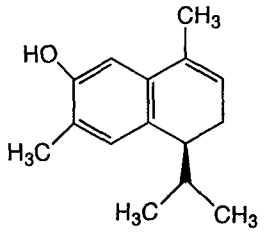

FIG. 1. Structure of sesquiterpene in *Heterotheca inuloides*.

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 constantly-packed cell preparation. A 10% suspension of erythrocytes in the 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 the same volume of 100 mM AAPH in the same buffer saline. The reaction mixture was gently shaken at 37°C. At intervals during incubation, two samples were taken out from the mixture; one sample was diluted with 20 vol 0·15 M NaCl and the other with distilled water to yield complete haemolysis. Both samples were centrifuged at 1000 *g* for 10 min. The absorbance of the supernatants was determined at 540 nm. The percent haemolysis was calculated according to the equation described by Miki et al (1986).

Results and Discussion

Membrane lipids are particularly 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 nonenzymic systems capable of generating free-radical species (Halliwell & Gutteridge 1990). Microsomes, especially smooth-surfaced endoplasmic reticulum, easily produce lipid peroxides and are thought to supply the peroxidation products to other tissues (Roders 1978). NADPH-P-450 reductase and cytochrome P-450 are involved in NADPH-induced microsomal lipid peroxidation. Lipid peroxidation, which can be measured by the thiobarbituric acid method, occurs when rat liver microsomes are incubated with Fe(III)-ADP/NADPH (Pederson et al 1973). The production of lipid peroxides induced by microsomal NADPH oxidation was inhibited by 7-hydroxy-3,4-dihydrocadalin with almost complete inhibition at 45 μM (Fig. 2).

Redox reactions frequently occur in mitochondria, which are constantly susceptible to oxidative stress (Wiswedel et al 1989). Lipid peroxidation by submitochondrial particles is supported by NADH or NADPH in the presence of ADP and Fe(III) (Takayanagi et al 1980). Mitochondrial lipid peroxidation was strongly inhibited by 7-hydroxy-3,4-dihydrocadalin with almost complete inhibition at 15 μM (Fig. 3).

The lipids in the red cell membrane are highly unsaturated and the red cells are exposed to a higher oxygen tension than all other tissues. Furthermore, the red cells are packed with

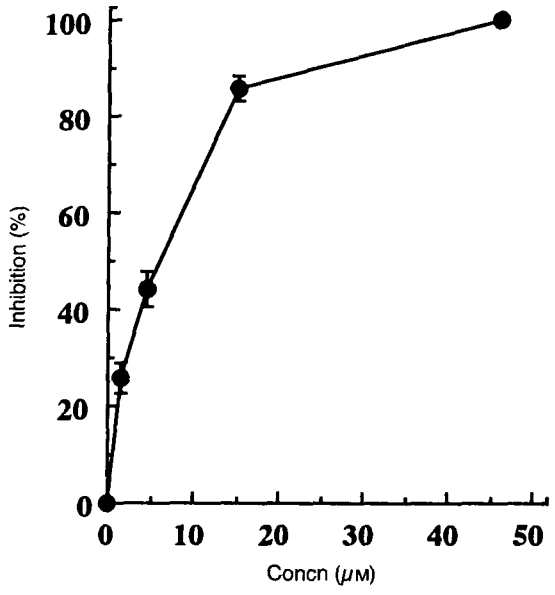

FIG. 2. Effects of 7-hydroxy-3,4-dihydrocadalin on rat liver microsomal lipid peroxidation. Mean ± s.d. of triplicate determinations is given.

haemoglobin, one of the most powerful catalysts of lipid peroxidation (Chiu et al 1982). When human 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 an azo-initiator AAPH induce the free-radical chain oxidation in erythrocyte membranes (Miki et al 1986). During a 4-h incubation with AAPH, 80% of erythrocytes were damaged. 7-Hydroxy-3,4-dihydrocadalin inhibited the lysis of human red cells due to the peroxy radical attack with almost 80% inhibition at 45 μM (Fig. 4).

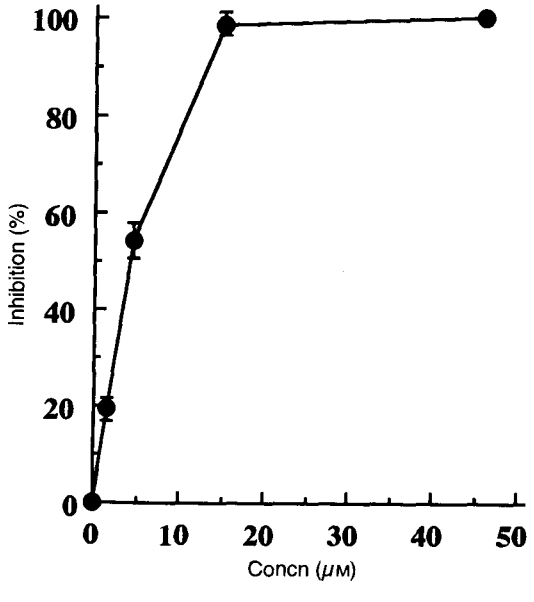

FIG. 3. Effects of 7-hydroxy-3,4-dihydrocadalin on rat liver mitochondrial lipid peroxidation. Mean ± s.d. of triplicate determinations is given.

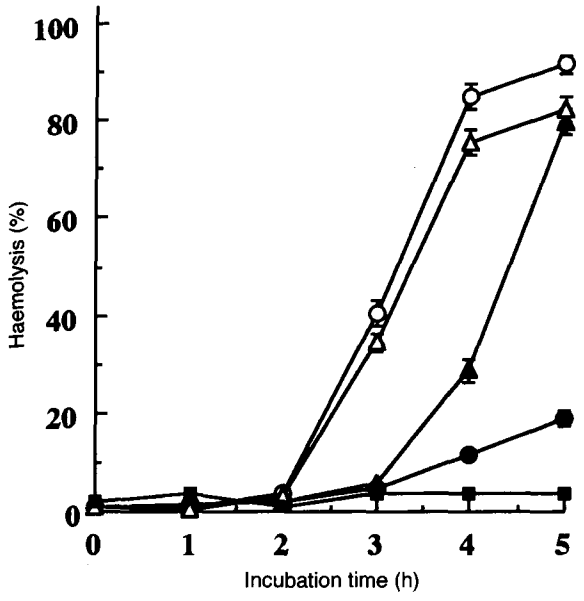

FIG. 4. Inhibition of oxidative haemolysis by 7-hydroxy-3,4-dihydrocadalin in human erythrocytes. Mean ± s.d. of triplicate determinations is given. ● 45 μM, ▲ 15 μM, △ 4·5 μM, ○ control, ■ without AAPH.

Our present results on biological systems showed that 7-hydroxy-3,4-dihydrocadalin was effective in to protecting tissues and cells against various oxidative stresses.

References

Buege, J. A., Aust, S. D. (1978) Microsomal lipid peroxidation. In: Fleischer, S., Packer, L. (eds) Methods in Enzymology. Vol. 52, Academic Press, New York, pp 302–310

Chiu, D., Lubin, B., Shohet, S. B. (1982) Peroxidative Reactions in Red Cell Biology. In: Pryor, W. A. (ed.) Free Radicals in Biology. Vol. 5, Academic Press, New York, pp 115–160

Halliwell, B., Gutteridge, J. M. C. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. In: Packer, L., Glazer, A. N. (eds) Methods in Enzymology. Vol. 186, Academic Press, New York, pp 1–85

Kok, F. J., Popple, G., Melse, J., Verheul, E., Schouten, E. G., Kruyssen, D. H. C. M., Hofman, A. (1991) Do antioxidants and polyunsaturated fatty acids have a combined association with coronary atherosclerosis? Atherosclerosis 86: 85–90

Kubo, I., Kinst-Hori, I., Ishiguro, K., Chaudhuri, S. K., Sanchez, Y., Ogura, T. (1994a) Tyrosinase inhibitory flavonoids from *Heterotheca inuloides* and their structural functions. Bioorg. Med. Chem. Lett. 4: 1443–1446

Kubo, I., Muroi, H., Kubo, A., Chaudhuri, S. W., Sanchez, Y., Ogura, T. (1994b) Antimicrobial agents from *Heterotheca inuloides*. Planta Med. 60: 218–221

Lin, T-J., Liu, G-T., Liu, Y., Xu, G-Z. (1992) Protection by salvianolic acid A against adriamycin toxicity on rat heart mitochondria. Free Radical Biol. Med. 12: 347–351

Lowry, O. H., Rosebrough, H. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275

Machlin, L., Bendich, A. (1987) Free radical tissue damage: protective role of antioxidant nutrients. Fed. Am. Soc. Exp. Biol. J. 1: 441–445

Miki, M., Tamai, H., Mino, N., Yamamoto, Y., Niki, E. (1986) Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. Arch. Biochem. Biophys. 258: 373–380

Pederson, T. C., Buege, J. A., Aust, S. D. (1973) Microsomal electron transport. J. Biol. Chem. 248: 7134–7141

Roders, M. K. (1978) NADPH-dependent microsomal lipid peroxidation and the problem of pathological action at a distance: new data on induction of red cell damage. Biochem. Pharmacol. 27: 437–443

Rousseau, E. J., Davison, A. J., Dunn, B. (1992) Protection by β-carotene and related compounds against oxygen-mediated cytotoxicity and genotoxicity; implications for carcinogenesis and anticarcinogenesis. Free Radical Biol. Med. 13: 407–433

Slater, T. F., Cheeseman, K. H. (1987) Free radical mechanisms in relation to tissue injury. Proc. Nutr. Soc. 46: 1–12

Sugawara, H., Tobise, K., Minami, H., Uekita, K., Yoshie, H., Onodera, S. (1992) Diabetes mellitus and reperfusion injury increase the level of active oxygen-induced lipid peroxidation in rat cardiac membranes. J. Clin. Exp. Med. 163: 237–238

Takayanagi, R., Takeshige, K., Minakami, S. (1980) NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. Dependence on the rate of electron flow in the respiratory chain and antioxidant role of ubiquinol. Biochem. J. 192: 853–860

Wiswedel, I., Ulbricht, O., Augustin, W. (1989) Studies of lipid peroxidation in isolated rat heart mitochondria. Biomed. Biochim. Acta 2: 73–76

Yagi, K. (1987) Lipid peroxides and human disease. Chem. Phys. Lipids 45: 337–341