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European Journal of Pharmacology 540 (2006) 46–52
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Effects of quinones and flavonoids on the reduction of all-*trans* retinal to all-*trans* retinol in pig heart

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Received 28 November 2005; received in revised form 20 April 2006; accepted 28 April 2006
Available online 7 May 2006

Abstract

We have recently purified a tetrameric carbonyl reductase from the cytosolic fraction of pig heart (pig heart carbonyl reductase). Since pig heart carbonyl reductase efficiently reduces all-*trans* retinal as the endogenous substrate, it probably plays an important role in retinoid metabolism in the heart. The purpose of the present study was to evaluate the inhibitory effects of quinones and flavonoids on the reduction of all-*trans* retinal to all-*trans* retinol catalyzed by pig heart carbonyl reductase, using pig heart cytosol. Of quinones tested, 9,10-phenanthrenequinone, a component of diesel exhaust particles, was the most potent inhibitor for the all-*trans* retinal reduction, and a significant inhibition was also observed for plumbagin and menadione. The order of the inhibitory potencies for flavonoids was kaempferol > quercetin > genistein > myricetin = apigenin = daidzein. However, the inhibitory potencies of flavonoids were much lower than that of 9,10-phenanthrenequinone. 9,10-Phenanthrenequinone competitively inhibited the all-*trans* retinal reduction, whereas kaempferol exhibited a mixed-type inhibition. It is likely that 9,10-phenanthrenequinone strongly inhibits the reduction of all-*trans* retinal to all-*trans* retinol by acting as the substrate inhibitor of pig heart carbonyl reductase present in pig heart cytosol.

Keywords: All-*trans* retinal; Carbonyl reductase; Quinone; Flavonoid; Pig heart

1. Introduction

All-*trans* retinol (vitamin A) is an important nutrient and biotransformed to an active form all-*trans* retinoic acid via all-*trans* retinal (Napoli, 1999; Duester, 2000). All-*trans* retinoic acid is a critical signaling molecule in the development and growth of vertebrate embryo (Ross et al., 2000), especially in early heart development (Niederreither et al., 2001; Zile, 2004; Keegan et al., 2005). Furthermore, recent investigations have demonstrated that small physiological doses of all-*trans* retinoic acid induce ventricular remodeling in adult rats (de Paiva et al., 2003), and a significant upregulation in the protein expression of angiotensin-converting enzyme 2 is observed in the heart of adult spontaneously hypertensive rats treated with all-*trans* retinoic acid (Zhong et al., 2004). Thus, it is possible that all-*trans* retinoic acid is responsible for the regulation of cardiac structure and function throughout life. However, in mature vertebrates, the contribution of all-*trans* retinoic acid to cardiac structure and function remains to be established.

We have recently purified a tetrameric form of carbonyl reductase (EC 1.1.1.184) from the cytosolic fraction of pig heart (pig heart carbonyl reductase), using 4-benzoylpyridine as a substrate (Usami et al., 2003). This enzyme reduces a variety of exogenous ketones, aldehydes and quinones (Shimada et al., 2003). Interestingly, pig heart carbonyl reductase, unlike other monomeric carbonyl reductases, has the ability to reduce effectively all-*trans* retinal to all-*trans* retinol (Usami et al., 2003), suggesting that this enzyme plays an important role in retinoid metabolism in the heart. Thus, it is interesting to examine effects of various inhibitors on the reduction of all-*trans* retinal catalyzed by pig heart carbonyl reductase.

In the present study, quinones and flavonoids were chosen as inhibitors of pig heart carbonyl reductase. Quinones have been reported to cause several toxic effects, including cardiotoxicity and neurotoxicity (Monks et al., 1992; Bolton et al., 2000). For example, doxorubicin and daunorubicin containing quinone moiety can produce severe cardiotoxicity

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doi:10.1016/j.ejphar.2006.04.050

that limits the therapeutic effect as anticancer drugs (Buzdar et al., 1985; Olson and Mushlin, 1990). Our previous papers (Usami et al., 2003; Shimada et al., 2004; Oginuma et al., 2005) have also shown that 9,10-phenanthrenequinone, a component of diesel exhaust particles, is the best substrate for pig heart carbonyl reductase, and mediates superoxide formation through its redox cycling in pig heart. Flavonoids such as quercetin and (+)-catechin are natural compounds that are widely distributed in vegetables, fruits, and green tea. These natural compounds exhibit enzyme inhibition and antioxidant activity (Zhang and Das, 1994; van Acker et al., 1996; Middleton et al., 2000; Shimada et al., 2005). Quercetin is well known as a potent inhibitor of carbonyl reductases purified from various tissues (Ikeda et al., 1984; Imamura et al., 1993, 1999). We report herein the inhibitory effects of 10 quinones including 9,10-phenanthrenequinone and 19 flavonoids on the reduction of all-*trans* retinal to all-*trans* retinol catalyzed by pig heart carbonyl reductase, using the cytosolic fraction of pig heart. The kinetic mechanism for the inhibition of all-*trans* retinal reduction by these compounds is also examined.

2. Materials and methods

2.1. Chemicals

The chemicals were obtained from the following sources: all-*trans* retinal, all-*trans* retinol, 9,10-phenanthrenequinone, 2-hydroxy-1,4-naphthoquinone, barbital (sodium salt), morin, myricetin, genistein, taxifolin (racemate), kaempferol, daidzein, and (−)-epigallocatechin-3-gallate (Sigma Chemical Co., St. Louis, MO); 1,2-naphthoquinone, 1,4-naphthoquinone, 9,10-anthraquinone, juglone, naringenin, apigenin, galangin, chrysin, and (−)-epicatechin, (Aldrich, Milwaukee, WI); menadione, plumbagin, toluquinone, quercetin, and luteolin (Wako Pure Chemicals, Tokyo, Japan); fisetin and quercitrin (Tokyo Kasei, Tokyo, Japan); 1,4-benzoquinone (Nacalai Tesque, Kyoto, Japan). Genistin, (+)-catechin, and rutin were donated by Dr. J. Kinjo (Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan). NADPH, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were of reagent grade.

2.2. Preparation of cytosolic fraction

Pig hearts were supplies from a slaughterhouse and stored at −20 °C. The tissues were homogenized in 3 volumes of 10 mM sodium potassium phosphate buffer containing 1.15% KCl (pH 6.0). The homogenates were centrifuged at 105,000×*g* for 60 min to obtain the cytosolic fraction.

2.3. Reduction of all-trans retinal to all-trans retinol

The reduction of all-*trans* retinal was estimated by measuring all-*trans* retinol formed in pig heart cytosol. The reaction mixture consisted of substrate (0.5 mM all-*trans* retinal), NADPH-generating system (50 μM $NADP^+$, 1.25 mM glucose-6-phosphate, 50 munits glucose-6-phosphate dehydrogenase and 1.25 mM $MgCl_2$), enzyme preparation (pig heart cytosol) and 100 mM sodium potassium phosphate buffer (pH 7.4) in a final volume of 0.5 ml. All-*trans* retinal was dissolved in methanol each time just before its use and the final concentration of methanol in the reaction mixture did not exceed 2% (v/v). The preparation of the reaction mixture and the incubation was carried out in dark room. In the case of inhibition experiments, quinones and flavonoids dissolved in dimethyl sulfoxide and methanol, respectively, were added to the reaction mixture at concentrations of 10 μM for quinones and 50 μM for flavonoids. The final concentration of dimethyl sulfoxide or methanol did not exceed 2% (v/v), and this concentration did not affect the enzyme reaction. The reaction mixture was incubated at 37 °C for 10 min, and ethanol containing butyl hydroxytoluene (100 μg/ml) was added to stop the reaction (Kedishvili et al., 2002). After centrifugation at 2000×*g*, the supernatant was subjected to a solid phase extract column (Sep-Pak Light C18, Waters) for extracting retinoids and then eluted with methanol (Kedishvili et al., 2002). The aliquot of 20 μl was subjected to high performance liquid chromatography (HPLC) for the determination of the reduction product, all-*trans* retinol, of all-*trans* retinal (Crosas et al., 2003). HPLC was carried out using a Shimadzu LC-10AD HPLC apparatus (Shimadzu, Tokyo, Japan) equipped with a Tosoh ODS-80Ts column and a Jasco 875-UV monitor (340 nm). Mixture of acetonitrile–1% ammonium acetate (4 : 1, v/v) was used as a mobile phase at flow rate of 1.0 ml/min. The IC_{50} (concentration of 50% inhibition) value was determined from linear regression of at least four points in different concentrations. The inhibition of all-*trans* retinal reduction by 9,10-phenanthrenequinone and kaempferol were kinetically analyzed using Lineweaver–Burk plots. Velocity (v) was expressed as nmol/min/mg protein. The inhibition constant (K_i) was determined from Dixon plots. Protein concentrations were determined with bovine serum albumin as the standard by the method of Lowry et al. (1951).

2.4. Reduction of 9,10-phenanthrenequinone, plumbagin, kaempferol, and quercetin

The reduction of 9,10-phenanthrenequinone, plumbagin, kaempferol, and quercetin were measured spectrophotometrically by monitoring the decrease in the absorbance of NADPH at 340 nm. The reaction mixture consisted of substrate (9,10-phenanthrenequinone, plumbagin, kaempferol, or quercetin), 0.3 mM NADPH, pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 7.4) in a final volume of 0.5 ml. The enzyme reaction was initiated by the addition of 9,10-phenanthrenequinone, plumbagin, kaempferol, or quercetin at a concentration of 10 μM to the reaction mixture. The kinetic parameters (K_m and V_{max}) of enzyme reaction for 9,10-phenanthrenequinone and plumbagin were analyzed using Lineweaver–Burk plots. One unit of enzyme activity was defined as the amount catalyzing the oxidation of 1 μmol of NADPH/min at 37 °C.

3. Results

3.1. Reduction of all-trans retinal in the reaction system of pig heart cytosol

The reduction of all-*trans* retinal was examined in the reaction system of pig heart cytosol. The reduction product generated from all-*trans* retinal appeared as a main peak corresponding to authentic all-*trans* retinol on HPLC (Fig. 1), indicating that all-*trans* retinal is mainly reduced to all-*trans* retinol in pig heart cytosol.

3.2. Inhibition of all-trans retinal reduction by quinones

We examined the effects of 10 quinones, listed in Fig. 2, on the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol. Fig. 3 shows the percentage of inhibition of all-*trans* retinal reduction by quinones at a concentration of 10 μM. Of quinones tested, 9,10-phenanthrenequinone was the most potent inhibitor for the all-*trans* retinal reduction. In addition, a significant inhibition was also observed for plumbagin and menadione.

3.3. Inhibition of all-trans retinal reduction by flavonoids

The inhibitory effects of 19 flavonoids, listed in Fig. 4, at a concentration of 50 μM on the reduction of all-*trans* retinal were examined in pig heart cytosol. As shown in Fig. 5, the order of inhibitory potencies was kaempferol > quercetin > genistein > myricetin = apigenin = daidzein. Several flavonoids such as galangin and morin were poor inhibitors. On the other hand, (+)-catechin or (−)-epicatechin showed little inhibitory effect.

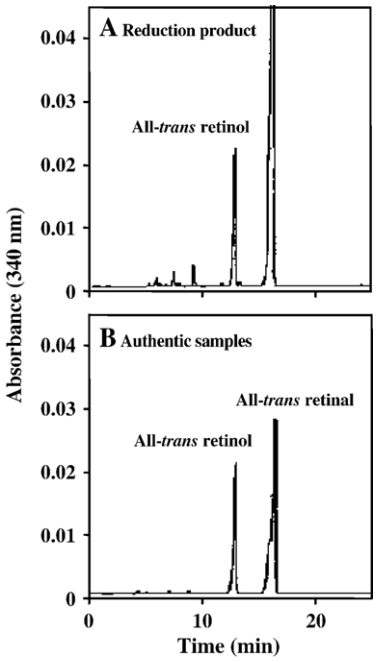

Fig. 1. HPLC chromatogram of all-*trans* retinol and all-*trans* retinal. (A) All-*trans* retinol generated from all-*trans* retinal (0.5 mM) in the reaction system of pig heart cytosol. (B) Authentic all-*trans* retinol and all-*trans* retinal.

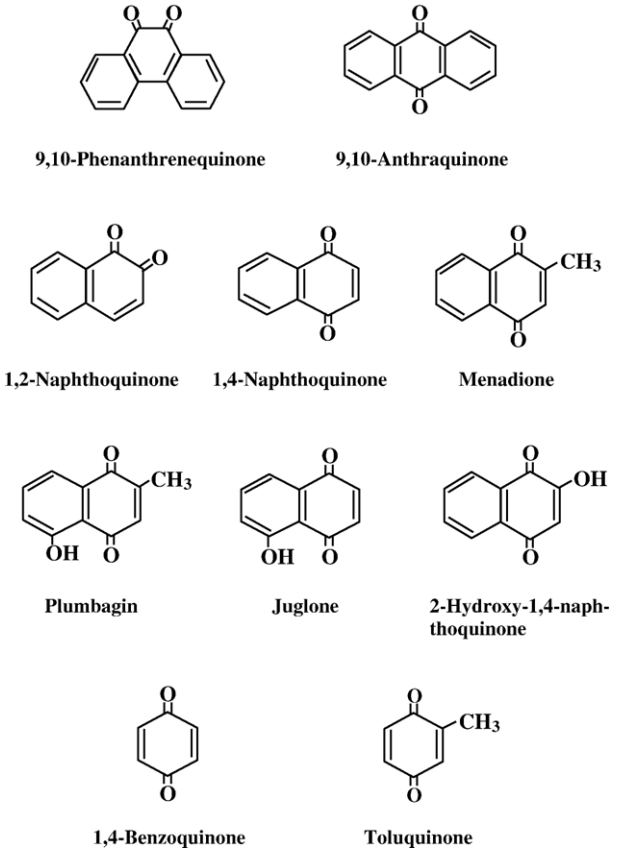

Fig. 2. Chemical structures of quinones.

3.4. IC50 values of 9,10-phenanthrenequinone and kaempferol

The inhibitory potencies of flavonoids appeared to be much lower than that of 9,10-phenanthrenequinone. Thus, the IC_{50} values for 9,10-phenanthrenequinone and kaempferol, which is

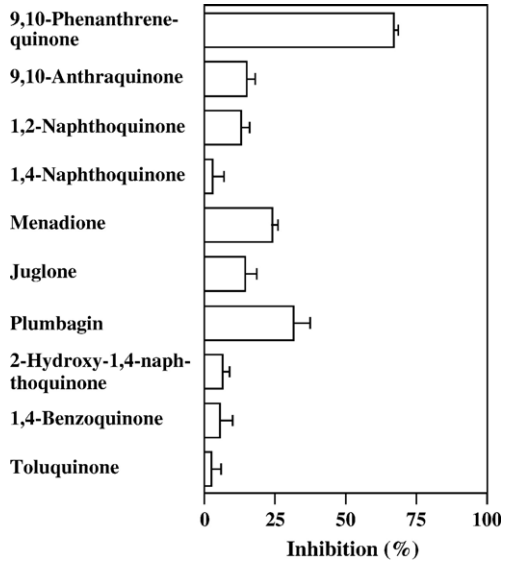

Fig. 3. Effects of quinones on the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol. The concentration of quinones was 10 μM. Each bar represents the mean ± S.D. of three to eight experiments.

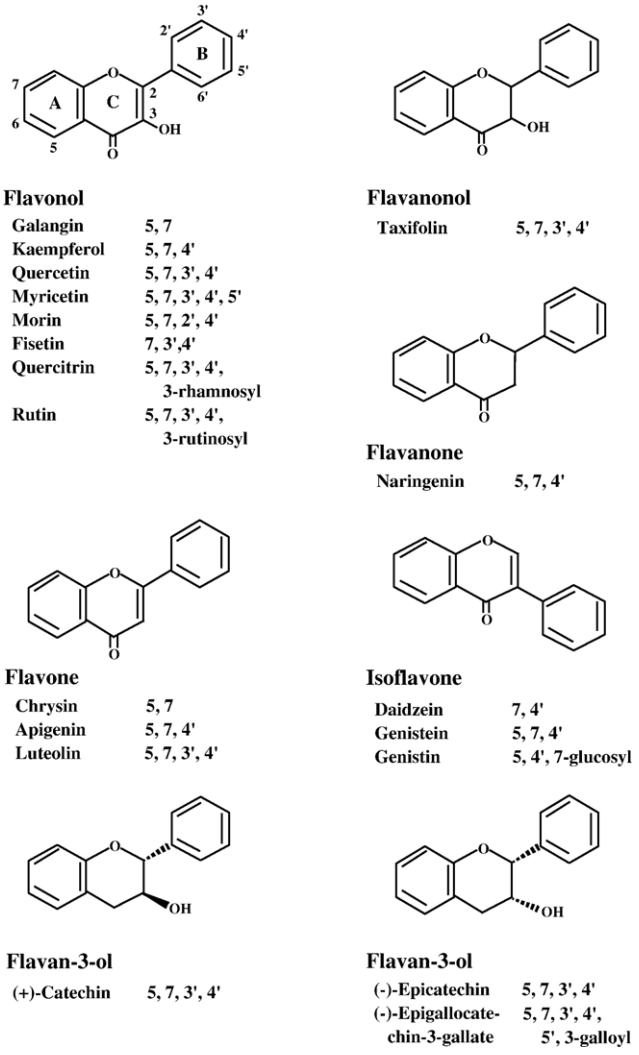

Fig. 4. Chemical structures of flavonoids. The numbers are hydroxylation pattern.

the most potent inhibitor among flavonoids, were determined. 9,10-Phenanthrenequinone and kaempferol exhibited concentration-dependent inhibition and the IC_{50} values for 9,10-phenanthrenequinone and kaempferol were 6.0±0.4 and 49.7±2.9 μM, respectively (Fig. 6).

3.5. Kinetic mechanism for the inhibition of all-trans retinal reduction by 9,10-phenanthrenequinone and kaempferol

The inhibition of all-*trans* retinal reduction by 9,10-phenanthrenequinone and kaempferol was kinetically examined in pig heart cytosol. As shown in Fig. 7, 9,10-phenanthrenequinone competitively inhibited the reduction of all-*trans* retinal (K_i=0.74 μM), whereas kaempferol exhibited a mixed-type inhibition.

3.6. Kinetic parameters for the reductive reaction of 9,10-phenanthrenequinone and plumbagin

We examined spectrophotometrically the oxidation of NADPH during the reduction of quinones (9,10-phenanthrenequinone and plumbagin) and flavonoids (kaempferol and quercetin) at a concentration of 10 μM in pig heart cytosol. 9,10-Phenanthrenequinone and plumbagin caused significant decreases in the absorbance of NADPH at 340 nm, indicating NADPH-dependent reduction of these quinones (data not

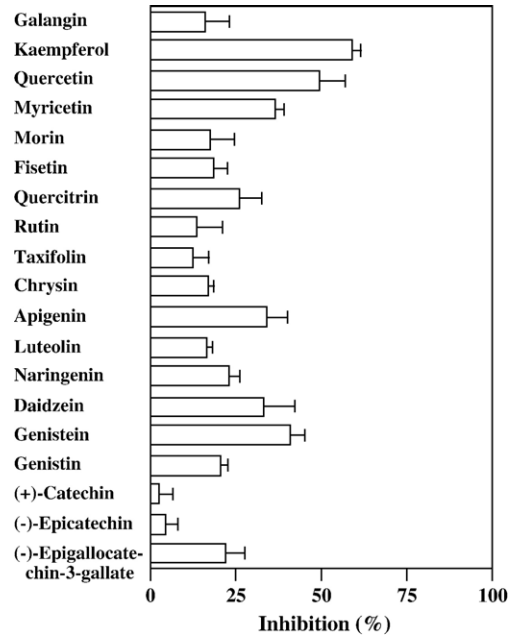

Fig. 5. Effects of flavonoids on the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol. The concentration of flavonoids was 50 μM. Each bar represents the mean±S.D. of three to six experiments.

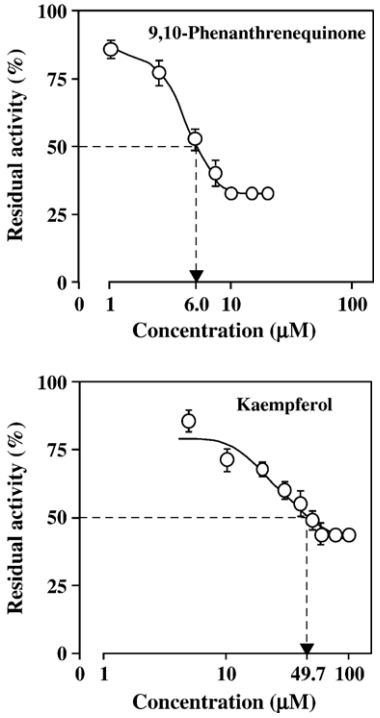

Fig. 6. Effects of 9,10-phenanthrenequinone and kaempferol at various concentrations on the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol. The arrow shows the IC_{50} value. Each point represents the mean± S.D. of three to six experiments.

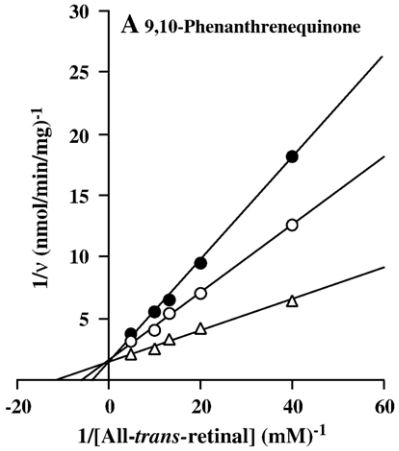

Fig. 7. Lineweaver–Burk plots for the reduction of all-*trans* retinal to all-*trans* retinol in the absence and in the presence of 9,10-phenanthrenequinone and kaempferol. (A) △, in the absence of 9,10-phenanthrenequinone; ○, in the presence of 9,10-phenanthrenequinone (1 μM); ●, in the presence of 9,10-phenanthrenequinone (2 μM). (B) △, in the absence of kaempferol; ○, in the presence of kaempferol (5 μM); ●, in the presence of kaempferol (20 μM). Each point represents the mean±S.D. of three to seven experiments.

shown). On the other hand, no decrease was observed for kaempferol or quercetin. The kinetic parameters for the reductive reaction of 9,10-phenanthrenequinone and plumbagin are summarized in Table 1. The V_{max}/K_m value for 9,10-phenanthrenequinone was larger than that for plumbagin.

3.7. Inhibition of all-trans retinal reduction by barbital

Barbiturates including barbital and phenobarbital are known as specific inhibitors of aldehyde reductase (Turner and Hick, 1976; Felsted et al., 1977). Thus, the effect of barbital on the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol was compared with that of quercetin. Barbital, unlike quercetin, had little effect on the all-*trans* retinal reduction (Fig. 8).

Table 1
Kinetic parameters for the reductive reaction of 9,10-phenanthrenequinone and plumbagin in pig heart cytosol

Compounds	K_m (mM)	V_{max}/K_m (units/mg/mM)
9,10-Phenanthrenequinone	0.0015±0.0003	12.4±2.6
Plumbagin	0.0024±0.0002	7.4±0.6

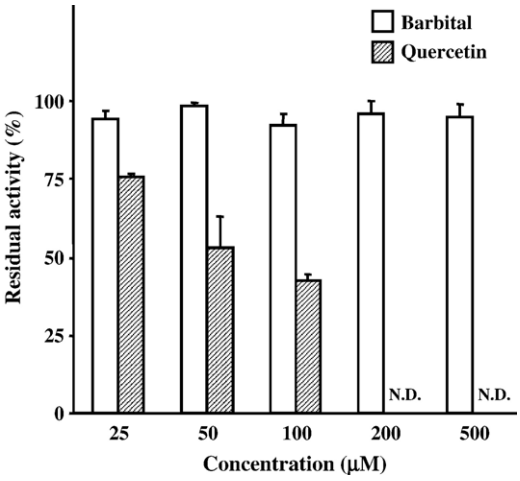

Fig. 8. Effects of barbital and quercetin on the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol. Each bar represents the mean±S.D. of three to seven experiments. N.D., not determined.

4. Discussion

We at first attempted to evaluate the inhibitory effects of 10 quinones on the reduction of all-*trans* retinal to all-*trans* retinol in the reaction system of pig heart cytosol. Of quinones tested, 9,10-phenanthrenequinone was the most potent inhibitor for the reduction of all-*trans* retinal to all-*trans* retinol. 9,10-Phenanthrenequinone is produced from phenanthrene, which comprises 6% of the total organic extract of diesel exhaust particles (Barfknecht et al., 1981; Tsien et al., 1997), by photooxidation and is known as a relatively abundant quinone in diesel exhaust particles (Schuetzle, 1983; Cho et al., 2004). Interestingly, diesel exhaust particles has been reported to induce cardiotoxicity including electrocardiographic alterations (Minami et al., 1999; Hirano et al., 2003). It is possible that 9,10-phenanthrenequinone is involved in diesel exhaust particles-induced cardiotoxicity, by inhibiting the reduction of all-*trans* retinal to all-*trans* retinol. Plumbagin and menadione, which are naphthoquinones having a methyl group at the 2-position (see Fig. 2), also exhibited significant inhibitory effects for the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol. No information on cardiac toxicity of plumbagin is available. However, it should be noted that menadione is toxic to cultured cardiomyocytes (Tzeng et al., 1995). In adult rats, excess of all-*trans* retinoic acid signaling has been shown to result in cardiomyocyte abnormalities and dilated cardiomyopathy (Colbert et al., 1997; Subbarayan et al., 2000). Thus, it is conceivable that the strong inhibition of pig heart carbonyl reductase by 9,10-phenanthrenequinone and menadione may induce a variety of toxic effects in pig heart, through increased production of all-*trans* retinoic acid.

Flavonoids have been considered as potential protectors against chronic cardiotoxicity caused by doxorubicin (van

Acker et al., 2001). In the present study, several flavonoids such as kaempferol, quercetin and genistein were found to inhibit effectively the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol, although the inhibitory potencies of these flavonoids were lower than that of 9,10-phenanthrenequinone. The utility of flavonoids as protectors against doxorubicin-induced cardiotoxicity may be limited, because of their inhibitory effects on the reduction of all-*trans* retinal.

Furthermore, the structural characteristics of flavonoids necessary for inhibiting the all-*trans* retinal reduction were examined in pig heart cytosol. Rutin has identical numbers of hydroxyl group in the same positions with quercetin, but is glycosylated with rutinose at the 3-position of C ring (see Fig. 4). Expectedly, the inhibitory potency of rutin was lower than that of quercetin. A similar result was observed between quercitrin and quercetin, or between genistin and genistein. It is reasonable to assume that the relative lipophilicities of flavonoids play a role in their inhibitory potencies. We have recently demonstrated that a planar benzopyrone ring with a coplannar phenyl ring is a structural characteristic determining the inhibitory effects of flavonoids on the reduction of progesterone to 20α-hydroxyprogesterone in rat liver cytosol (Shimada et al., 2005). However, in the case of the inhibitory effects of flavonoids on the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol, such structural characteristic was not observed.

We have recently demonstrated that 9,10-phenanthrenequinone is the best substrate for pig heart carbonyl reductase (Usami et al., 2003; Shimada et al., 2004). Thus, it is reasonable to assume that this quinone is a good competitive inhibitor. In fact, 9,10-phenanthrenequinone was confirmed to inhibit competitively the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol, although the inhibition by kaempferol was mixed-type. However, further studies, using the recombinant enzyme, are necessary to clarify the detailed mechanism for inhibition of pig heart carbonyl reductase by 9,10-phenanthrenequinone. In an attempt to predict the inhibition mechanism of all-*trans* retinal reduction by 9,10-phenanthrenequinone and kaempferol, the catalytic properties of pig heart carbonyl reductase for quinones (9,10-phenanthrenequinone and plumbagin) and flavonoids (kaempferol and quercetin) were spectrophotometrically examined in pig heart cytosol. The obtained results proposed the possibility that plumbagin, like 9,10-phenanthrenequinone, is a good substrates of pig heart carbonyl reductase present in pig heart cytosol, although the V_{max}/K_m value for plumbagin is smaller than that for 9,10-phenanthrenequinone, as expected from their inhibitory potencies. Wermuth et al. (1986) and Jarabak (1991) have also pointed out that carbonyl reductase provides the enzymatic basis for the reduction of quinones. On the other hand, kaempferol or quercetin was not reduced in pig heart cytosol.

Since all-*trans* retinal has an aldehyde group in its chemical structure, it might be reduced to all-*trans* retinol not only by carbonyl reductase (pig heart carbonyl reductase), but also by aldehyde reductase present in pig heart cytosol. However, barbital, a specific inhibitor of aldehyde reductase, had little effect on the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol, indicating that the all-*trans* retinal reduction is not catalyzed by aldehyde reductase. A recent paper has also shown that pig aldehyde reductase has no catalytic activity for all-*trans* retinal (Crosas et al., 2003). Additional studies may be necessary to rule out the participation of enzymes other than pig heart carbonyl reductase present in pig heart cytosol.

In conclusion, the present study demonstrates that a number of quinones and flavonoids inhibit the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol. In particular, it is noteworthy that 9,10-phenanthrenequinone is the most potent competitive inhibitor for the reduction of all-*trans* retinal to all-*trans* retinol in pig heart cytosol.

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