Inhibition by chlorogenic acid of haematin-catalysed retinoic acid 5,6-epoxidation

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Chlorogenic acid (3-*O*-caffeoylquinic acid) inhibited haematin- and haemoglobin-catalysed retinoic acid 5,6-epoxidation. Some other phenol compounds (caffeic acid and 4-hydroxy-3-methoxybenzoic acid) also showed inhibitory effects on the haematin- and haemoglobin-catalysed epoxidation, but salicylic acid did not. Of the above compounds, caffeic acid and chlorogenic acid were potent inhibitors compared with the other two, suggesting that the *o*-hydroquinone moiety of chlorogenic acid and caffeic acid is essential to the inhibition of the epoxidation. Although caffeic acid inhibited retinoic acid 5,6-epoxidation requiring the consumption of O₂, formation of retinoic acid radicals was not inhibited on the addition of caffeic acid to the incubation mixture. The above results suggest that caffeic acid does not inhibit the formation of retinoic acid radicals but does inhibit the step of conversion of retinoic acid radical into the 5,6-epoxide.

INTRODUCTION

The biological generation and reaction of radical species are of great interest in the studies of cellular metabolism and of the pathogenesis of diverse cytotoxic phenomena [1]. Indeed, some radical species have been presumed to exist in many biological systems, e.g. lipid peroxidation catalysed by haemoproteins [2], conversion of linoleic acid hydroperoxide into hydroxy, oxo, epoxyhydroxy and trihydroxy fatty acid [3], and epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene [4] and retinoic acid [5,6]. The study of reaction mechanisms containing radical species is important and necessary, since many biological reactions seem to proceed via radical intermediates. Recently, an e.p.r. spin-trapping study of haematin-catalysed retinoic acid 5,6-epoxidation resulted in the direct detection of radical species [7].

On the other hand, chlorogenic acid, which occurs in plants, particularly in coffee beans, contains an *o*-hydroquinone moiety, and it has been assumed that this moiety is essential to its anti-oxygenic property. The mechanism of the inhibition of oxidation by chlorogenic acid, however, is not completely understood. It is necessary to clarify the mechanism of the inhibition of oxidation by chlorogenic acid, since there are many reactions in which chlorogenic acid participates, e.g. inhibition of lipxygenase activity in prostaglandin metabolism [8], inhibition of oxidation of vitamin A [9] and protection against oxidation of adrenaline [10].

In the present paper the inhibitory effect of caffeic acid on retinoic acid 5,6-epoxidation catalysed by haematin (or oxyhaemoglobin) is investigated by using the e.p.r. spin-trapping technique and h.p.l.c.–e.p.r. spectrometry. It is shown that chlorogenic acid (or its derivative caffeic acid) inhibits haematin- (or haemoglobin-) catalysed retinoic acid 5,6-epoxidation, and the inhibitory effect can be ascribed to the *o*-hydroquinone moiety in the

molecules. In addition, the relationship between the formation of retinoic acid radicals and the inhibition of the epoxidation by caffeic acid is discussed.

MATERIALS AND METHODS

Materials

Haematin, human haemoglobin (type IV) and all-*trans*-retinoic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Caffeic acid and chlorogenic acid (3-*O*-caffeoylquinic acid) were from Tokyo Kasei (Tokyo, Japan). 4-Hydroxy-3-methoxybenzoic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Salicylic acid was purchased from Nakarai Chemical Co. (Kyoto, Japan). All other chemicals used were commercial products of the highest grade available. Oxyhaemoglobin was prepared by the method of Mieyal & Blumer [11]. 5,6-Epoxyretinoic acid was synthesized by the method of John *et al.* [12], except for the step of methylation of retinoic acid. The methylation of retinoic acid was done with diazomethane.

Assay conditions

The standard reaction mixture contained, in a total volume of 0.22 ml, 4% (v/v) Triton X-100, 1.65 mM-retinoic acid (which was added as 10 µl of solution in dimethyl sulphoxide), 0.1 M-potassium phosphate buffer, pH 7.5, 5 µl of oxyhaemoglobin (24 µM-haem) or haematin (24 µM), and 10 µl of various concentrations of methanolic solutions of inhibitor. All reactions were carried out at 37 °C for 5 min under aerobic conditions and stopped by the addition of 1.6 ml of methanol containing 50 mM-2-mercaptoethanol. The peak height of the product was compared with those of three different concentrations of 5,6-epoxyretinoic acid standards in the range 5–15 pmol to determine the concentration of 5,6-epoxyretinoic acid formed [5,6]. The concentration of

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Fig. 1. H.p.l.c. elution profiles of incubation mixtures of haematin-catalysed retinoic acid 5,6-epoxidation with and without caffeic acid

Incubation conditions and h.p.l.c. procedure are as described in the text. (a) Without caffeic acid; (b) with caffeic acid ($30\ \mu\text{M}$). The peak of 5,6-epoxyretinoic acid is indicated by the arrow ($\downarrow$).

standard 5,6-epoxyretinoic acid was determined by use of $A_{346}^{1\%} = 1360$ in chloroform [12]. The amounts of the 5,6-epoxyretinoic acid in the assay solutions were in the range 5–15 pmol.

H.p.l.c. and e.p.r.

The h.p.l.c. was performed on a Jasco Trirotor V with a variable-wavelength u.v. detector and e.p.r. spectrometer. The u.v. detector was set at 310 nm. A column (250 mm $\times$ 4.6 mm internal diam.) packed with TSK ODS gel (5 μm particle size) was used at flow rate of 1.0 ml/min, with 10 mM-ammonium acetate and aq. 85%

(v/v) methanol as the mobile phase. The column was kept at 40°C throughout. An e.p.r. spectrometer (JEOL-FX2XG) was connected to the h.p.l.c. system with a Teflon tube that passed through the e.p.r. cell. The magnetic field of the e.p.r. spectrometer was fixed at the position $g = 2.017$. The e.p.r. spectrometer was operated at room temperature.

O_2 consumption

O_2 -consumption studies were performed with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.).

RESULTS

Inhibition by caffeic acid of haematin-catalysed retinoic acid 5,6-epoxidation

By using h.p.l.c., formation of 5,6-epoxyretinoic acid was measured on addition of caffeic acid (final concn. $30\ \mu\text{M}$) to the standard reaction mixture (haematin as catalyst). H.p.l.c. elution profiles of the incubation mixture are shown in Fig. 1. Peaks of 5,6-epoxyretinoic acid are indicated by arrows. When caffeic acid ($30\ \mu\text{M}$) was added to the standard reaction mixture of haematin-catalysed retinoic acid 5,6-epoxidation, formation of 5,6-epoxyretinoic acid was inhibited (Fig. 1b). Caffeic acid also inhibited the oxyhaemoglobin-catalysed retinoic acid 5,6-epoxidation (results not shown). To test whether or not O_2 consumption is influenced on addition of caffeic acid, O_2 -consumption studies were performed with a Clark-type oxygen electrode in the presence and in the absence of caffeic acid ($44\ \mu\text{M}$). When caffeic acid was added to the standard reaction mixture (haematin as catalyst), the O_2 consumption stopped.

Inhibition by some phenolic compounds of haematin-catalysed retinoic acid 5,6-epoxidation

The inhibitory effects of some phenolic compounds on the haematin-catalysed retinoic acid 5,6-epoxidation

Fig. 2. Inhibition by some phenolic compounds of haematin-catalysed retinoic acid 5,6-epoxidation

Various concentrations of phenolic compounds ($\bullet$, chlorogenic acid; $\circ$, caffeic acid; $\blacksquare$, 4-hydroxy-3-methoxybenzoic acid; $\square$, salicylic acid) were added to the incubation mixtures. Incubation and assay conditions were as described in the test.

Fig. 3. Double-reciprocal plots of initial velocity versus retinoic acid concentration in the presence and in the absence of 15 μM -cafeic acid

Assay conditions were as described in the text except for the concentration of retinoic acid. ○, In the presence of cafeic acid (15 μM); ●, in the absence of cafeic acid.

were compared (Fig. 2). When various concentrations of some phenolic compounds were incorporated into the standard reaction mixture, the formation of 5,6-epoxyretinoic acid was inhibited dose-dependently. The inhibitory effects of the phenolic compounds were in the following order: chlorogenic acid, cafeic acid $\gg$ 4-hydroxy-3-methoxybenzoic acid > salicylic acid. That order was also the case for the oxyhaemoglobin-catalysed retinoic acid 5,6-epoxidation.

Substrate-concentration-dependence of retinoic acid 5,6-epoxidation catalysed by haemoglobin

The substrate-concentration-dependence of retinoic acid 5,6-epoxidation catalysed by oxyhaemoglobin was measured in the presence and in the absence of cafeic acid (15 μM). Haemoglobin was used as catalyst in the place of haematin to test whether the reaction is enzyme-like. In both cases typical Michaelis-Menten curves were obtained. Double-reciprocal plots of initial velocity of retinoic acid 5,6-epoxidation catalysed by haemoglobin in the presence and in the absence of cafeic acid (15 μM) are shown in Fig. 3. These plots clearly showed that the cafeic acid inhibited the 5,6-epoxidation of retinoic acid by haemoglobin non-competitively. Least-squares regression analysis allowed us to estimate kinetic parameters as follows: $K_m = 1.6 \text{ mM}$ and $V_{\text{max}} = 0.55 \text{ mol/min per mol of haem}$ in the presence of cafeic acid (15 μM), and $K_m = 1.2 \text{ mM}$ and

Fig. 4. Inhibition by cafeic acid of haematin-catalysed retinoic acid 5,6-epoxidation

Cafeic acid (44 μM) was added to the incubation mixture at 5 min (●) and 10 min (Δ) after the reaction had been started. The incubation mixture contained 1.2 μM -haematin, 0.1 M-potassium phosphate buffer, pH 7.5, 4% (v/v) Triton X-100 and 1.65 mM-retinoic acid added in 100 μl of dimethyl sulphoxide, in total volume of 2.35 ml. The reaction was stopped by mixing 0.1 ml of incubation mixture with 0.8 ml of methanol containing 50 mM-2-mercaptoethanol. The result obtained with no addition of cafeic acid is also shown (○).

$V_{\text{max}} = 1.6 \text{ mol/min per mol of haem}$ in the absence of cafeic acid.

Effect of cafeic acid on the formation of 5,6-epoxyretinoic acid

As just mentioned above, cafeic acid and chlorogenic acid effectively inhibited the formation of 5,6-epoxyretinoic acid catalysed by haematin (or oxyhaemoglobin). These findings suggest two possible mechanisms, namely that cafeic acid (or chlorogenic acid) inhibits the reaction itself or that it degrades the epoxide formed. The addition of cafeic acid during an incubation (haematin as catalyst) inhibited production of the epoxide completely, but did not decrease the amount of the epoxide already formed (Fig. 4). This indicates that cafeic acid inhibits the formation of the epoxide.

E.p.r. measurement of the incubation mixture of haematin-catalysed retinoic acid 5,6-epoxidation

Haematin-catalysed retinoic acid 5,6-epoxidation was studied by using an e.p.r. spin-trapping technique. When the spin-trap reagent nitrosobenzene was added to the standard reaction mixture of haematin-catalysed retinoic acid 5,6-epoxidation, e.p.r. signals ($g = 2.006$) were detected (Fig. 5a). The signals were observed even in the absence of haematin, and their peak heights and hyperfine coupling constants were unchanged (Fig. 5b). The e.p.r. signals were not observed when nitrosobenzene was dissolved in the buffer alone or in the absence of nitrosobenzene (Figs. 5c and 5d).

Fig. 5. E.p.r. spectrum of spin adducts of nitrosobenzene with retinoic acid radicals formed in a reaction mixture containing retinoic acid and haematin

The e.p.r. spectrum was obtained after incubation for 10 min at 25 °C. The incubation mixture contained, in a total volume of 0.22 ml, 5 μ l of nitrosobenzene (0.1 M in methanol) and standard reaction mixture without 10 μ l of methanolic solution of inhibitors (described in the text). E.p.r. measurements were made at room temperature by using a Teflon tube instead of an aqueous-solution cell. The microwave power was 5 mW, and modulation frequency was 100 kHz with an amplitude of 63 μ T. The sweep time was 2.5 mT/min, and the receiver gain was 3.2×10^3 with a response time of 0.3 s. (a) Complete incubation mixture; (b) in the absence of haematin; (c) nitrosobenzene dissolved in the buffer alone; (d) in the absence of nitrosobenzene.

Effect of caffeic acid on e.p.r. signals of spin adducts of nitrosobenzene with retinoic acid radicals

To test whether formation of retinoic acid radicals is influenced on incorporation of caffeic acid into the incubation mixture (haematin as catalyst) or not, the time course of the intensity of e.p.r. signal was measured and h.p.l.c.-e.p.r. analysis of the spin adducts was performed. As shown in Fig. 6(a), time courses of the intensity of the e.p.r. signal were almost same both in the absence and in the presence of caffeic acid. H.p.l.c.-e.p.r. analysis [7] of the incubation mixture (haematin as catalyst) showed that three peaks of the spin adducts were observed both in the presence and in the absence of caffeic acid, and the intensities of the peaks were unchanged. In these measurements, magnetic field was fixed at the position ($g = 2.017$) where maximum intensity was observed in the e.p.r. signal. The results obtained in the above two experiments indicate that caffeic acid does not inhibit the formation of retinoic acid radicals.

DISCUSSION

Haematin- (or haemoglobin-)catalysed retinoic acid 5,6-epoxidation, a reaction that proceeds via radical intermediates, was inhibited by chlorogenic acid and caffeic acid to a great extent, by 4-hydroxy-3-methoxybenzoic acid to some extent and by salicylic acid hardly at all. This result suggests that the *o*-hydroquinone moiety in chlorogenic acid and caffeic acid may play an important role in the inhibitory effect, since 4-hydroxy-3-methoxybenzoic acid and salicylic acid molecules do not contain this moiety. Chlorogenic acid and its

positional isomers exist in coffee beans [13,14]. Dihydrocaffeic acid appears to be one of the metabolites of chlorogenic acid, and it has been detected in the human sera of subjects who drank coffee before blood sampling [15]. Dihydrocaffeic acid retains the *o*-hydroquinone moiety and seems to exhibit the same inhibitory effect as chlorogenic acid. Thus a high chlorogenic acid intake may result in inhibition of epoxidation via radical intermediates *in vivo*.

In a previous investigation [7] we presented the following scheme for the reaction mechanism of the haematin-catalysed retinoic acid 5,6-epoxidation:

where R and R $^{\bullet}$ are retinoic acid and its radical intermediate respectively. It was shown that O $_2$ reacts with retinoic acid in step (2), since retinoic acid radicals were detected only under anaerobic conditions [7]. In the present paper it has been shown that caffeic acid did not inhibit the formation of retinoic acid radical (R $^{\bullet}$) and did not degrade 5,6-epoxyretinoic acid. Therefore caffeic acid appears to inhibit step (2). Caffeic acid may inhibit the reaction of O $_2$ with retinoic acid radical, since addition of caffeic acid to the incubation mixture of haematin-catalysed retinoic acid 5,6-epoxidation resulted in complete cessation of O $_2$ consumption. Although haematin catalysed the 5,6-epoxidation of retinoic acid, the formation of retinoic acid radicals was not catalysed by haematin. Triton X-100 might be considered a candidate for the catalyst in the formation of the retinoic acid radical. However, the radical is also detected in the methanolic solution of retinoic acid (H. Iwahashi,

Fig. 6. Effect of addition of caffeic acid on the intensity of the e.p.r. signal and on the elution profile of the h.p.l.c.-e.p.r. analysis of spin adducts of nitrosobenzene with retinoic acid radicals formed in a reaction mixture containing retinoic acid and haematin

The intensity of the e.p.r. signal of the spin adduct of the spin-trap reagent nitrosobenzene with retinoic acid radicals was measured in the presence and in the absence of caffeic acid. The incubation mixture contained, in a total volume of 2.35 ml, 50 μ l of nitrosobenzene (20 mM in methanol) and 2.3 ml of standard reaction mixture (haematin as catalyst) containing 100 μ l of caffeic acid (0.1 M in methanol) or 100 μ l of methanol. A 2 ml portion of the incubation mixture, which was incubated for 10 min at 25 °C, was subjected to h.p.l.c.-e.p.r. analysis. H.p.l.c. conditions were as described in the text. E.p.r. conditions were as described in Fig. 5 legend except for the amplitude of modulation frequency and response time. The response time was 1.0 s and the amplitudes of modulation frequency were 5×10^3 for h.p.l.c.-e.p.r. analysis and 10×10^3 for time course of intensity of the e.p.r. signal. (a) Time course of intensity of e.p.r. signal was measured at 25 °C. Magnetic field was fixed at the position $g = 2.017$. (i) In the absence of caffeic acid; (ii) in the presence of caffeic acid. (b) H.p.l.c.-e.p.r. analysis of the incubation mixture. Magnetic field was fixed at the position $g = 2.017$. (i) In the absence of caffeic acid; (ii) in the presence of caffeic acid.

Y. Negoro, A. Ikeda, H. Morishita & R. Kido, unpublished work). Therefore Triton X-100 is not the catalyst. At this stage, we consider that retinoic acid and its radical are at the following equilibrium in the solution:

and the reaction may be autocatalytic or light-catalytic. Haematin appears to catalyse one and/or the other of the following reactions in step (2), namely reaction of O_2 molecules with retinoic acid radicals or formation of the epoxide. Dix & Marnett [3] presented the reaction mechanism of conversion of linoleic acid hydroperoxide into its epoxide. In their paper they showed that haematin catalysed the conversion of hydroperoxide into its epoxide. In the present retinoic acid 5,6-epoxidation catalysed by haematin a similar scheme seems very probable, namely that haematin catalyses the formation of 5,6-epoxyretinoic acid from retinoic acid hydro-

peroxide, which can be formed from the reaction of retinoic acid radical with O_2 .

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