Involvement of carbonyl reductase in superoxide formation through redox cycling of adrenochrome and 9,10-phenanthrenequinone in pig heart

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Abstract

The effects of adrenochrome, a metabolite of epinephrine (adrenaline), and 9,10-phenanthrenequinone (PQ), a component of diesel exhaust particles, on the stereoselective reduction of 4-benzoylpyridine (4-BP) were examined in pig heart cytosol. PQ was a potent inhibitor for the 4-BP reduction, while adrenochrome was a poor inhibitor. A similar result was observed in the effects of adrenochrome and PQ on the reduction of all-*trans* retinal. Furthermore, although PQ mediated efficiently the formation of superoxide anion radical through its redox cycling in pig heart cytosol, adrenochrome had no ability to mediate the superoxide formation. These may be because the reactivity for adrenochrome, catalyzed by pig heart carbonyl reductase (PHCR), is much lower than that for PQ. The optimal pH for the reduction of PQ in pig heart cytosol was around 5.5. Dicumarol, a potent inhibitor of DT-diaphorase, had little effect on the time course of NADPH oxidation during the reduction of PQ. Therefore, it is concluded that PHCR plays a critical role in superoxide formation through redox cycling of PQ.

Keywords: Adrenochrome; 9,10-Phenanthrenequinone; Carbonyl reductase; Superoxide formation; Pig heart

1. Introduction

Quinones are widely distributed in nature and environment. Quinones cause a variety of toxic effects, including cardiotoxicity, neurotoxicity, immunotoxicity and carcinogenesis [1, 2]. The toxic effects of quinones depend mainly on the production of reactive oxygen species through redox cycling mediated by microsomal NADPH-cytochrome P450 reductase (EC 1.6.2.4). The flavin enzyme catalyzes the one-electron reduction of quinones to semiquinones that would result in redox cycling. On the other hand, cytosolic DT-diaphorase (EC 1.6.99.2) and carbonyl reductase (EC 1.1.1.184) have the ability to reduce quinones to hydroquinones through two-electron transfer mechanism [3–7], leading to a detoxification pathway because the resulting hydroquinones serve as substrates for glucuronidation and sulfation reactions. Thus, it is likely that DT-diaphorase and carbonyl reductase play a role in protection against toxic effects of quinones. However, when secondary reactions such as glucuronidation and sulfation become limiting, autoxidation of the hydroquinones could contribute to the generation of reactive oxygen species [8].

Catecholamines can be enzymatically or non-enzymatically oxidized to the corresponding *o*-quinones [9–11]. For example, epinephrine (adrenaline) is biotransformed to adrenochrome (Fig. 1A). Adrenochrome is known to induce cardiotoxicity and neurotoxicity [9, 10, 12]. We have recently demonstrated that 9,10-phenanthrenequinone (PQ) shown in Fig. 1B, a component of diesel exhaust particles, not only inhibits the reduction of 4-benzoylpyridine (4-BP) and all-*trans*

retinal in the cytosolic fraction of pig heart, but also mediates superoxide formation through its redox cycling [13]. That is, PQ disturbs the homeostasis of retinoid metabolism and induces oxidative stress by acting probably as a substrate inhibitor of carbonyl reductase present in pig heart (pig heart carbonyl reductase, PHCR). Since adrenochrome, like PQ, is one of *o*-quinones, it may be reduced to its *o*-hydroquinone by PHCR, and mediate superoxide formation through its redox cycling. The purpose of this study is to examine the involvement of carbonyl reductase in superoxide formation through redox cycling of adrenochrome and PQ in pig heart.

2. Materials and methods

2.1. Materials

The chemicals were purchased from the following sources. Adrenochrome, PQ, all-*trans* retinal and all-*trans* retinol were from Sigma Chemical Co. (St. Louis, MO); 4-BP, cytochrome *c*, superoxide dismutase (SOD, from bovine erythrocyte) and dicumarol were from Wako Pure Chemical Industries (Osaka, Japan); NADPH, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Oriental Yeast Co. (Tokyo, Japan). *S*(-)-α-Phenyl-4-pyridylmethanol [*S*(-)-PPOL] was synthesized from 4-BP as reported previously [14]. All other chemicals were of reagent grade.

2.2. Preparation of pig heart cytosol

The pig hearts were supplied 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 105,000g for 60 min to obtain the cytosolic fraction.

2.3. Stereoselective reduction of 4-BP

The stereoselective reduction of 4-BP was estimated by measuring *S*(-)-PPOL formed in pig heart cytosol [13]. The reaction mixture consisted of substrate (0.5 mM 4-BP), NADPH-generating system (50 μM $NADP^+$, 1.25 mM glucose-6-phosphate, 50 munits glucose-6-phosphate dehydrogenase and 1.25 mM $MgCl_2$), pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 0.5 ml. In the case of inhibition experiments, adrenochrome or PQ dissolved in dimethyl sulfoxide was added at a concentration of 10 μM. The reaction mixture was incubated at 37 ˚C for 10 min and boiled for 2 min to stop the reaction. After centrifugation at 5,000 rpm, the supernatant (20 μl) was subjected to HPLC for the determination of the reduction product, *S*(-)-PPOL, of 4-BP. HPLC was carried out using a Waters 600E HPLC apparatus (Japan Waters, Tokyo, Japan) equipped with a Daicel Chiralpak AD-RH column (Daicel, Tokyo, Japan) and a Waters 484 UV monitor (254 nm). Mixture of 20 mM borate buffer (pH 9.0)-acetonitrile (6:4, v/v) was used as a mobile phase at a flow rate of 0.5 ml/min.

2.4. Reduction of all-trans retinal

The reduction of all-*trans* retinal was estimated by measuring all-*trans* retinol

formed in pig heart cytosol [13]. The reaction mixture consisted of substrate (0.5 mM all-*trans* retinal), NADPH-generating system, pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 7.4) in a final volume of 0.5 ml. In the case of inhibition experiments, adrenochrome or PQ dissolved in dimethyl sulfoxide was added at the concentration of 10 μM. The reaction mixture was incubated at 37 ˚C for 10 min and boiled for 2 min to stop the reaction. The preparation of the reaction mixture and the incubation was carried out in dark room. After centrifugation at 5,000 rpm, the supernatant (20 μl) was subjected to HPLC for the determination of the reduction product, all-*trans* retinol, of all-*trans* retinal. HPLC was carried out using a Tosoh DP-8020 HPLC apparatus (Tosoh, Tokyo, Japan) equipped with a Tosoh ODS-80Ts column and a Tosoh UV-8020 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.

2.5. Determination of superoxide anion radical

Superoxide anion radical was determined by the method of McCord and Fridovich [15] using cytochrome *c*. The reaction mixture consisted of NADPH-generating system, 0.1 mM EDTA, 50 μM cytochrome *c*, pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 1.0 ml. The reaction was started by the addition of adrenochrome or PQ at a concentration of 10 μM. The reduction of ferricytochrome *c* (Fe^{3+}) to ferrocytochrome *c* (Fe^{2+}) in the enzyme reaction system was measured by recording the absorbance at 550 nm.

2.6. Reduction of adrenochrome and PQ

The reduction of adrenochrome and PQ was measured spectrophotometrically by monitoring the decrease in the absorbance of NADPH at 340 nm. The reaction mixture consisted of substrate (adrenochrome or PQ), 0.3 mM NADPH, pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 0.5 ml. In the case of determination of the optimal pH, 100 mM sodium potassium phosphate buffers at pH 5.0-9.0 were used. The enzyme reaction was initiated by the addition of adrenochrome or PQ at various concentrations to the reaction mixture. The kinetic parameters (K_m and V_{max}) of enzyme reaction for adrenochrome and PQ 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. Protein concentration was determined by the method of Lowry et al. [16] with bovine serum albumin as the standard.

2.7. Statistical analysis

Statistical analysis of data were performed using Student's *t*-test, and $P < 0.05$ was considered to be significant.

3. Results

3.1. Effects of adrenochrome and PQ on the stereoselective reduction of 4-BP

Figure 2 shows the effects of adrenochrome and PQ on the stereoselective reduction

of 4-BP in pig heart cytosol. PQ was a potent inhibitor for the 4-BP reduction. Although adrenochrome at a concentration of 100 μM also exhibited a significant inhibition in the stereoselective reduction of 4-BP, the inhibition was smaller than that by PQ at a concentration of 10 μM.

3.2. Effects of adrenochrome and PQ on the reduction of all-trans retinal

All-*trans* retinal is known to be an endogenous substrate of PHCR [17]. Thus, we attempted to elucidate the effects of adrenochrome and PQ on the reduction of all-*trans* retinal in pig heart cytosol. As shown in Fig. 3, PQ potently inhibited the reduction of all-*trans* retinal, while ardenochrome, even at a concentration of 100 μM, had little effect on the reduction of all-*trans* retinal.

3.3. Adrenochrome- and PQ-mediated reduction of cytochrome c

Whether adrenochrome and PQ induce the formation of superoxide anion radical was examined in pig heart cytosol. The absorbance of cytochrome *c* at 550 nm was increased with the time by adding PQ, based on the reduction of ferricytochrome *c* to ferrocytochrome *c* (line a of Fig.4). Furthermore, SOD was confirmed to decrease the increased absorbance of cytochrome *c* (line b of Fig. 4), indicating that PQ mediates the formation of superoxide anion radical through its redox cycling in pig heart cytosol. SOD could not fully abolish the increased absorbance of cytochrome *c* at 550 nm. This may be because the semiquinone generated in this reaction system also reduces ferricytochrome *c*, as has been pointed out by Winterbourn [18]. On the other hand,

even though adrenochrome slightly increased the absorbance of cytochrome *c* at 550 nm, superoxide dismutase had no effect on the increased absorbance of cytochrome *c* (lines c and d of Fig. 4). These findings suggest that adrenochrome has no ability to mediate the formation of superoxide anion radical through its redox cycling in pig heart cytosol.

3.4. The kinetic parameters of reductive reaction for adrenochrome and PQ

The kinetic parameters of reductive reaction for adrenochrome and PQ in pig heart cytosol are summarized in Table 1. The K_m values for adrenochrome and PQ were 0.083 ± 0.021 and 0.003 ± 0.001 mM, respectively. Furthermore, the V_{max}/K_m value for adrenochrome was much smaller than that for PQ, indicating that the reactivity for adrenochrome is much lower than that for PQ.

3.5. pH dependency of PQ reduction

The pH dependency of PQ reduction was examined in pig heart cytosol. The optimal pH for the PQ reduction was around 5.5 (Fig. 5).

3.6. Effect of dicumarol on the time course of NADPH oxidation during the reduction of adrenochrome and PQ

The effect of dicumarol on the reduction of adrenochrome and PQ was spectrophotometrically examined in pig heart cytosol. Dicumarol was used at a concentration of 4 μM, which has been shown to produce complete inhibition of DT-diaphorase [19]. As shown in lines a and b of Fig. 6, dicumarol did not affect the

decrease in the absorbance of NADPH at 340 nm during the reduction of adrenochrome. A similar result was observed for the reduction of PQ in pig heart cytosol (lines c and d of Fig. 6).

4. Discussion

We have recently found that carbonyl compounds including 4-BP and all-*trans* retinal are reduced by PHCR present in the cytosolic fraction of pig heart [13, 14]. Thus, the effects of adrenochrome and PQ on the reduction of 4-BP and all-*trans* retinal were examined using the cytosolic fraction of pig heart. The results obtained in this study established that PQ not only inhibits strongly the reduction of 4-BP and all-*trans* retinal, which is an endogenous substrate of PHCR, but also mediates superoxide formation through its redox cycling. Our previous paper has shown that PQ competitively inhibits 4-BP reduction (K_i = 0.57 μM) [13]. Furthermore, the present study demonstrated that PQ is efficiently reduced in the cytosolic fraction of pig heart. On the basis of these results, we propose that PQ disturbs the homeostasis of retinoid metabolism and induces oxidative stress by acting as a potent substrate inhibitor of PHCR. Adrenochrome, like PQ, is one of *o*-quinones. However, adrenochrome was a poor inhibitor for the reduction of all-*trans* retinal in pig heart cytosol, although adrenochrome at a high concentration of 100 μM exhibited a significant inhibition in the reduction of 4-BP. In addition, adrenochrome had no ability to mediate the formation of superoxide anion radical through its redox cycling in pig heart cytosol.

To elucidate the reason for the differential effects of PQ and adrenochrome on the reduction of 4-BP and all-*trans* retinal, the kinetic parameters of reductive reaction for these two *o*-quinones were determined in pig heart cytosol. As expected, the V_{max}/K_m value for adrenochrome was much smaller than that for PQ, indicating that adrenochrome can not act efficiently as a substrate inhibitor of PHCR.

Recently, the optimal pH for the reduction of recombinant PHCR has been reported to be around pH 5.5 [17]. In the present study, the optimal pH for PQ reduction in pig heart cytosol was confirmed to be around pH 5.5. In addition, PQ strongly inhibited the reduction of carbonyl compounds such as 4-BP and all-*trans* retinal in pig heart cytosol. We conclude that in pig heart cytosol, carbonyl reductase (PHCR) plays a critical role in the two-electron reduction of PQ to its hydroquinone.

It has been accepted that DT-diaphorase, like carbonyl reductase, is a cytosolic enzyme and catalyzes two-electron reduction of quinones [3, 20, 21]. Thus, whether DT-diaphorase also contributes to the reduction of adrenochrome and PQ in pig heart cytosol was examined using dicumarol known as a potent inhibitor of DT-diaphorase [19, 22]. However, dicumarol did not affect the time course of NADPH oxidation during the reduction of adrenochrome and PQ. As described above, the optimal pH for PQ reduction in pig heart cytosol was around pH 5.5. On the other hand, DT-diaphorase has been pointed out to exhibit maximal activity in the pH range 7-9 [23, 24]. Therefore, it is reasonable to assume that DT-diaphorase has little ability to reduce adrenochrome and PQ under the experimental conditions of this study. Further studies are necessary to demonstrate whether high enough 4-BP can abolish PQ redox

cycling as failure of this to happen would imply a second enzyme is involved in PQ reduction aside from PHCR.

In conclusion, carbonyl reductase present in pig heart, namely PHCR, is mainly involved in superoxide formation through redox cycling of PQ. In the case of adrenochrome, however, the enzyme is not because the reactivity for adrenochrome is much lower than that for PQ. We are currently investigating the contribution of carbonyl reductase to cardiotoxicity of quinones other than adrenochrome and PQ.

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Figure captions:

Fig. 1. Chemical structures of adrenochrome and PQ

Fig. 2. Effects of adrenochrome and PQ on the stereoselective reduction of 4-BP in pig heart cytosol. 4-BP at a concentration of 0.5 mM was used as the substrate. Each bar represents the mean ± S.D. of three experiments. ***$P < 0.001$, significantly different from adrenochrome at a concentration of 10 μM.

Fig. 3. Effects of adrenochrome and PQ on the reduction of all-*trans* retinal in pig heart cytosol. All-*trans* retinal at a concentration of 0.5 mM was used as the substrate. Each bar represents the mean ± S.D. of four experiments. ***$P < 0.001$, significantly different from adrenochrome at a concentration of 10 μM.

Fig. 4. Adrenochrome- and PQ-mediated reduction of cytochrome *c* in pig heart cytosol. a, PQ (10 μM); b, PQ (10 μM) + SOD (300 units); c, adrenochrome (10 μM); d, adrenochrome (10 μM) + SOD (300 units).

Fig. 5. pH dependency of PQ reduction in pig heart cytosol. PQ at a concentration of 10 μM was used as the substrate. Each point represents the mean ± S.D. of three experiments.

Fig. 6. Effect of dicumarol on the time course of NADPH oxidation during the reduction of adrenochrome and PQ in pig heart cytosol. The reaction mixture consisted of substrate (adrenochrome or PQ), 0.3 mM NADPH, pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 0.5 ml. a, adrenochrome (10 μM) + dicumarol (4 μM); b, adrenochrome (10 μM); c, PQ (10 μM) + dicumarol (4 μM); d, PQ (10 μM).