

**Inhibitory effects of diesel exhaust components and flavonoids on  
20 $\alpha$ -hydroxysteroid dehydrogenase activity in mouse tissues**

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## **Abstract**

The inhibitory effects of diesel exhaust components and flavonoids on  $20\alpha$ -hydroxysteroid dehydrogenase ( $20\alpha$ -HSD) activity were examined in cytosolic fractions from the liver, kidney and lung of male mice. 9,10-Phenanthrenequinone (9,10-PQ) and 1,2-naphthoquinone (1,2-NQ), which are contained in diesel exhaust particles (DEPs), potently inhibited  $20\alpha$ -HSD activity in liver cytosol. 9,10-PQ also inhibited the enzyme activity in lung cytosol. However,  $20\alpha$ -HSD activity in kidney cytosol was little inhibited by 9,10-PQ or 1,2-NQ. Flavonoids such as quercetin, fisetin and kaempferol exhibited high inhibitory potencies for  $20\alpha$ -HSD activity in liver cytosol, whereas these flavonoids were poor inhibitors for the enzyme activity in kidney cytosol. It is likely that several diesel exhaust components and flavonoids augment the signaling of progesterone in the liver cells, by inhibiting potently  $20\alpha$ -HSD activity in mouse liver cytosol. The possibility that there are distinct enzymes catalyzing  $20\alpha$ -HSD activity in non-reproductive tissues of male mice is also discussed.

**Keywords:**  $20\alpha$ -Hydroxysteroid dehydrogenase activity, diesel exhaust components, flavonoids, mouse tissues, inhibitory potency

## Introduction

20 $\alpha$ -Hydroxysteroid dehydrogenase (20 $\alpha$ -HSD) was originally purified from rat ovary and characterized [1-3]. This enzyme belongs to the aldo-keto reductase (AKR) superfamily [4] and catalyzes the reduction of progesterone to its inactive metabolite 20 $\alpha$ -hydroxy-4-pregnen-3-one, as shown in Figure 1. Progesterone is essential for maintaining pregnancy in mammals, and its metabolism to 20 $\alpha$ -hydroxy-4-pregnen-3-one is associated with the termination of pregnancy. However, progesterone at high levels has adverse effects on the development of fetuses [5]. 20 $\alpha$ -HSD probably plays a role in regulating the amount of progesterone and in protecting its toxic effect. Recently, the ratio of 5 $\alpha$ -pregnanes produced by 5 $\alpha$ -reductase to 4-pregnenes produced by 20 $\alpha$ -HSD or 3 $\alpha$ -HSD from progesterone has been demonstrated to provide a hormonal basis for breast cancer [6, 7]. Thus, the inhibition of 20 $\alpha$ -HSD by environmental pollutants and xenobiotic compounds can increase this ratio and may promote breast cancer by stimulating cell proliferation and detachment.

Exposure of diesel exhaust particles (DEPs) to experimental animals and humans has been reported to cause lung cancer, allergic inflammation, asthma, and cardiopulmonary diseases [8-10]. DEPs contain a variety of quinones that are capable of catalyzing the generation of reactive oxygen species in biological systems [11, 12]. Among quinones, 9,10-phenanthrenequinone (9,10-PQ) is known as a relatively abundant quinone in DEPs [13, 14]. We have recently shown that 20 $\alpha$ -HSD activity is

observed in cytosolic fractions from the lung in addition to the liver and kidney of male mice [15]. Since 9,10-PQ is a good substrate of 20 $\alpha$ -HSD [3], it may inhibit 20 $\alpha$ -HSD activity as a substrate inhibitor.

Flavonoids are naturally occurring compounds that are widely found in plants. Much attention has been paid to their antioxidant activities that affect the formation of reactive oxygen species and lipid peroxidation [16, 17]. However, there is also evidence that flavonoids exhibit adverse health effects [18-20]. Our previous paper [15] has found that flavonoids such as fisetin and quercetin potently inhibit 20 $\alpha$ -HSD activity in cytosolic fraction from the liver of male mice, suggesting that the inhibition by flavonoids may result in disordered progesterone signaling. Furthermore, in our preliminary experiment, the inhibitory potency of a typical flavonoid quercetin for 20 $\alpha$ -HSD activity was found to be distinguished clearly between liver and kidney cytosols.

The purpose of the present study was to elucidate whether diesel exhaust components including 9,10-PQ inhibit 20 $\alpha$ -HSD activity in cytosolic fractions from the liver, kidney and lung of male mice. Furthermore, the inhibitory potencies of flavonoids for 20 $\alpha$ -HSD activity in kidney cytosol were compared with those for the enzyme activity in liver cytosol.

## **Materials and methods**

### *Materials*

Progesterone (4-pregnene-3,20-dione) and 20 $\alpha$ -hydroxy-4-pregnen-3-one were purchased from Sigma (St. Louis, MO, USA). Diesel exhaust components were obtained from the following sources: 9,10-PQ (Sigma); 1,2-naphthoquinone (1,2-NQ), 1,4-naphthoquinone (1,4-NQ), 9,10-anthraquinone (9,10-AQ), phenanthrene, and pyrene (Aldrich, Milwaukee, WI); anthracene and  $\beta$ -naphthoflavone ( $\beta$ -NF) (Wako Pure Chemicals, Tokyo, Japan). Flavonoids were obtained from the following sources: morin, myricetin, genistein, taxifolin (racemate), kaempferol and daidzein (Sigma); naringenin, apigenin and (-)-epicatechin (Aldrich); quercetin and luteolin (Wako Pure Chemicals); fisetin and quercitrin (Tokyo Kasei, Tokyo, Japan). Genistin, (+)-catechin and rutin were donated by Dr. J. Kinjo (Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan). Sulfobromophthalein (SBP) and succinic semialdehyde (SSA) were obtained from Sigma, and 2-carboxybenzaldehyde (2-CBA) was obtained from Wako Pure Chemicals. NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were of reagent grade.

### *Animals*

Male ddY mice at 8 weeks of age were purchased from Japan SLC (Shizuoka, Japan). The animals had free access to a diet of standard laboratory chow and water. All animal experiments were undertaken in compliance with the guideline principles and procedures of Kumamoto University for the care and use of laboratory animals.

### *Preparation of cytosolic fraction*

The animals were slightly anesthetized and killed by decapitation. The liver, kidney and lung were quickly excised, and homogenized in a Potter-Elvehjem homogenizer with three volumes of 10 mM sodium-potassium phosphate buffer containing 1.15% KCl (pH 7.4). All subsequent procedures were performed at 3–5°C. The homogenates were centrifuged at 10,000g for 20 min and the resulting supernatants were centrifuged at 105,000g for 60 min to obtain the cytosolic fraction. The cytosolic fractions from the liver, kidney and lung of male mice were used as enzyme preparations.

### *Assay of 20 $\alpha$ -HSD activity*

The enzyme activity was conducted in an NADPH-generating system consisting of progesterone (0.1 mM), NADP (0.25 mM), glucose-6-phosphate (6.25 mM), glucose-6-phosphate dehydrogenase (0.25 units), MgCl<sub>2</sub> (6.25 mM), enzyme preparation (cytosolic fraction) and 100 mM sodium-potassium phosphate buffer (pH 7.4) in a final volume of 2.0 ml. In the case of determination of the optimal pH, 100 mM sodium-potassium phosphate buffers at 5.0–9.0 were used. The mixture was incubated at 37 °C for 30 min under aerobic condition. The reaction was stopped by addition of 0.5 ml of 1.0 N HCl to the mixture. The reduction product (20 $\alpha$ -hydroxy-4-pregnen-3-one) of progesterone was determined by HPLC according to a slightly modified method of Swinney et al [21]. HPLC was carried out using a Shimadzu LC-10AD HPLC apparatus (Shimadzu, Kyoto, Japan) equipped with a Tosoh

ODS-80Ts column (Tosoh, Tokyo, Japan) and a JASCO 875-UV monitor (240 nm) (JASCO, Tokyo, Japan). Mixture of water-acetonitrile-methanol-tetrahydrofuran (44:28:17:11, v/v) was used as a mobile phase at a flow rate of 0.6 ml/min. Protein concentration was estimated by the method of Lowry et al. [22] with bovine serum albumin as the standard.

#### *Inhibition experiments*

Inhibitors were dissolved in dimethyl sulfoxide (DMSO) or methanol, and then added to the reaction mixture. The final concentration of DMSO or methanol did not exceed 2 % (v/v), and this concentration did not affect the enzyme reaction. The final concentration of inhibitors was 10  $\mu$ M for diesel exhaust components, 20 or 50  $\mu$ M for flavonoids and 100  $\mu$ M for SBP, 2-CBA and SSA.

## **Results**

#### *Effects of diesel exhaust components on 20 $\alpha$ -HSD activity*

The inhibitory effects of diesel exhaust components at a concentration of 10  $\mu$ M on 20 $\alpha$ -HSD activity were examined in the cytosolic fractions from the liver, kidney and lung of male mice (Figure 2). Among diesel exhaust components tested, 9,10-PQ and 1,2-NQ were potent inhibitors for 20 $\alpha$ -HSD activity in liver cytosol, although diesel exhaust components other than these two *o*-quinones had little ability to inhibit 20 $\alpha$ -HSD activity in liver cytosol. Interestingly, 9,10-PQ also inhibited 20 $\alpha$ -HSD

activity in lung cytosol. On the other hand, the enzyme activity in kidney cytosol was little inhibited by diesel exhaust components including 9,10-PQ or 1,2-NQ.

#### *Effects of flavonoids on 20 $\alpha$ -HSD activity*

The inhibitory effects of flavonoids on 20 $\alpha$ -HSD activity in the cytosolic fraction from the kidney of male mice were compared with those from the liver [15]. As shown in Figure 3, the inhibitory potencies of several flavonoids were much lower for 20 $\alpha$ -HSD activity in kidney cytosol than that in liver cytosol. For example, although quercetin, fisetin and kaempferol at a concentration of 20  $\mu$ M exhibited high inhibitory potencies for 20 $\alpha$ -HSD activity in liver cytosol, these flavonoids, even at a concentration of 50  $\mu$ M, were poor inhibitors for the enzyme activity in kidney cytosol. Furthermore, naringenin potently inhibited 20 $\alpha$ -HSD activity in liver cytosol, whereas it had no ability to inhibit 20 $\alpha$ -HSD activity in kidney cytosol.

#### *Optimal pH of 20 $\alpha$ -HSD activity*

Figure 4 shows pH-dependent profiles of 20 $\alpha$ -HSD activity in mouse liver and kidney cytosols. The optimal pH of 20 $\alpha$ -HSD activity in liver cytosol was 6.5 – 7.0. On the other hand, the optimal enzyme activity in kidney cytosol was observed at around pH 6.0.

#### *Effects of specific inhibitors on 20 $\alpha$ -HSD activity*

The inhibitory effects of SBP, 2-CBA and SSA at a concentration of 100  $\mu$ M on



20 $\alpha$ -HSD activity were examined in the cytosolic fractions from the liver and kidney of male mice. SBP is a potent inhibitor of a mouse aldo-keto reductase (AKR) AKR1C20 [23]. 2-CBA and SSA were used as substrate inhibitors because of good substrates for AKR7A1 and AKR7A5 [24, 25]. As shown in Figure 5, SBP strongly inhibited 20 $\alpha$ -HSD activity in kidney cytosol, whereas 2-CBA and SSA had little ability to inhibit the enzyme activity in kidney cytosol. These three inhibitors had little effect on 20 $\alpha$ -HSD activity in liver cytosol.

## **Discussion**

It has been reported that 9,10-PQ and 1,2-NQ serve as inhibitors of enzymes such as nitric oxide synthase [26], carbonyl reductase [27, 28] and glyceraldehyde-3-phosphate dehydrogenase [29]. In the present study, 9,10-PQ was demonstrated to be a potent inhibitor for 20 $\alpha$ -HSD activity in the cytosolic fractions from the liver and lung of male mice. It should be noted that 9,10-PQ inhibits 20 $\alpha$ -HSD activity in lung cytosol. However, 20 $\alpha$ -HSD activity in kidney cytosol, unlike those in liver and lung cytosols, was little inhibited by 9,10-PQ. Although 1,2-NQ, one of *o*-quinones, also inhibited 20 $\alpha$ -HSD activity in liver cytosol, it had little ability to inhibit 20 $\alpha$ -HSD activity in kidney and lung cytosols. 20 $\alpha$ -HSD catalyzes the reduction of progesterone to its inactive metabolite 20 $\alpha$ -hydroxy-4-pregnen-3-one and is involved in regulating the amount of progesterone that binds to its nuclear receptor. Therefore, 9,10-PQ and 1,2-NQ contained in DEPs probably augments the

signaling of progesterone in the liver cells, by inhibiting potently  $20\alpha$ -HSD activity. 9,10-PQ is produced from phenanthrene, which comprises 6% of the total organic extract of DEPs, by photooxidation in the atmosphere [30]. However, phenanthrene was found to have little ability to inhibit  $20\alpha$ -HSD activity in cytosolic fractions of mouse liver, kidney and lung.

Very recently, evidence has been provided that flavonoids such as fisetin, quercetin and naringenin potently inhibit  $20\alpha$ -HSD activity in cytosolic fraction from the liver of male mice [15]. Thus, it is reasonable to assume that these flavonoids, like 9,10-PQ and 1,2-NQ, augment progesterone signaling by inhibiting potently hepatic  $20\alpha$ -HSD activity. In this study, we further attempted to evaluate the inhibitory effects of flavonoids on  $20\alpha$ -HSD activity in cytosolic fraction of mouse kidney. However, the inhibitory potencies of flavonoids for the enzyme activity were much lower in kidney cytosol than in liver cytosol.

On the basis of the tissue-dependent inhibitory potencies of *o*-quinones and flavonoids for  $20\alpha$ -HSD activity as described above, we propose the possibility that there are several distinct enzymes catalyzing  $20\alpha$ -HSD activity in tissues of mice. In addition, the optimal pH of  $20\alpha$ -HSD activity in mouse liver cytosol was found to be distinguished from that in mouse kidney cytosol. This finding also supports the idea that in cytosolic fractions of mouse liver and kidney, different enzymes catalyze the  $20\alpha$ -HSD activity.

In mice,  $17\beta$ -HSD type5 (AKR1C6) has been demonstrated to exhibit low  $20\alpha$ -HSD activity [31, 32]. Thus, it is possible that enzymes belonging to the AKR

subfamily other than mouse 20 $\alpha$ -HSD (AKR1C18) play a role in the reduction of progesterone to 20 $\alpha$ -hydroxy-4-pregnen-3-one (20 $\alpha$ -HSD activity) in cytosolic fractions of mouse liver and kidney. AKR1C20 exhibits the highest sequence identity with AKR1C6, and its mRNA is specifically expressed in the liver [23]. This enzyme also reduces *S*-camphorquinone, showing a broad pH optimum from 6.5 to 7.5, similarly to the pH-dependent profile of 20 $\alpha$ -HSD activity in mouse liver cytosol. Thus, AKR1C20 may be an enzyme responsible for 20 $\alpha$ -HSD activity in mouse liver cytosol. However, SBP, a potent inhibitor of AKR1C20 [23], had little effect on 20 $\alpha$ -HSD activity in mouse liver cytosol. Unexpectedly, SBP strongly inhibited 20 $\alpha$ -HSD activity in mouse kidney cytosol, even though AKR1C20 mRNA is not expressed in the kidney [33]. Furthermore, SSA and 2-CBA, substrate inhibitors of AKR7A5 [25], had little effect on the enzyme activity in mouse liver or kidney cytosol. Additional studies are progress to identify the AKR enzymes that catalyze 20 $\alpha$ -HSD activity in cytosolic fractions from the non-reproductive tissues of male mice.

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Figure legends:

Figure 1. Reduction of progesterone to 20 $\alpha$ -hydroxy-4-pregnen-3-one by 20 $\alpha$ -HSD.

Figure 2. Inhibitory effects of diesel exhaust components on 20 $\alpha$ -HSD activity in cytosolic fractions from the liver, kidney and lung of male mice. Progesterone at a concentration of 100  $\mu$ M was used as the substrate. The concentration of diesel exhaust components was 10  $\mu$ M. Each bar represents the mean  $\pm$  S.D. of three to seven experiments.

Figure 3. Inhibitory effects of flavonoids on 20 $\alpha$ -HSD activity in cytosolic fractions from the liver and kidney of male mice. Progesterone at a concentration of 100  $\mu$ M was used as the substrate. The concentrations of flavonoids were 20 and 50  $\mu$ M. Each bar represents the mean  $\pm$  S.D. of three experiments. Data in cytosolic fraction from the liver were cited from reference [15].

Figure 4. pH dependence of 20 $\alpha$ -HSD activity in cytosolic fractions from the liver and kidney of male mice. Progesterone at a concentration of 100  $\mu$ M was used as the substrate. Each point represents the mean  $\pm$  S.D. of three experiments.

Figure 5. Inhibitory effects of specific inhibitors on 20 $\alpha$ -HSD activity in cytosolic fractions from the liver and kidney of male mice. Progesterone at a concentration of



100  $\mu\text{M}$  was used as the substrate. The concentration of specific inhibitors was 100  $\mu\text{M}$ . Each bar represents the mean  $\pm$  S.D. of three experiments.