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Comparison Between TCDD and 3MC Action on CYP1A1 Expression and EROD Activity in the Isolated Perfused Female Rat Liver

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ABSTRACT *: In order to understand the mechanism of the regulation of CYP1A1 gene expression and ethoxyresorufin deethylase (EROD) activity in ex vivo system, we have studied the action of TCDD and 3MC in the isolated perfused female rat liver. CYP1A1 mRNA level and EROD activity were measured in rat liver that was isolated and perfused with various chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methylcholanthrene (3MC), 17β-estradiol (E_2), morin. TCDD or 3MC alone perfusion into female rat liver resulted in increase of CYP1A1 mRNA level and the magnitude of stimulation was six times higher with TCDD treatment than 3MC treatment. However E_2 perfusion into female rat liver showed inhibition of CYP1A1 mRNA level. When 10^{-8} M E_2 was administered concomitantly with either 10^{-9} M TCDD or 10^{-9} M 3MC, stimulated CYP1A1 mRNA by either TCDD or 3MC was inhibited. Morin was examined for its effects on CYP1A1 mRNA level and result was similar to that was observed with estrogen. EROD activity was also stimulated with either TCDD or 3MC perfusion, and the magnitude of EROD stiumlation was smaller than that of CYP1A1 mRNA stimulation in response to TCDD or 3MC perfusion. Unlike CYP1A1 mRNA level, stimulation of EROD activity was greater with 3MC than TCDD. Concomitant perfusion either E2 or morin with TCDD or 3MC inhibited 3MC perfusion or TCDD perfusion stimulated EROD activity. These data suggested that TCDD and 3MC might act diffrently in terms of regulation of CYP1A1 gene expression in rat liver.*

Key Words *: TCDD, 3MC, EROD, Morin, CYP1A1*

I. INTRODUCTION

The cytochrome P450-dependent monooxygenase system catalyzes oxidative metabolism of a wide variety of substrates including endogenous as well as exogenous compounds. As a preliminary detoxification step, many compounds are first converted to polar metabolites by cytochrome P450, which facilitates their elimination. However, some compounds may also be inadvertently bioactivated by cytochrome P450 to reactive intermediates that produced adverse biological effects (Amdur *et al.*, 1996; Yang *et al.*, 1978). For example carcinogenic polycyclic aromatic hydrocarbons (PAH) such as benzo(a)pyrene (B[a]P) undergo metabolic activation by cytochrome P450 and expoxide hydrolase to chemically reactive ultimate carcinogen diol expoxides (Chou *et al.*, 1986). The *CYP1* family, which consists of at least three enzymes, *CYP1A1, CYP1A2* and *CYP1B1* has been shown to be important in the metabolism of several xenobiotics such as PAH and heterocyclic amines, and expression of these enzymes is inducible by PAHs such as TCDD. TCDD induction of CYP1 transcription is mediated by the cytosolic arylhydrocarbon receptor (AhR), which is known as a ligand-activated transcription factor. Activation of AhR involves ligand binding, dissociation of heat-shock protein-90, nuclear translocation, and dimerization with the AhR nuclear translocator protein (Arnt) followed by binding to DRE (for XRE) enhancer elements in the 5-noncoding region of the responsive gene (Carrier *et al.*, Riddick *et al.*, 1994; Denison *et al.*, 1988). The mechanism of action of this compound is to activate the arylhydrocarbon receptor (AhR) to a form that binds to specific gene regulatory sequence

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elements, called xenobiotic responsive elements (XREs), through heterodimerization with Arnt (Ko *et al.*, 1996; Mason *et al.*, 1994; Dolwick *et al.*, 1993). AhR and Arnt have a similar overall structure and belong to the basic helix-loop-helix class of transcription factors (Poland and Gloven, 1974; Pendurthi *et al.*, 1993). Upon binding XREs, the AhR-Arnt complex activates transcription of adjacent structural genes which encodes enzymes that are involved in the oxidative metabolism of these compounds (Miksicek, 1995; Sousa and Marletta, 1995). Thus far, we assumed that arylhydrocarbons such as 3MC, and TCDD would affect drug metabolism via identical mechanism of action. However, very little study have been reported to confirm if it is in deed true that 3MC and TCDD act in an identical mechanism of action on the regulation of *CYP1A1* gene expression. Therefore we have undertaken study to examine how 3MC and TCDD would affect *CYP1A1* gene expression and EROD activity. After livers from female sprague dawely rats were isolated and perfused with TCDD or 3MC or E2 or morin, the level of CYP1A1 mRNA was measured via RT-PCR and EROD activity was determined. Our finding showed that the levels of CYP1A1 mRNA and EROD activity in female liver were increased with either 3MC or TCDD treatment. The magnitude of TCDD stimulation of CYP1A1 mRNA was ten times higher than that of 3MC stimulation whereas the magnitude of TCDD stimulation of EROD was less than that of 3MC stimulation. This study indicated that 3MC and TCDD might act differently on CYP1A1 expression and EROD activity.

II. MATERIALS AND METHODS

1. Materials

3MC was purchased from Aldrich Chemical Co (Milwaukee, WI, USA). Quercetin, morin, dimethylsulfoxide (DMSO), 17β-estradiol, β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), bovine serum albumin (BSA), ethoxyresorufin, resorufin, potassium phosphate, sodium phosphate, sodium bicarbonate, sodium chloride, potassium chloride, glucose, calcium chloride, magnesium sulfate, phenol, mercaptoethanol, and chloroform were obtained from Sigma Chemical Co (St. Louis, MO, USA) and diethylpyrocarbonate, guanidinium thiocyanate, dATP, dCTP, dGTP, dTTP, moloney murine leukemia virus reverse transcriptase, dithiothreitol were from GIBCO (Gaithersburg, MD, USA). Oligo d(T)15, Taq DNA polymerase, RNasin were purchased from Promega (Madison, WI, USA). TCDD was a gift from Dr. Chae K. at NIEHS, NIH, USA.

2. Isolated liver perfusion

Female Sprague-Dawley rats were anesthesized by ketamine chloride (100 mg/kg, i.p.). The portal vein of rat was cannulated by polyethylen catheter (18 guage) and isolated into perfusion chamber. The isolated liver was perfused with Krebs-Henseleit Bicarbonate buffer (KHB, pH 7.4) saturated with air (95% O_2, 5% CO_2). The isolated liver was perfused with KHB for the first 10 minutes to maintain a stable physiological condition before the perfusion with various chemicals for 4 hrs.

3. Preperation of microsomes

Liver tissue was homogenized in phosphate buffer (pH 7.6) using polytron. Microsomal fraction was prepared by differential centrifugation and resuspended in pyrophosphate buffer (pH 7.6) and stored at -70°C until use. Total protein was measured using the bicinchonic acid (BCA) method (Pierce, Rockville, IL USA) using BSA as a standard.

4. Measurement of ethoxyresorufin O-deethylase activity

Liver microsomes were prepared as previously reported (Fujino *et al.*, 1984). Microsomes were incubated with 5 μM ethoxyresorufin for 1 minute at 37°C and 250 μM NADPH, enzymatic activity was monitored via change in fluorescence based on time change. After the 15 minutes, measurement of enzymatic activity was calculated from the area under the peak. (excitation 530 nm, emission 579 nm).

5. Total RNA isolation

Total RNA was isolated using method by Chomczynski and Sacchi (1987). Liver tissue was homogenized with denaturing solution and extrated with phenol and chloroform. The RNA pellet was reconstituted in diethylpyrocarbonate treated water.

6. Reverse transcription and Polymerase chain reaction (RTPCR)

For conversion of total RNA to cDNA, a reaction mixture was prepared containing reverse transcriptase (RT) buffer, 10 mM dithiothreitol, 0.05 mM dNTPs, oligo d(T)15 primer, 20U RNasin, 200U MLV-RT and 3 μg of total RNA. The reaction was incubated for 1hr at 37°C followed by inactivation of the enzyme at 95°C for 10 min. For the PCR amplification of cDNA, a reaction mixture was prepared containing RT mix, polymerase reaction buffer, 2.5 mM $MgCl_2$, 0.125 mM dNTP, 1U *Taq* DNA polymerase, and 3 pmol each of the forward and reverse primers. *CYP1A1* forward primer; 5'CCATGACCAGGAACTATGGG3' *CYP1A1* reverse primer; 5'TCTGGTGAGCATCCAGGACA3' : β-Actin forward primer; 5'CCTCTATGCCAACACAGT3' β-*Actin* reverse primer; 5'AGCCACCAATCCACACAG3' PCR products were analyzed on in 2% agarose gels (PCR product sizes; *CYP1A1*, 341 bps; β-*Actin*, 153 bps) and quantified by a Gel Doc 1000 video imaging analysis system.

III. RESULTS AND DISCUSSION

1. Change of CYP1A1 mRNA level with 3MC or TCDD treatment

Expression of *CYP1A1* gene in response to either 3MC or TCDD was examined by measuring CYP1A1 mRNA level via RTPCR from the isolated perfused rat liver system *in ex vivo*. As shown in Fig. 1, 1 nM 3MC perfusion into female rat liver resulted in 160% of CYP1A1 mRNA level when that of 0.01% DMSO perfused liver was set at 100%. In the case of 1 nM TCDD perfusion, CYP1A1 mRNA level was increased over control female rat liver by 1000%

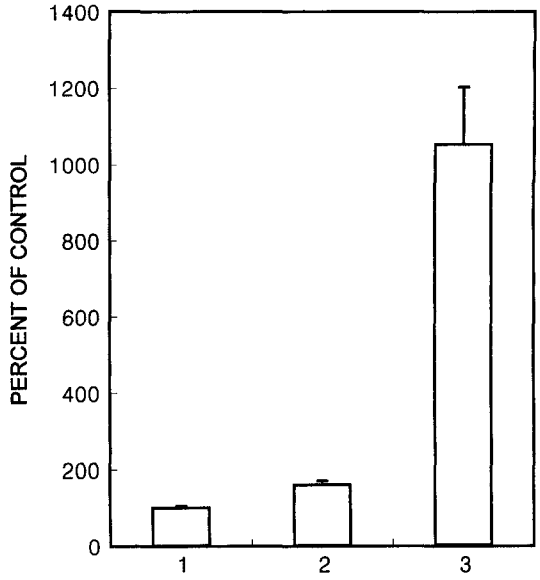

Fig. 1. Quantitation of the CYP1A1 mRNA expression by 3-MC and TCDD stimulation in the perfused female rat liver. CYP1A1 mRNA was measured by RTPCR followed by agarose gel electrophoresis. 341 bp PCR product was quantified by image analysis system as described in Methods. Data represent mean±S.E. (n=5). 1: 0.01% DMSO in KHB, 2: 10^{-9} M 3-MC, 3: 10^{-9} M TCDD.

(Fig. 1). This data clearly showed differences between 3MC and TCDD in terms of the regulation of xenobiotic metabolising enzyme *CYP1A1* gene expression in female rat liver.

2. Change of EROD activity with 3MC or TCDD treatment

The EROD activity was determined in the 1 nM 3MC or 1nM TCDD perfused female rat liver microsome. 1 nM 3MC increased EROD activity over that of control rat liver with similar magnitude compare to CYP1A1 mRNA increase in response to 1 nM 3MC (Figs. 1, 2). But 1 nM TCDD treatment in female rat liver showed less effect on EROD activity which was 106% of control than on CYP1A 1 mRNA level which was about 1000% that of control. 3MC is known to stimulate both CYP1A1 mRNA and CYP1A2 mRNA, whereas TCDD stimulate only CYP1A1 mRNA (Gonzales, 1988). Since EROD activity represented both *CYP1A1* and *CYP1A2*, EROD activity alone could not be a best indicator to monitor *CYP1A1* gene expression. When we compare this data with other study (Ahn and Sheen, 1998) it is shown that the basal activities

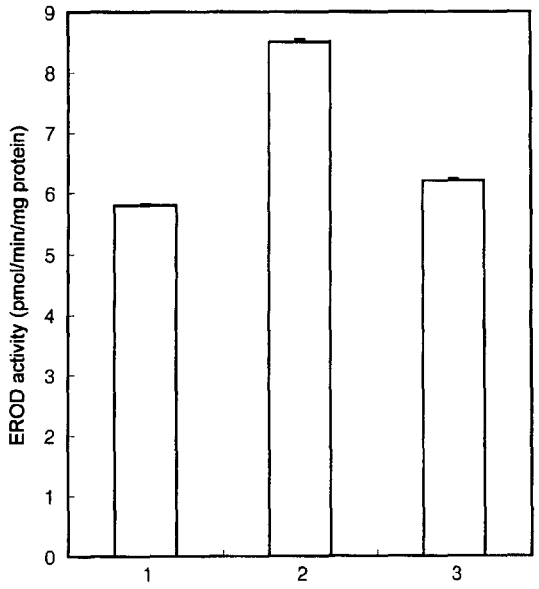

Fig. 2. EROD activity by 3-MC and TCDD stimulation in the perfused female rat liver. Fluorometry was carried out to measure EROD activity in microsome containing 80 μg of total protein that was prepared from female rat liver was perfused with various chemicals as described in Methods. Data represent mean±S.E. (n=6). 1: 0.01% DMSO in KHB, 2: 10^{-9} M 3-MC, 3: 10^{-9} M TCDD.

of EROD between male and female liver are different, female liver appears to have three times more EROD activity than male liver does. This high level of basal EROD activity might mask the effect of TCDD so that fold stimulation by TCDD appeared to be limited.

3. Effects of morin and estrogen on TCDD stimulated CYP1A1 mRNA

In female rat liver, 1 nM TCDD perfusion resulted in ten fold increase of CYP1A1 mRNA level compared to control female liver (Figs. 3, 4). Either morin or estradiol alone treatment did not change the level of CYP1A1 mRNA in comparison to that of 0.01 M DMSO treated liver. Either morin or estradiol administration along with TCDD showed inhibition of CYP1A1 mRNA level stimulated by TCDD. If we compare this data with results reported by Ahn and Sheen (1998) the inhibiting power of morin or estradiol was not as strong as that was shown in male liver. Based on literature and this result, one might argue that male liver and female liver showed distinct results in response to TCDD, estrogen and morin.

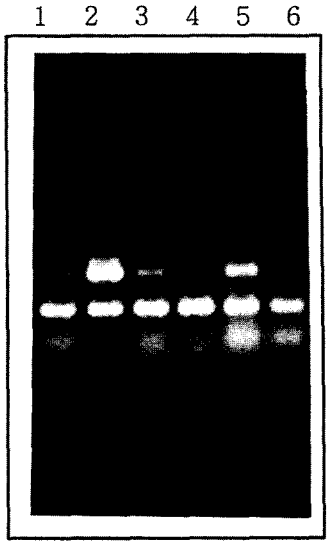

Fig. 3. Effects of morin and estrogen on the CYP1A1 mRNA expression by TCDD stimulation in the perfused female rat liver. Livers of S.D. female rats were perfused with 10^{-6} M morin or 10^{-8} M estradiol for 1.5 hrs and then perfused with 10^{-9} M TCDD for 4hrs and analysed by RTPCR as described in methods. Upper band shows 341bps PCR products of *CYP1A1* and lower band shows 153 bps PCR products of β-*Actin*. lane 1: 0.01% DMSO in KHB, lane 2: 10^{-9} M TCDD, lane 3: 10^{-6} M Morin, lane 4: 10^{-8} M Estradiol, lane 5: 10^{-6} M Morin → 10^{-9} M TCDD, lane 6: 10^{-8} M Estradiol → 10^{-9} M TCDD.

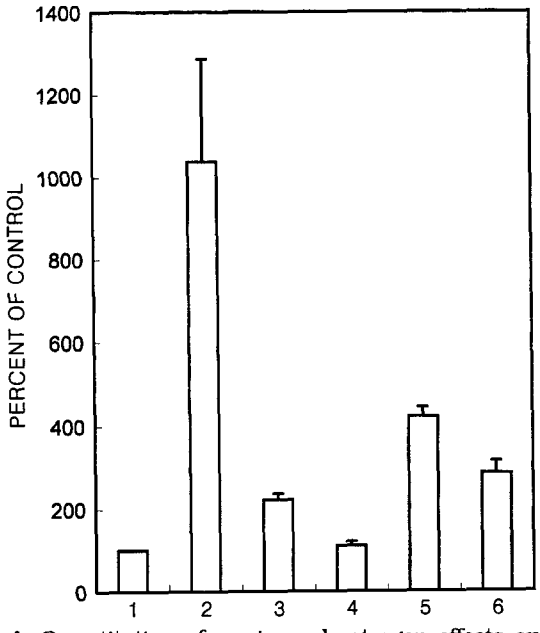

Fig. 4. Quantitation of morin and estrogen effects on the CYP1A1 mRNA expression by TCDD stimulation in the perfused female rat liver. CYP1A1 mRNA was measured by RTPCR followed by agarose gel electrophoresis. 341 bp PCR product was quantified by image analysis system as described in Methods. Data represent mean±S.E. (n=6). 1: 0.01% DMSO in KHB, 2: 10^{-9} M TCDD, 3: 10^{-6} M Morin, 4: 10^{-8} M Estradiol, 5: 10^{-6} M Morin → 10^{-9} M TCDD, 6: 10^{-8} M Estradiol → 10^{-9} M TCDD.

4. Effects of morin and estrogen on 3MC stimulated CYP1A1 mRNA

1 nM 3MC perfusion into female rat liver showed slight increase in CYP1A1 mRNA (149% that of control) over control liver. This 3MC stimulated CYP1A1 mRNA level in female rat liver was decreased by either estradiol or morin concomitant treatment with 3MC (Figs. 5, 6). When we compare this result with that has been reported (Ahn and Sheen, 1998), 3MC treated female rat liver showed slightly better responsiveness to arylhydrocarbon than male rat liver. This phenomenon was similar to the results of TCDD treatment. At the same, time estrogen and flavonoid inhibition on arylhydrocarbon induce CYP1A1 mRNA level was not as prominent as that has been shown in male rat liver.

5. Effects of morin and estrogen on TCDD stimulated EROD activity

Female rat liver seems to show high basal level of EROD activity which was 5.8 pmole/min/mg protein. 1 nM TCDD perfusion into female liver showed almost no change in EROD activity, although CYP1A1 mRNA level was increased by 10 fold (Fig. 7). This is quite different from the male liver based on result reported by Ahn and Sheen

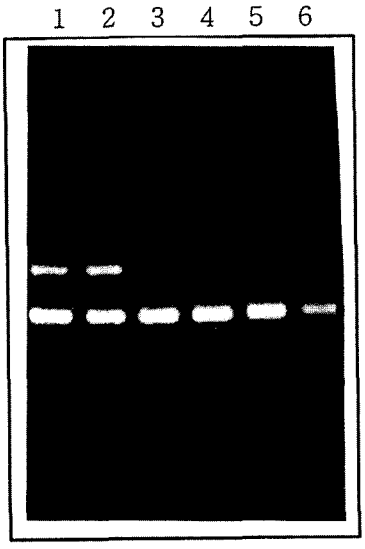

Fig. 5. Effects of morin and estrogen on the CYP1A1 mRNA expression by 3-MC stimulation in the perfused female rat liver. Livers of S.D. female rats were perfused with 10^{-6} M morin or 10^{-8} M estradiol for 1.5 hrs and then perfused with 10^{-9} M 3-MC for 4hrs and analysed by RTPCR as described in methods. Upper band shows 341 bps PCR products of *CYP1A1* and lower band shows 153 bps PCR products of β-*Actin*. lane 1: 0.01% DMSO in KHB, lane 2: 10^{-9} M 3-MC, lane 3: 10^{-6} M Morin, lane 4: 10^{-8} M Estradiol, lane 5: 10^{-6} M Morin → 10^{-9} M 3-MC, lane 6: 10^{-8} M Estradiol → 10^{-9} M 3-MC.

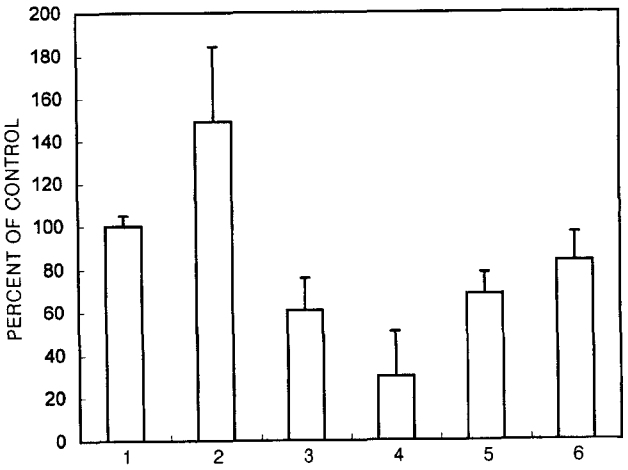

Fig. 6. Quantitation of morin and estrogen effects on the CYP1A1 mRNA expression by 3-MC stimulation in the perfused female rat liver. CYP1A1 mRNA was measured by RTPCR followed by agarose gel electrophoresis. 341 bp PCR product was quantified by image analysis system as described in Methods. Data represent mean± S.E. (n=4). 1: 0.01% DMSO in KHB, 2: 10^{-9} M 3-MC, 3: 10^{-6} M Morin, 4: 10^{-8} M Estradiol, 5: 10^{-6} M Morin → 10^{-9} M 3-MC, 6: 10^{-8} M Estradiol → 10^{-9} M 3-MC.

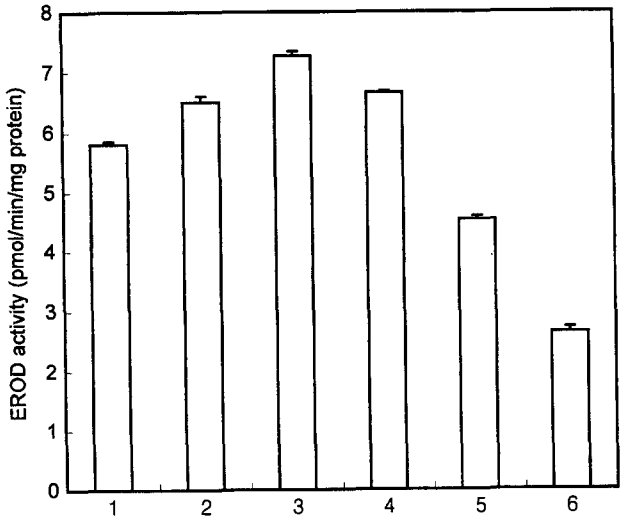

Fig. 7. Effects of morin and estrogen on the EROD activity by TCDD stimulation in the perfused female rat liver. Fluorometry was carried out to measure EROD activity in microsome containing 80 μg of total protein that was prepared from female rat liver was perfused with various chemicals as described in Methods. 1: 0.01% DMSO in KHB. Data represent mean±S.E. (n=5). 2: 10^{-9} M TCDD, 3: 10^{-6} M Morin, 4: 10^{-8} M Estradiol, 5: 10^{-6} M Morin → 10^{-9} M TCDD, 6: 10^{-8} M Estradiol → 10^{-9}M TCDD.

(1998). Morin or esradiol perfusion into female liver showed slight increase in EROD activity compared to that of control liver. When either estradiol or morin was administered concomitantly with TCDD, EROD activity was decreased to 45% and 77% thah of control liver respectively (Fig. 7). This shows estrogen and morin antogonize the effect of TCDD on EROD activity.

6. Effects of morin and estrogen on 3MC stimulated EROD activity

1 nM 3MC perfusion into female rat liver showed increase in EROD activity by one and half fold of control rat which shows more potent effect than that of TCDD treatment (Fig. 8). In the case of CYP1A1 mRNA level, TCDD treatment showed much stronger effect than 3MC, but EROD activity shoewd better response to 3MC than TCDD. Estradiol or morin treatment along with 3MC showed inhibitory effect on 3MC stimulated EROD. This shows estrogen and morin antogonize the effect of 3MC on the stimulation of EROD activity. There is

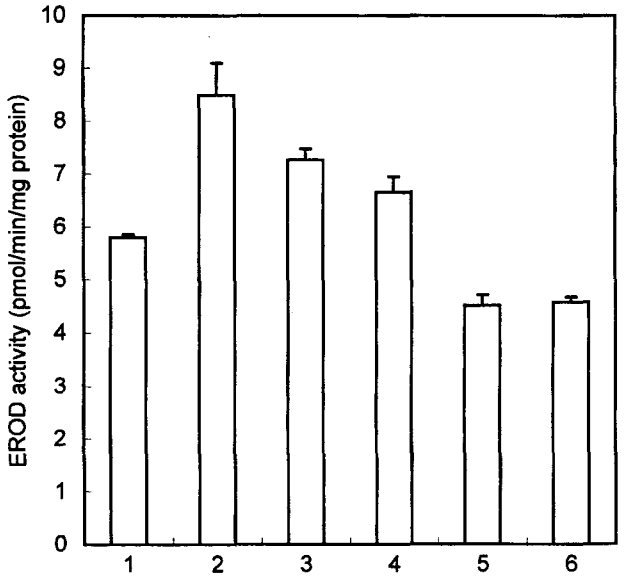

Fig. 8. Effects of morin and estrogen on the EROD activity by 3-MC stimulation in the perfused female rat liver. Fluorometry was carried out to measure EROD activity in microsome containing 80 μg of total protein that was prepared from female rat liver was perfused with various chemicals as described in Methods. Data represent mean±S.E. (n=4). 1: 0.01% DMSO in KHB, 2: 10^{-9} M 3-MC, 3: 10^{-6} M Morin, 4: 10^{-8} M Estradiol, 5: 10^{-6} M Morin → 10^{-9} M 3-MC, 6: 10^{-8} M Estradiol → 10^{-9} M 3-MC.

little information about how estrogen affects drug metabolism in terms of CYP1A1 gene expression and EROD activity. Although there are some reports showing that arylhydrocarbon antagonize the estrogen action. In mice and rats, TCDD exposure counteracts the effects of estrogens, such as 17-estradiol, on uterine hypertrophy, peroxidase activity, ER binding activity, progesterone receptor binding activity, and epidermal growth factor binding activity (Astroff and Safe, 1990). In human mammary cell lines, TCDD exposure results in decreased secretion of tissue plasminogen activator (Gierthy *et al.*, 1987), and decreased secretion of estrogen induced proteins, such as cathepsin D (Krishinan *et al.*, 1994). TCDD exposure also blocks the estradiol dependent cell proliferation response (Gierthy *et al.*, 1993), and occurrence of multicellular foci in postconfluent cultures of human mammary cell line, MCF-7 (Spink *et al.*, 1994).

The flavonoids display a remarkable array of biochemical and pharmacological actions of some of which suggests that certain members of this group of compounds may significantly affect the function of multiple mammalian cellular system. Especially they are known to modulate activities of monooxygenase of drug metabolism (Canivenc-Lavier MC *et al.*, 1996). Earlier studies demonstrated that synthetic flavonoids inhibit the microsomal enzymatic activity of drug metabolism and later study also showed that hydroxyl group of flavonoids was important for the inhibition of hydroxylation of benzo(a)pyrene whereas flavonoid without hydroxyl group increase in the hydroxylation of benzo(a)pyrene (Buening et.al. 1981). It is intriguing to know the mechanism of action of flavonoids in drug metabolizing enzymes. Previous studies demonstrated that 7,8-benzoflavone seemed to increase the interaction of cytochrome P450 dependent NADPH reductase and cytochrome P450 (Sousa and Marletta, 1985). This study showed hydroxylated flavonoids such as morin inhibited the 3MC or TCDD stimulated ethoxyresorufin O-deethylase and CYP1A1 mRNA level. However, further studies of regulation of gene expression of cytochrome P4501A would be necessary to know the mechanism of action of hydroxylated flavonoids on drug metabolizing enzymes.

ACKNOWLEDGEMENTS

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