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Effects of Hydroxylated Flavonoids on the Ethoxyresorufin O-deethylase and Benzo(α)pyrene Hydroxylase

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In order to understand the mechanism of action of flavonoids on the drug metabolizing enzyme, cytochrome P450IA1, this study was undertaken to examine the effect of chrysin, morin, myricetin and aminopyrine on the activities of ethoxyresorufin O-deethylase and benzo(α) pyrene hydroxylase in the liver. In the isolated perfused rat liver that was pretreated with 3-methylcholanthrene (3MC), chrysin, morin, myricetin and aminopyrine inhibited the activity of ethoxyresorufin O-deethylase with concentration dependent manner. The isolated liver perfusion with chrysin, morin, myricetin and aminopyrine showed inhibition on the induction of ethoxyresorufin O- deethylase by 3MC. And also, in mouse liver hepa I cells, 3MC-stimulated the benzo(α)pyrene hydroxylase activity which was inhibited by chrysin, morin, myricetin and aminopyrine. These results strongly suggested that hydoxylated flavonoids interfered not only the induction of cytochrome P450IA1 enzymes by 3MC but also the interaction of substrates and enzyme.

Key words : Ethoxyresorufin O-deethylase, Benzo(a)pyrene hydroxylase, 3-methylcholanthrene, Cytochrome P450, Flavonoid

INTRODUCTION

Over 4,000 chemically unique flavonoids have been identified in plant sources. These low molecular weight substances, found in all vascular plants, were phenylbenzopyrones (phenylchromes) with an assortment of basic structures. The flavonoids were prominent components of citrus fruits and other food sources (Timberlake and Henry, 1986). Resurgence of interest in traditional medicine during the past two decades together with an expanded effort in pharmacognosy have rekindled interest in the flavonoids and the need to understand their interaction with mammalian cells and tissues. The flavonoids have long been recognized to possess antiallergic, antiinflammatory, antiviral, antiproliferative and anticarcinogenic activities as well as to affect some aspects of mammalian metabolism (Cody *et al.*, 1988; Das, 1989; Farkas *et al.*, 1986; selway, 1986). The flavonoids displayed a remarkable array of biochemical and pharmacological actions of some of which suggested that certain members of this group of compounds might significantly affect the function of multiple mammalian cellular system. Especially they were known to modulate activities of monooxygenase of drug metabolism (Canivenc-Lavier *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(α)pyrene whereas flavonoid without hydroxyl group increased in the hydroxylation of benzo(α)pyrene (Buening *et al.*, 1981). It was 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). However, the action mechanism of flavonoids in cytochrome P450 was not known. Thus, in this study, we examined the effect of chrysin, morin, myricetin and aminopyrine on the effect of cytochrome p450 IA1 activity via monitoring O-deethylation of ethoxyresorufin and hydroxylation of benzo(α)pyrene. In order to understand the mechanism of action of those flavonoids in the regulation of cytochrome P450 IA1, experiments were carried out both in the isolated perfused rat liver and mouse hepa I cells.

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MATERIALS AND METHODS

Animals

C57B2/6N and DBA/2N mice, and sprague-dawley

rats were obtained from Korea Research Institute of Bioscience and Biothechnology. Experimental animals were maintained at least for two weeks under normal nutrition and environmental conditions with 12 hours light 12 hours dark, and 24hours prior to the experiment, animals were starved.

Cell culture

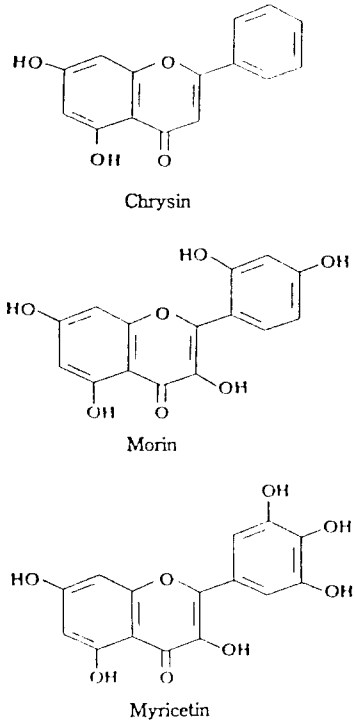
Hepa I mouse liver cells were cultured in 10% fetal bovine serum containing minimum essential medium at 37°C in humidified 5% CO_2 incubator. Cells were divided weekly with trypsin-EDTA. Various concentrations of 3-methylcolanthrene (3MC), chrysin, morin, myricetin, aminopyrine were administered into hepa I cells and control cells were treated with 0.1% DMSO.

The Isolated liver perfusion

Under the ether anesthesia, after the hepatic vein of male sprague-dawley rat was cannulated and catheter was inserted into the vein, and the liver was isolated and transferred into perfusion block. Krebs-Henseleit buffer, pH 7.4 was perfused for various time periods in the presence or absence of various chemicals.

Measurement of ethoxyresorufin O-deethylase activity

Liver microsomes were prepared as previously reported (Fujino *et al.*, 1984). Microsomes were incubated with 5uM ethoxyresorufin in the presence or absence of various flavonoids for 1 minute at 37°C and 250 uM 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).



Chemical structure of various flavonoids

Measurement of benzo(α)pyrene hydroxylase activity

Experiments were carried out as previously described in "measurement of ethoxyresorufin O-deethylase" except the fact that benzo(α)pyrene was used as a substrate and fluorescence was determined (excitation at 396 nm and emission at 522 nm).

RESULTS AND DISCUSSION

Effect of 3MC on the ethoxyresorufin O-deethylase activity in both C57BL/6N and DBA/2N mice

3MC (60 mg/kg) was given to C57BL/6N and DBA/2N mice for 48hrs, and microsomal fractions were prepared from both mice. Enzymatic activity of ethoxyresorufin O-deethylase was measured as described in materials and methods. As shown in fig. 1, in C 57BL/6N mouse 3MC treatment increased in the O-deethylase activity 2.79 fold over untreated mouse microsome, whereas in DBA/2N mouse, 3MC treated mouse microsome showed no increase in enzymatic activity. The high level of arylhydrocarbon receptor

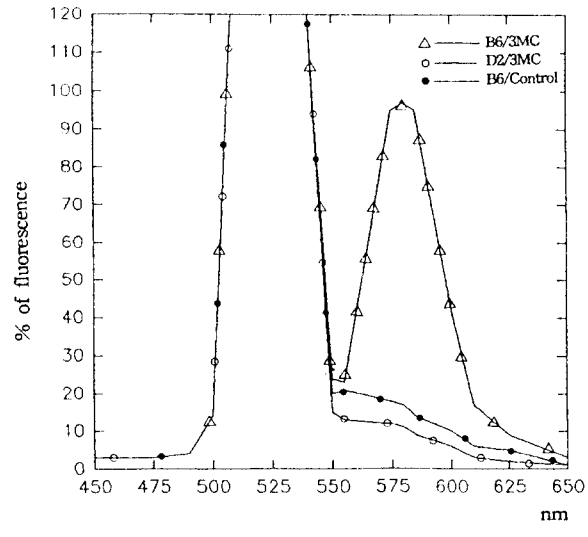


Fig. 1. Fluorometry of ethoxyresorufin O-deethylase activity. Microsome (protein 80 ug) was prepared from C57BL/6N mice treated with either 1 mM 3MC or corn oil and DBA/2N treated with 1 mM 3MC. Assay of O-deethylase activity was conducted as described in methods.

were identified in C57BL/6N mouse liver, but not detected in DBA/2N mouse liver based on [^{3}H]TCDD binding assay (Okey, *et al.*, 1980). In consistent with the earlier findings from many laboratories (Whitlock, 1986; Yao and Denison, 1992; Lir *et al.*, 1992; Hapgood, 1989), this data confirmed that arylhydrocarbon receptor system might play an important role for the induction of ethoxyresorufin O-deethylase, cytochrome P450IA1 by inducers such as 3MC.

Effect of flavonoids on the ethoxyresorufin O-deethylase activity that was stimulated by 3MC

In order to gain insight into the regulation of ethoxyresorufin O-deethylase activity by flavonoids, experiments were carried out whether flavonoids inhibit the stimulated ethoxyresorufin O-deethylase activity by 3MC. After the microsomes from 3MC treated C 57BL/6N mouse liver were incubated with various concentrations of chrysin, the ethoxyresorufin O-deethylase activity was measured as previously. Results were shown in fig. 2 and 3, where as increased the concentrations of chrysin, the O-deethylase activities were decreased with concentration dependent manner. In the case of morin, ethoxyresorufin O-deethylase activity was inhibited by morin in a way similar to the inhibition by chrysin, except the fact that morin exhibited more potent action than chrysin did (Fig. 4, 5). Myricetin also inhibited O-deethylase activity similar to the action of chrysin but the ability of myricetin to inhibit O-deethylase activity was much weaker compared to that of chrysin (Fig. 6). Synthetic flavonoids such as 7,8-benzoflavon were known to stimulate the activities of cytochrome P4501A1 (Buening *et al.*, 1981) and β-naphthoflavone resulted in the pretranslational induction of cytochrome P4501A in the periportal region of liver (Teija *et al.*, 1994). It was interesting to know the derivatives of flavonoids also affect the activity of drug metabolizing enzyme. As shown in fig.s 2-6, chrysin, morin, myricetin, which were flavonoids with hydroxyl group at their chemical structure, inhibited the 3MC stimulated drug metabolizing enzyme activity. The mechanism of distinct actions of 7, 8-benzonflavone and hydoxyflavonoids were not understood, therefore, further researches would require

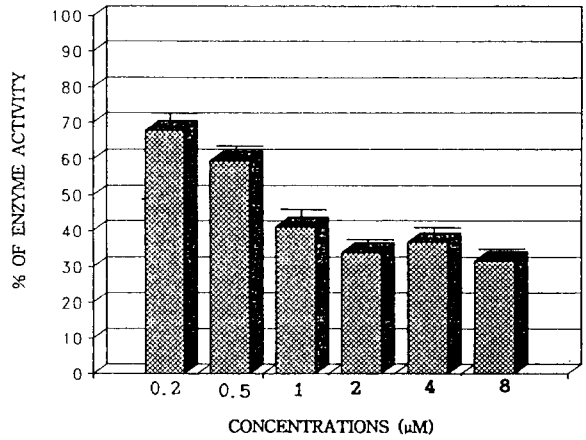


Fig. 3. The quantification of fluorometry of ethoxyresrufin O-deethylase activity in 1 nM 3MC-treated C57BL/6N mice microsome (protein 80 ug) with different concentrations of chrysin. Microsome without chrysin (protein 80 ug) was set as 100%.

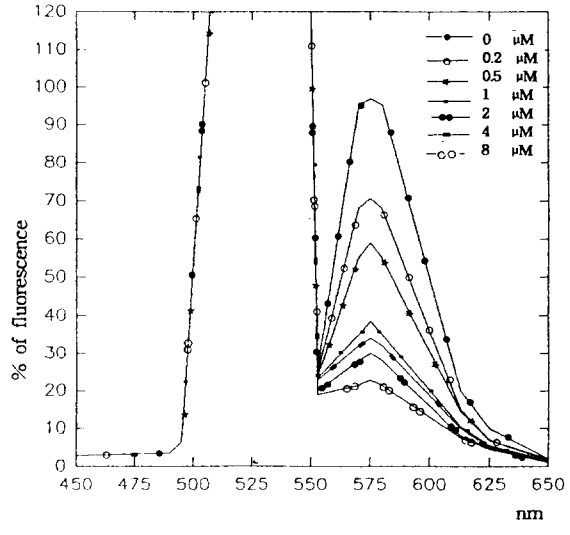


Fig. 2. The effect of various concentrations of chrysin on ethoxyresorufin O-deethylase activity in microsome (protein 80 ug) that was prepared from C57BL/6N mice treated with 3MC (60 mg/kg) based on fluorometry. Various concentrations of chrysin were examined as described in methods.

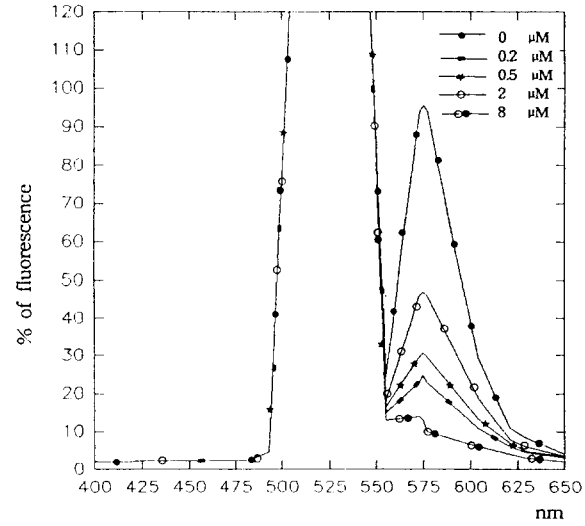


Fig. 4. The effect of various concentrations of morin on ethoxyresorufin O-deethylase activity of microsome (protein 80 ug) that was prepared from C57BL/6N mice treated with 3MC (60 mg/kg) was measured based on fluorometry. Various concentrations of morin were tested as described in methods.

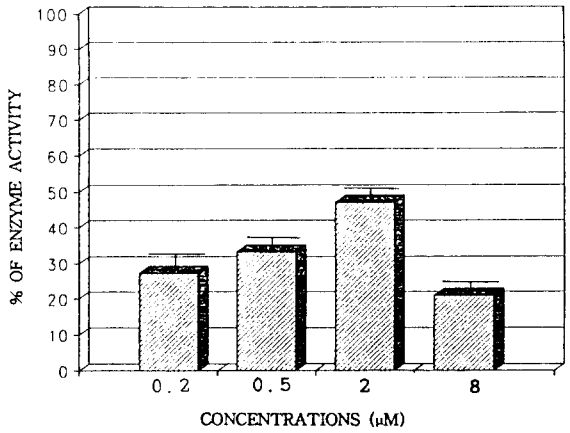


Fig. 5. The quantification of fluorometry of ethxyresorufin O-deethylase activity in 3MC-treated C57BL/6N mice microsome (protein 80 ug) with different concentrations of morin. Microsome without morin (protein 80 ug) was set as 100%.

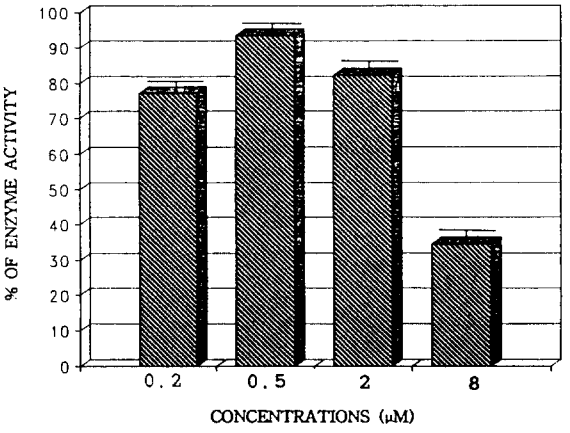


Fig. 6. The quantification of fluorometry of ethxyresorufin O-deethylase activity in 3MC-treated C57BL/6N mice microsome (protein 80 ug) with different concentrations of myricetin. Microsome without myricetin (protein 80 ug) was set as 100%.

to enlighten the mechanism of action of flavonoids on drug metabolism.

The effect of aminopyrine on the ethoxyresorufin O-deethylase activity

In order to find out whether the inhibition of ethoxyresorufin O-deethylase activity was flavonoid specific, nonflavonoid chemical, aminopyrine which was known to inhibit the activity of arylhydrocarbon hydroxylase (Guengerich, 1988), was examined to see how it would affect O-deethylase activity. As shown in fig. 7, aminopyrine also inhibited the ethoxyresorufin O-deethylase activity that was stimulated by 3MC. The machanism of aminopyrine action on enzyme inhibition was not known, but there were reports that 7,8-

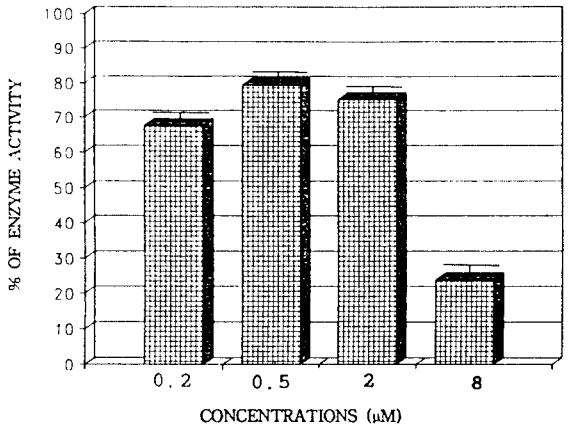


Fig. 7. The quantification of fluorometry of ethxyresorufin O-deethylase activity in 3MC-treated C57BL/6N mice microsome (protein 80 ug) with different concentrations of aminopyrine. Microsome without aminopyrine (protein 80 ug) was set as 100%.

benzoflavone enhanced microsomal hydroxylation of benzo(α)-pyrene via improvement of interaction between cytochrome P450 dependent reductase and cytochrome P450 (Sousa and Marletta, 1985; Buening *et al.*, 1981). This data suggested that the inhibition of ethoxyresorufin O-deethylase was not flavonoid specific phenomenon although the mechanism of inhibition was not understood. Comparisons between flavonoids and aminopyrine in term of the inhibition of O-deethylase activity showed hydroxylated flavonoids seemed to have potent activity (Fig. 3, 5, 6, 7).

Flavonoids effects on ethoxyresorufin O-deethylase activity in the isolated perfused rat liver

Flavonoids and aminopyrine were perfused into the isolated rat liver in order to examine if the effect of flavonoid on ethoxyresorufin O-deethylase in vitro is consistent with that of flavonoids in situ system. 10^{-6}M Myricetin or morin perfusion 1.5 hour prior to 10^{-9}M 3MC perfusion for 4 hours resulted in about 30% decrease in enzymatic activity of O-deethylase compared to that of 3MC alone treatment (Fig. 8). This data strongly suggested that flavonoids, such as morin and myricetin, aminopyrine decreased in the ethoxyresorufin O-deethylase activity not only at the level of enzyme action but also at the level of regulation of expression of this enzyme. To elucidate the exact mechanism of action of flavonoids on the regulation of the expression of cytochrome P450, further studies would need to be done.

Effect of Flavonoids on ethoxyresorufin O-deethylase and benzo(α)pyrene hydroxylase in hepa I cells

On the contrary to the in vitro experiment and the isolated perfused rat liver, mouse hepa I cells did not

Fig. 8. The quantification of fluorometry of ethxyresorufin O-deethylase activity in microsome (protein 500 ug) that was prepared from the isolated liver of sprague-dawley perfused with various drugs for 4 hours. 1, 3-MC (10^{-9}M); 2, Chrysin (10^{-6}M)+3-MC (10^{-9}M); 3, 3-MC (10^{-9}M); 4, Myricetin (10^{-6}M)+3-MC (10^{-9}M)

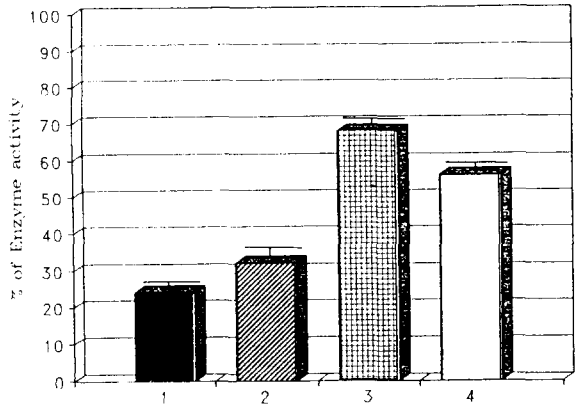


Fig. 9. The quantification of fluorometry of benzo(α)pyrene hydoxylase activity in microsome (protein 100 ug) that was prepared from Hepa-I cell treated with various drugs. Microsome with 1 nM 3-MC was set as 100%. 1, Chrysin+3-MC; 2, morin+3-MC; 3, myricetin+3-MC; 4, aminopyrine+3-MC

show the inhibition of ethoxyresorufin O-deethylase by either flavonoids, such as morin, myricetin, chrysin or aminopyrine (data not shown). Therefore, instead of O-deethylase activity, the benzo(α)pyrene hydroxylase activity was monitored in order to examine the effect of flavonoids on cytochrome P450IA 1. As shown in fig. 9, 10^{-6}M morin or 10^{-6}M chrysin treatment into hepa I cells resulted in magnificent decrease of benzo(α)pyrene hydroxylase activity compared to that of 10^{-9}M 3MC treated cells. In the case of myricetin and aminopyrine, similar inhibitory effects to those of morin and chrysin were observed (fig. 9). From these data, one could speculate that flavonoids might interfere the induction of cytochrome P450IA1 enzyme by 3MC specifically as well as the interaction of substrate and enzyme. Based on this study, further studies to examine how flavonoid affect the gene expression of cytochrome P450 IA1 would be necessary to answer the exact mechanism of action of flavonoids and gain insight into how 3MC and flavonoids interplay to regulate the activity of the drug metabolizing enzymes.

CONCLUSION

Hydroxylated flavonoids such as morin, chrysin, myricetin and aminopyrine inhibited the 3MC stimulated ethoxyresorufin O-deethylase and benzo(α) pyrene hydroxylase activities and the enzyme induction by 3MC. 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.

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