Korean J Physiol Pharmacol
Vol 1: 591−595, October, 1997

Thiobenzamide S-oxidation in Perfused Rat Liver: Ex Vivo Determination of Hepatic Flavin-Containing Monooxygenase Activity

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An ex vivo assay determining the flavin-containing monooxygenase (FMO) activity in perfused rat liver has been developed by assessing the rate of thiobenzamide S-oxide (TBSO) formation from the infused thiobenzamide (TB). The hepatotoxicity by TB or TBSO was not a critical factor for maintaining the FMO activity for up to 50 min. The FMO activity expressed in nmoles TBSO produced/g liver/min was the same for the recycling and non-recycling perfusion. This implies that reduction of the oxidized TBSO back to the parent compound (TB) is negligible. Hydrolysis of the collected perfusates with either β-glucuronidase or arylsulfatase did not increase the TBSO level and thus, TBSO does not appear to undergo conjugation either to glucuronide or sulfate esters. Thus, measuring the rate of TB S-oxidation in the isolated perfused liver with 1 mM TB for 50 min provides a useful tool for evaluation of the hepatic FMO activity in the absence of hepatic necrosis and without the interferences caused by further conjugation or back reduction of the TBSO to the parent TB.

Key Words: Flavin-containing monooxygenase (FMO), Thiobenzamide S-oxidation, Isolated perfused rat liver, Thiobenzamide perfusion, Cycling and non-recycling perfusion

INTRODUCTION

Flavin-containing monooxygenase (FMO; EC 1.14. 13.8) oxidizes a large number of drugs, pesticides and environmental chemicals containing soft nucleophilic atoms like nitrogen, sulfur, phosphorus and selenium (Ziegler, 1988). However, the FMO remains as an orphan enzyme mainly due to inability to define its physiological functions except for the suggestive formation of sulfoxide metabolites from cysteine S-conjugates under in vitro conditions (Elfarra, 1995). One of the reason why the in vivo function of FMO is uncertain is that it has been difficult to assess the in vivo metabolism of drugs oxidized by FMO due to the lack of a proper assay method to determine the enzyme activity in intact animal until very recently. Utilizing cimetidine and nicotine, Cashman (1995) has described the in vivo properties of mammalian FMO activity in intact animal and human models. In particular, the primary metabolites produced by FMO are known to undergo back reduction to the parent compound in vivo situation (Ziegler, 1988) and thus, identification of an FMO substrate whose oxidation metabolite is not the subject of back reduction to the parent compound in the in vivo situation is necessary for an assessment of the physiological function of FMO.

Several in vitro assays have been developed to determine the FMO activity by monitoring the rates of NADPH- and oxygen- consumptions as well as the rates of producing the signature metabolites which are detected either by radioactivity, absorption in spectrophotometer, or emission in fluorometer by using the well known FMO substrates like N,N-dimethylaniline (Ziegler, 1980), thiobenzamide (Cashman & Hanzlik, 1981), guanethidine (McManus et al, 1983), methimazole (Dixit & Roche, 1984) and benzyda-

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mine (Kawaji et al, 1993). These methods utilized liver microsomes or purified FMO as the enzyme sources. In the present study, we demonstrate the establishment of an ex vivo assay method for assessment of FMO activity in the isolated perfused rat liver by infusing TB as an intermediate step for eventual development of an in vivo assay.

METHODS

Chemicals

TB was obtained from Fluka (Buchs, Switzerland). Arylsulfatase, dichlorophenol indophenol (DCPIP), dicoumarol, β-glucuronidase, NADH and most other chemicals utilized in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals and preparation of isolated perfused liver

Male Sprague-Dawley rats weighing 200 to 250 g (liver weights; 7~10 g) were obtained from the Animal Breeding Laboratory of Inha University. Rats were anesthetized by an intraperitoneal injection of sodium secobarbital (30 mg/kg). After the abdominal cavity was opened, a polyethylene cannula was inserted in the portal vein according to the method described earlier (Dong & Cha, 1987). The cannula was then connected to a peristaltic perfusion pump flowing with modified Krebs-Henseleit bicarbonate buffer containing 5.5 mM glucose (referred as KHB hereafter) at a rate of 30 ml/min (3~4 ml/min/g liver). The KHB was saturated with O_2 and CO_2 (95:5) and stirred by continuous bubbling at 37°C. The liver was then carefully isolated from surrounding tissues while being perfused with the KHB and transferred to an insulated plastic perfusion block.

Hepatotoxicity monitoring in perfused liver

Perfusion with KHB was maintained for 10 min equilibration and the perfusate was not recirculated during this equilibration period. Subsequently, the infusion medium was switched from the KHB to reservoirs of 100 ml KHB solutions containing various concentrations of TB or 0.1 mM oxidized DCPIP (a well known model hepatotoxic agent) and the perfusates were recirculated. One ml of the perfusate was collected at every 5 min interval for up to 50 min and they were used to determine the activities of lactate dehydrogenase (LDH) released from hepatocytes undergoing cell death (Dong & Cha, 1987). From the collected perfusates, a duplicate of 50 μl aliquots were taken and added to 1 ml of the LDH assay solution containing 80 mM Tris buffer (pH 7.2), 0.25 mM NADH, 10 mM pyruvate, 200 mM NaCl and 0.1 mM dicoumarol (to inhibit the released NAD(P)H:quinone reductase activity). The decreases of NADH absorbance at 340 nm were measured after 5 min incubation at room temperature. From this NADH consumption rate, the amount of LDH released into perfusates were calculated and for the present study, infusion of 1 mM TB did not appear to cause hepatotoxicity.

Metabolism of thiobenzamide by isolated perfused liver

After the initial equilibration with KHB for 10 min, the infusion medium was switched to the KHB containing 1 mM TB. For the recycling perfusion, the volume of reservoir was 100 ml and for the non-recycling perfusion, the reservoir volume was 1,000 ml. From both perfusion modes, 1 ml perfusates were collected at every 5 min interval for up to 50 min and they were used for the quantification of TB S-oxide (TBSO) using the molar extinction coefficient of 2930 $M^{-1}cm^{-1}$ at 370 nm as described by Cashman and Hanzlik (1981).

In order to determine whether the produced TBSO was conjugated to form its glucuronide or sulfate ester, 0.3 ml of the collected perfusates were hydrolyzed either with 20 units of β-glucuronidase or with 20 units of β-glucuronidase plus 0.15 units of arylsulfatase in 0.1 M Tris buffer (pH 7.2) in a total volume of 1 ml. After 2 hr incubation at 37°C, TBSO was determined as described above.

RESULTS AND DISCUSSION

FMO contained in liver plays a vital role in the metabolism of xenobiotics including many clinically useful drugs, pesticides and plant alkaloids that contain nitrogen and sulfur (Ziegler, 1988). However, the contribution of FMO in the metabolism of xenobiotics in intact animal has been difficult to assess

due to presence of species and tissue variations in the expressed FMO isozymes having different substrate specificities. Furthermore, because the oxidized metabolites produced in vivo by FMO are usually very susceptible either to reduction back to the parent compound, to further oxidation by other oxidases or to conjugation, the metabolites excreted in bile or urine do not normally reflect the initial oxidative metabolites produced by FMO in vivo (Ziegler, 1988). Therefore, an in vivo assay for determining the activity of hepatic FMO using a substrate whose oxidized metabolite is not subject either to the recycling reduction, to further oxidation or conjugation is needed. In the present study, we have developed an ex vivo assay determining the FMO activity by measuring the release of TBSO which is produced from the infused TB (1 mM) in the isolated perfused rat liver.

As TB and its FMO catalyzed oxidation product (TBSO) are known to be hepatotoxic (Hanzlik et al, 1980), one of the main concerns in infusing TB to the liver is to keep the hepatocytes from cell death while maintaining the hepatic drug metabolism function intact. Apparently, the working conditions established by recycling infusion of 1 mM TB does not cause significant hepatic cell death (Fig. 1) and appears to be good enough to measure the linear production of TBSO for up to 50 min (Fig. 2).

The cumulative amounts of TBSO present in the collected perfusates produced by the perfused liver in a recirculation mode have increased gradually in almost a linear manner for up to 50 min (Fig. 2). This linearity proves that the drug metabolism function of hepatocytes in the perfused liver is maintained at least for 50 min and supports the result obtained in Fig. 1, indicating that the infused TB or its oxidized metabolite (TBSO) are not hepatotoxic. When the infusion medium is not recirculated, the rate of TBSO formation remained at almost the constant rate from beginning to termination of the infusion at 50 min (Fig. 2). This implies that the FMO activity in the perfused liver is maintained without any decline throughout the 50 min assay and again supports the result obtained in Fig. 1.

In order to compare the rates of TBSO production obtained under recycling and non-recycling perfusion modes, the rate of TBSO production is expressed in nmole/g liver/min (Fig. 3). The rates of TBSO for-

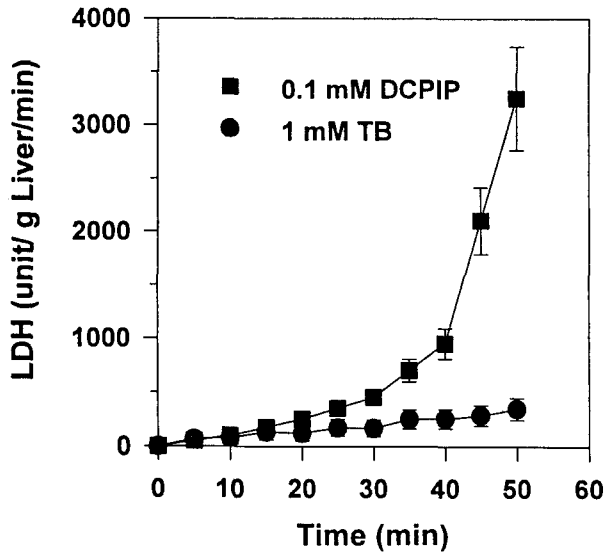

Fig. 1. Effect of thiobenzamide infusion on hepatic cell death in perfused liver. (Comparison of hepatotoxicity caused by infusing 0.1 mM DCPIP or 1 mM TB into isolated perfused liver was made by estimation of the released LDH activity from recycling perfusion experiments. Data obtained from 4 determinations are expressed in mean ± SD).

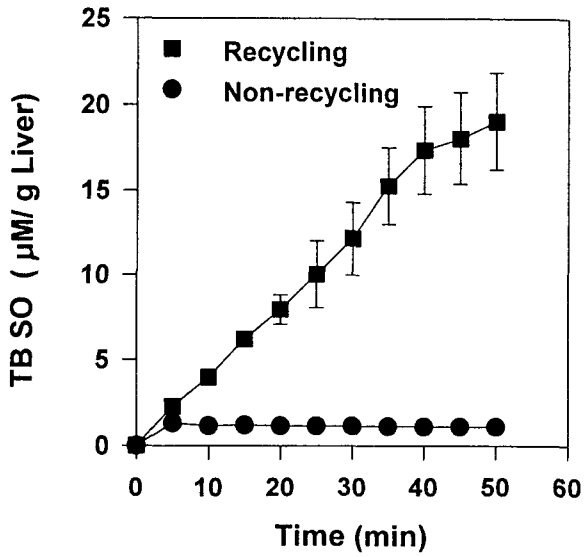

Fig. 2. Rates of hepatic TB S-oxidation in recycled or non-recycled perfusion. (Time profile of TB S-oxidation in isolated perfused rat livers infused with 1.0 mM TB in either a recycling or a non-recycling perfusion mode. Data obtained from 4 determinations are expressed in mean ± SD).

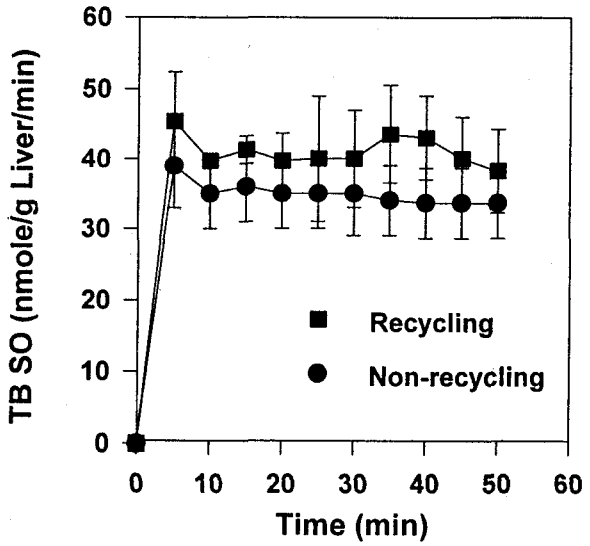

Fig. 3. Comparison of TB S-oxidation rates obtained by recycled and non-recycled perfusion modes. (Comparison of TBSO production rates in the isolated perfused rat livers infused with 1 mM TB in a recycling or a non-recycling perfusion mode. Data shown in Fig. 2 are re-calculated to express the FMO activity in nmole TBSO/g liver/min and expressed in mean±SD).

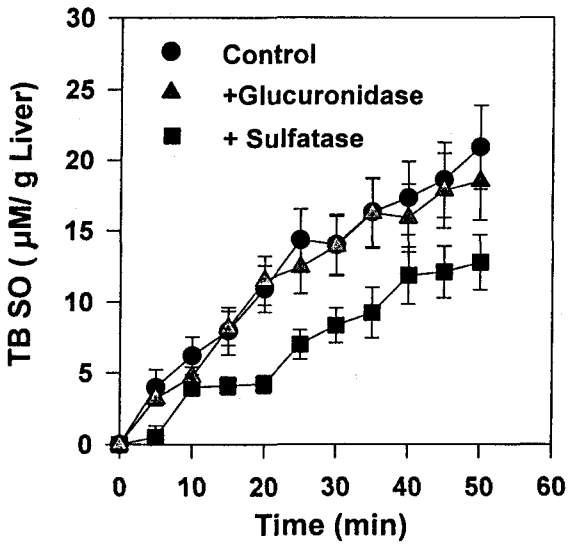

Fig. 4. Effect of hydrolyzing the collected perfusates with β-glucuronidase or arylsulfatase to release additional TBSO. (Effect of hydrolyzing the perfusates collected from the perfused rat livers in a recycling mode with β-glucuronidase or arylsulfatase. Data obtained from 4 determinations are expressed in mean±SD).

mation between the two perfusion systems are not significantly different. This means that both the infused TB (1 mM) or the produced TBSO do not cause significant damage to the hepatic drug metabolism function. This result also implies that the produced mono-oxygenation metabolite TBSO (C_6H_5-$C(NH_2)$=S=O) does not undergo further oxygenation to the di-oxygenated TB imminesulfinate (C_6H_5-C(=NH)SO_2H) or reduction back to the parent compound TB. In previous studies using other FMO substrates, the presence of multiple reducing enzyme systems in intact liver and kidney has caused reduction of the oxidized metabolites back to parent compounds and this recycling reduction has been the source of miss-interpretation of FMO activity measured in vivo (Ziegler, 1982).

Another metabolic processing of the oxidized metabolites produced from the initial phase I oxidation in the in vivo situation is the phase II conjugation reaction. When the collected perfusates are hydrolyzed with β-glucuronidase, the amount of TBSO is not increased (Fig. 4). This implies that glucuronidation does not occur at least at the S- or O- position of TBSO. This result, however, cannot rule out the possibility of N-glucuronide formation because the detection of TBSO at 370 nm is not likely to detect the N-glucuronidation product which may have been formed. When the collected perfusates are hydrolyzed with arylsulfatase to release the TBSO from its potential sulfate ester conjugate, the amount of TBSO is not increased but decreased uniformly by 30% (Fig. 4). Thus, the produced TBSO may not be a substrate of sulfate conjugation or, if the TBSO is sulfated to form its N-sulfated conjugate, it is not hydrolyzed by the arylsulfatase. Most probably, the uniform decline by 30% in the TBSO absorption at 370 nm may reflect the destruction of TBSO caused by arylsulfatase in an unknown mechanism.

In conclusion, by infusing a nontoxic concentration of TB (1 mM) into the isolated perfused rat liver, we have developed an ex vivo assay assessing the FMO1 activity in intact rat liver which may serve as a good candidate for a quantitative measurement of the enzyme activity in vivo. From the results obtained utilizing this ex vivo hepatic FMO assay, the mono-oxidized metabolite of TB (TBSO) generated by FMO in intact hepatocytes does not appear to undergo further metabolism either by secondary oxidation to its di-oxygenated metabolite, by reduction back to the parent compound, or by conjugation.

Thus, with this ex vivo assay, we are now in a position to evaluate the in vivo role of FMO1 in hepatic drug oxidation as well as its regulation under varying physiological and environmental conditions.

ACKNOWLEDGEMENTS

We would like to thank Mr. Sung-Wan Ahn for his superb technical assistance.

This study was supported by an intramural research grant from Inha University (1995).

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