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Purification of Thiazole- and Pyrazine-inducible Microsomal Epoxide Hydrolase: Induction of Epoxide Hydrolase-related Novel 43 kDa Protein

Sang Geon Kim

College of Pharmacy, Duksung Women's University, Seoul, Korea
and Institute of Chemical Toxicology, Wayne State University, Detroit, MI 48201, U.S.A.

ABSTRACT

Liver microsomal epoxide hydrolase (mEH) is active in the detoxification of epoxide-containing reactive intermediate. Previous studies in this laboratory have shown that thiazole and pyrazine are efficacious inducers of mEH in rats with large increases in mEH mRNA levels (Carcinogensis, Kim *et al*, 1993). mEH was purified to electrophoretic homogeneity from thiazole-induced rat hepatic microsomes using DEAE-cellulose column chromatography whereas another protein (~43 kDa) was co-purified with mEH from pyrazine-induced rat hepatic micrsomes (200 mg/kg body weight/day, ip, 3d). The antibody raised from a rabbit against mEH protein purified from thiazole-induced rat hepatic microsomes appeared to specifically recognize mEH protein in rat hepatic microsomes, as assessed by immunoblotting analysis. Immunoblotting analyses revealed a 10- and 7-fold increase in mEH levels in the hepatic microsomes isolated from thiazole- and pyrazine-treated rats, respectively. Moreover, immunoblotting analysis showed cross-reactivity of the mEH antibody with a 43 kDa protein in pyrazine-induced rat hepatic microsomes and with co-purified 43 kDa protein in purified fractions. The ratio between the 43 kDa protein and mEH in pyrazine-induced rat microsomes or in purified fractions was ~1 to 15. N-terminal amino acid sequence analysis of both purified rat mEH and 43 kDa protein revealed that 10 out of 12 amino acids in N-terminus of the 43 kDa protein were identical with the mEH sequence with two amino acid residues of the 43 kDa protein undetermined. Either thiazole or pyrazine treatment, however, failed to increase the levels of mEH protein in rabbits while pyrazine caused elevation of the 43 kDa protein in this species, as determined by immunoblotting analysis. These results demonstrated that treatment of rats with either thiazole or pyrazine causes elevation in hepatic mEH expression whereas pyrazine treatment results in induction of another mEH-related 43 kDa protein and that a distinct species difference exists between rats and rabbits in the induction of mEH by these xenobiotics.

Key Words: Epoxide Hydrolase, Thiazole, Pyrazine, Induction

Abbreviations: mEH, microsomal epoxide hydrolase; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

INTRODUCTION

Evidence that supports the existence of multiple forms of epoxide hydrolase has been reported, although some aspects of this multiplicity remain unclear (Guengerich, 1989). Currently, at least three forms of epoxide hydrolase have been identified. These are a cytosolic form inducible by clofibrate administration (Gill & Hammock, 1979;

Hammock & Ota, 1983) and two microsomal forms: one which is specific for the hydration of cholesterol 5,6-epoxide and related compounds (Levin *et al.*, 1983), and the other which catalyzes the hydration of a wide variety of xenobiotic oxides. The form responsible for the stereospecific hydration of arene and alkene oxides to trans-dihydrodiols is microsomal epoxide hydrolase (mEH[1]) (Lu & Miwa, 1980; Seidegard & DePierre, 1983). In general, the resulting diols are considered to be detoxification products.

The three forms of epoxide hydrolase appeared to be enzymatically and immunologically distinct. The presence and/or induction of epoxide hydrolase in different subcellular compartments has also been reported (Guenthner, 1986). For example, 3-methylcholanthrene induces the nuclear envelope enzyme, whereas the microsomal enzyme is unaffected: phenobarbital and trans-stilbene oxide also showed difference in the inducibility of microsomal and nuclear epoxide hydrolase (Guenthner, 1986).

Thiazole and pyrazine form part of the molecular structure of a number of natural products, drugs and industrial chemicals (Mussinan *et al.*, 1973; Mega & Sizer, 1973; Nishie *et al.*, 1970; Rance, 1989; Schumacher *et al.*, 1977; Spies *et al.*, 1987; Vitzthum & Werkhoff, 1974; Wilson *et al.*, 1991). In fact, the presence of 28 pyrazine derivatives has been identified in tobacco and tobacco smoke (Schmeltz & Hoffmann, 1977) and typically has been associated with foods that either toasted or roasted during their preparation (e.g. coffee, cocoa products and popcorn) (Mega & Sizer, 1973; Mussinan *et al.*, 1973; Vitzthum & Werkhoff, 1974).

Previous studies in this laboratory have shown that treatment of rats with thiazole or pyrazine causes an increase in mEH levels and that a large increase in mEH mRNA is associated with the induction of the protein (Kim *et al.*, 1991a). In the present paper, I report the purification of mEH protein from thiazole-induced rat hepatic microsomes and co-purification of both mEH and a 43 kDa protein from the hepatic microsomes isolated from pyrazine-exposed rats. Antibody generated against purified hepatic mEH protein from thiazole-exposed rats was employed to quantify the levels of mEH protein in rat hepatic microsomes and to examine whether any immunochemical and structural similarity exists between mEH and the 43 kDa protein, the hepatic level of which was found to be elevated in pyrazine-exposed rats. This study shows that thiazole causes elevated expression in mEH protein whereas pyrazine treatment results in induction of both mEH and another protein which is related with mEH, as assessed by chromatographic profile, immunochemical analysis and N-terminal amino acid analysis. To this end, species difference in the induction of mEH and the 43 kDa protein was examined in rabbits in response to these xenobiotics.

MATERIALS AND METHODS

Materials and animals

Thiazole and pyrazine were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Male Sprague-Dawley rats (~200 g) obtained from Harlan (Indianspolis, IN, U.S.A.) and New Zealand white rabbits (2.0~2.5 kg) purchased from Langshaw Farms (Augusta, MI, U.S.A.) were fed ad libitum on a 12 h light and dark cycle. Animals were treated with either thiazole or pyrazine for 3 days (200 mg/kg body weight/day, ip) and subjected to the isolation of hepatic microsomes. Animals were fasted 16 h prior to sacrifice.

Isolation of microsomal proteins

Hepatic microsomes prepared by differential centrifugation were washed in pyrophosphate buffer and stored in 50 mM Tris acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glyerol, and stored at −80℃ until used. Protein was assayed by the method of Lowry et al using lysozyme as a protein standard (Lowry *et al.*, 1951).

Gel electrophoresis and immunoblotting analysis

SDS-PAGE analysis (7.5%) was performed, as described by Laemmli (Laemmli, 1970) using a BioRad Mini-protean II apparatus. Immunoblotting analysis was carried out according to the previously published procedures (Davis *et al.*, 1986; Kim *et al.*, 1991b).

Protein purification

Hepatic mEH protein was purified from either thiazole- or pyrazine-induced rat hepatic micro-

somes according to the published procedure (Guengerich, 1989), as modified in this laboratory. Briefly, liver microsomes isolated from thiazole- or pyrazine-treated rats (~20 rats in each group) were suspended in 0.2 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA. A 10 % tergitol NP-10 was added to yield a final concentration of 1% while stirring and stirred for an additional 30 min. Unsolubilized microsomes were sedimented by centrifuging for 1 h at 100,000 g and the supernatant was dialyzed against 5 mM potassium phosphate buffer (pH 7.25) containing 0.05% tergitol NP-10 (buffer A) overnight with two changes. The dialysate was applied to a DEAE-cellulose column (Whatman DE-52) equilibrated with buffer A and the column was washed with buffer A. The absorbance at 290 nm of the eluate was monitored, which contained mEH protein or both mEH and the 43 kDa protein. The homogeneity was determined electrophoretically by SDS-PAGE analysis and Coomassie brilliant blue staining. The eluate was pooled and concentrated using an Amicon ultrafiltration apparatus (Amicon, Beverly, MA). Protein concentration was determined by staining intensities of the bands in SDS-PAGE using bovine serum albumin as a standard, followed by densitometric scanning analysis.

Antisera production

Antibody was produced in a rabbit using purified mEH protein by Bethyl Laboratories (Montgomery, TX, U.S.A.). Specificity of the antibody was tested by immunoblotting analysis of hepatic microsomes and purified mEH protein and compared with that of rabbit anti-rat mEH antibody kindly provided by Dr C.B. Kasper, McArdle Laboratory for Cancer Research, University of Wisconsin (Madison, WI, U.S.A.).

Amino acid sequence analysis

N-terminal amino acid sequencing was performed using a gas-phase sequencer at the macromolecular core facility, Wayne State University. The purified or co-purified proteins were transferred to Immobilon PVDF membrane (Millipore, Bedford, MA, U.S.A.) following gel electrophoresis (LeGendre & Matsudaira, 1988). The membrane was stained with Coomassie brilliant blue solution containing 50% methanol and 10% acetic acid. After destaining of the membrane with 50% methanol and 10% acetic acid, the region containing mEH or the 43 kDa protein was cut out and subjected to direct sequencing.

RESULTS AND DISCUSSION

The SDS-PAGE of hepatic microsomes isolated from rats treated with thiazole or pyrazine revealed a significant increase in intensity of a band migrating in the region of mEH. Purification of this induced mEH protein from thiazole- or pyrazine-induced rat hepatic microsomes was initiated with DEAE-cellulose column chromatography. The absorbance profile of eluted mEH protein from DEAE-cellulose column is shown in Fig. 1A. mEH protein in thiazole-induced microsomes was purified from the other microsomal proteins to electrophoretic homogeneity at low phosphate concentration, as detected by SDS-PAGE analysis (Fig. 1B). When pyrazine-induced rat hepatic microsomes were subjected to DEAE-cellulose column chromatography, both mEH and the 43 kDa protein were simultaneously eluted with the same washing solution, as monitored by SDS-PAGE and immunoblotting analysis (Fig. 1B). Thus, mEH was purified to electrophoretic homogeneity from thiazole-induced rat hepatic microsomes by the single step column chromatography whereas another protein (Mr=~43 kDa) was co-purified with mEH from pyrazine-induced rat hepatic microsomes. Purity of the mEH was confirmed by sequence analysis of N-terminal amino acid (Table 1).

Antibody was raised from a rabbit against the mEH protein purified from thiazole-induced rat hepatic microsomes. This rabbit anti-rat mEH antibody appeared to bind specifically with mEH in rat hepatic microsomes (Fig. 2). Immunoblotting analyses revealed a 10- and 7-fold increase in mEH levels in hepatic microsomes isolated from thiazole- and pyrazine-treated animals, respectively. The result of this increase in mEH protein levels, as assessed by immunoblotting analysis, is consistent with the epoxide hydrolase activity toward 2-cyanoethylene oxide, as determined previously using these xenobiotic-induced rat hepatic

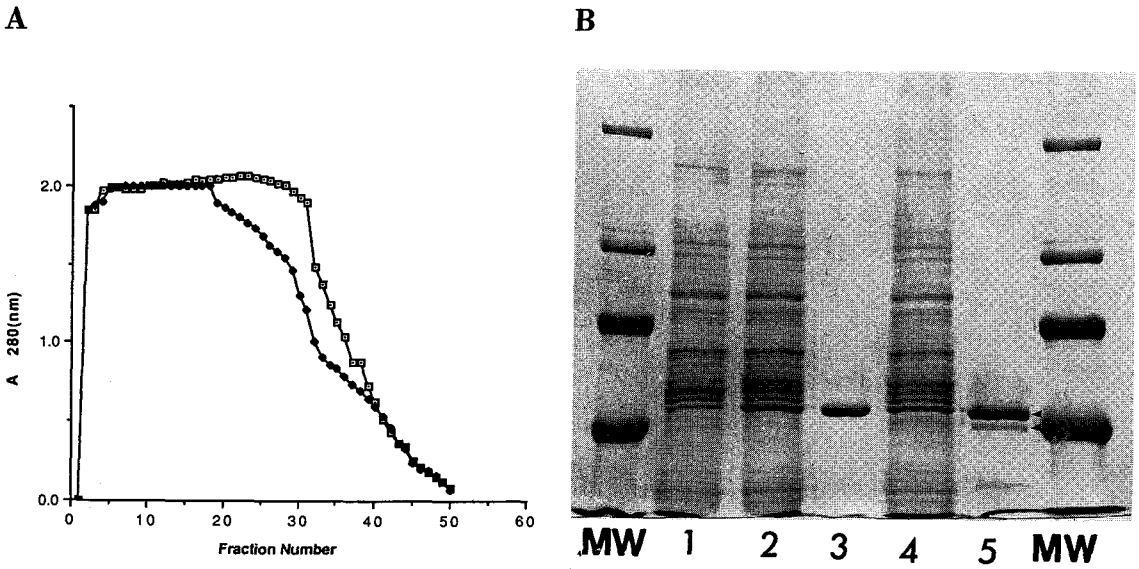

Fig. 1. A) The absorbance profile of eluted mEH or/and mEH-related protein from thiazole-induced (○) or pyrazine-induced (◆) rat hepatic microsomes using DEAE-cellulose column chromatography (bed volume, ~300ml). An ~3000 mg of solubilized hepatic microsomes was applied to the column. Fractional volume of the eluates was 15 ml.

B) SDS-PAGE (7.5%) of uninduced (lane 1, 12 μg), thiazole-induced hepatic microsomes (lane 2, 12 μg), purified mEH from thiazole-induced hepatic microsomes (lane 3, 1.4 μg), pyrazine-induced hepatic microsomes (lane 4, 12 μg), and co-purified mEH and the 43 kDa protein from pyrazine-induced hepatic microsomes (lane 5, 2.4 μg). The purified samples pooled from fraction number 5 through 45 were concentrated using an Amicon ultrafiltration apparatus and applied to SDS-PAGE analysis. The proteins for molecular weight standards were myosin (200 K), phosphorylase (97 K), bovine serum albumin (68 K) and ovalbumin (43 K).

microsomes (a 10− and 6-fold increase in enzymatic hydrolysis of 2-cyanoethylene oxide in thiazole- and pyrazine-induced microsomes, respectively, relative to control) (Kim *et al.*, 1993). Previous studies in this laboratory have shown that the levels of mEH mRNA increase 15- to 20-fold following either thiazole or pyrazine treatment, as compared with those in untreated animals (Kim *et al.*, 1991a; Kim *et al.*, 1993). Thus, the increase in mEH protein by these xenobiotics is primarily associated with the accumulation of mEH mRNA.

Western immunoblotting analysis revealed cross-reactivity of the mEH antibody with another protein in pyrazine-induced rat hepatic microsomes, the molecular weight of which was ~43 kDa. The ratio of band intensities between the 43 kDa protein and mEH was ~1 to 15 in pyrazine-induced rat hepatic microsomes. The band intensity of the immunoblotted 43 kDa protein was significantly diminished when diluted microsomal sample was employed (Fig. 2). These results provide evidence that another protein in pyrazine-induced rat hepatic microsomes has immunochemical cross-reactivity with the antibody raised against mEH protein. When immunoblotting analysis was performed with the purified fractions from pyrazine-induced microsomes, the co-purified lower molecular weight protein exhibited immunochemical cross-reactivity with mEH antibody (Fig. 3).

N-terminal amino acid sequence was determined using a gas-phase microsequencer in order to examine the structural similarity between the 43 kDa protein and mEH. The sequence analysis revealed that 10 out of 12 amino acids of N-terminus in the 43 kDa protein was identical to mEH sequence with two amino acid residues of the 43 kDa protein undetermined (Table 1) (Porter *et al.*,

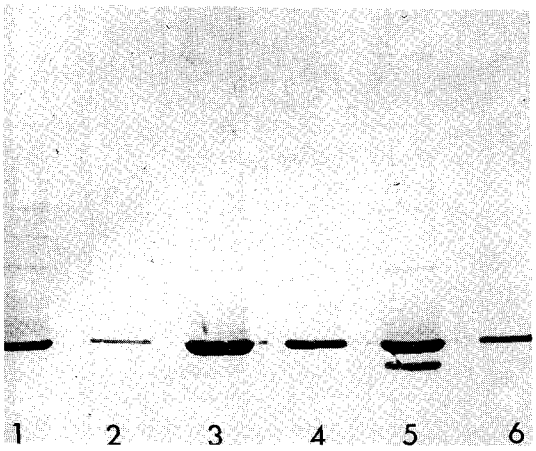

Fig. 2. Immunoblotting analysis of rat hepatic microsomes with rabbit anti-rat mEH antibody. Immunoblotting analysis exhibits the levels of hepatic mEH protein in microsomes isolated from rats treated with thiazole or pyrazine. Shown above are the lanes containing uninduced (lane 1, 5 μg; lane 2, 0.5 μg), thiazole-induced (lane 3, 5 μg; lane 4, 0.5 μg) and pyrazine-induced (lane 5, 5 μg; lane 6, 0.5 μg) rat hepatic microsomes. This immunoblot is a representative blot among the four analyses performed. This blot exhibits significant increases of hepatic mEH protein and another protein, which cross-reacted with mEH antibody, in thiazole- or pyrazine-induced hepatic microsomes.

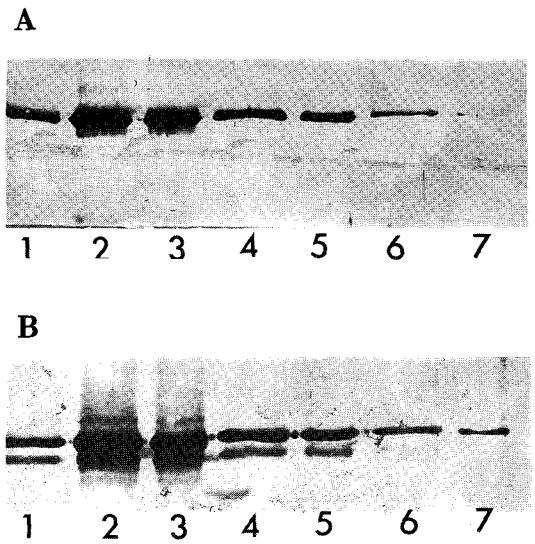

Fig. 3. Immunoblotting analysis of purified fractions from thiazole- and pyrazine-induced microsomes. Panel A) Thiazole-induced rat hepatic microsomes (lane 1, 1 μg) and purified mEH (lanes 2 through 7, 1.4, 0.7, 0.14, 0.07, 0.014 and 0.007 μg, respectively) were immunoblotted with rabbit anti-rat mEH antibody. Panel B) Pyrazine-induced rat hepatic microsomes (lane 1, 1 μg), and co-purified mEH and the 43 kDa protein (lanes 2 through 7, 2.0, 1.0, 0.2, 0.1, 0.02 and 0.01 μg, respectively) were immunoblotted with rabbit anti-rat mEH antibody.

1986). The level of the 43 kDa protein was approximately 7% of the induced mEH protein level in purified fractions, as determined by the levels of amino acids in N-terminal amino acid sequence analyses.

Incubation of either microsomes or purified mEH protein with α-chymotrypsin caused the formation of cleavage product which appeared to migrate in the region of 43 kDa with a concomitant decrease in mEH band intensity, as monitored by immunoblot analysis (data not shown). Treatment of the proteins with either trypsin or Staphylococcus aureus V8 protease yielded different patterns in proteolytic digestion (data not shown). Incubation of either purified mEH or thiazole-induced microsomes with CNBr was carried out in order to examine whether cleaved peptide fragments of mEH include the protein migrating in the region of 43 kDa. The cleaved protein products obtained from purified mEH protein after incubation with CNBr failed to exhibit a protein band migrating at 43 kDa, as assessed by SDS-PAGE and immunoblotting (data not shown). Additional experiments were conducted to examine whether it is possible that either pyrazine or metabolite(s) produced from pyrazine chemically cleaves mEH protein to produce the 43 kDa protein. Thiazole-induced microsomes were incubated with pyrazine in the presence or absence of NADPH. Neither of the incubations resulted in the formation of cleaved protein pruduct which migrates in the region of 43 kDa (data not shown). Given the present results obtained, the possiblity of post-translational modification for the formation of 43 kDa protein cannot be excluded (i.e. as partly suggested from proteolytic digestion). How-

Table 1. N-terminal amino acid sequence analysis

Cycle #	mEH	pmol[a]	43 kDa	pmol[b]
1	Met	31.8	Met	3.8
2	Trp	18	X	
3	Leu	31	Leu	4.7
4	Glu	27.8	Glu	4.7
5	Leu	30.7	Leu	5.8
6	Val	20.5	Val	3.0
7	Leu	–	Leu	5.7
8	Ala	21	X	
9	Ser	3.6	Ser	1.3
10	Leu	26.6	Leu	5.7
11	Leu	37.3	Leu	6.6
12	Gly	21	Gly	6.2
13	Phe	18.7		
14	Val	14.7		
15	Ile	13		
16	Tyr	12.9		

Three lanes of gel purified strips[a] were employed for analysis of mEH protein whereas 10 lanes[b] were used for the 43 kDa protein.

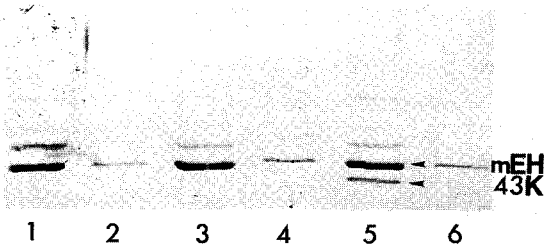

Fig. 4. Immunoblotting analysis of rabbit hepatic microsomes with rabbit anti-rat mEH antibody. Immunoblotting analysis exhibits the levels of hepatic mEH protein in microsomes isolated from rabbits treated with either thiazole or pyrazine. Shown above are the lanes containing uninduced (lane 1, 5 μg; lane 2, 0.5 μg), thiazole-induced (lane 3, 5 μg; lane 4, 0.5 μg) and pyrazine-induced (lane 5, 5 μg; lane 6, 0.5 μg) hepatic microsomes. The extra high molecular weight band was only detectable in rabbit hepatic microsomes due to non-specific cross-reactivity.

ever, it is also possible that the 43 kDa protein is expressed from a different gene after pyrazine treatment. The mechanism(s) underlying the 43 kDa protein induction should be further established at the molecular level.

Multiple forms of proteins related with epoxide hydrolase have been reported (Friedberg *et al.*, 1989; Griffin & Noda, 1980). Studies by Friedberg et al have shown that peroxisomes contain organelle proteins immunologically related to mEH yet with molecular weights clearly distinct from the mEH present in endoplasmic reticulum (Friedberg *et al.*, 1989). The peroxisomal proteins appeared not to be generated from a common precursor protein by post-translational modification either in vivo or in vitro during the preparation of peroxisomes, as evidenced by the presence of the proteins in the in vitro translation system which is deviod of several post-traslational modification system (Friedberg *et al.*, 1989). In addition, Griffin and Noda reported the presence of mEH-related 43 kDa protein in liver, the level of which is undetectable in normal hepatic microsomes but elevated in hepatic nodule microsomes (Griffin & Noda, 1980). Although it remains to be determined whether the nodular protein is the same as that found to be increased by treatment of animals with pyrazine, it is possible that the expression of the 43 kDa protein in nodule microsomes may share common regulatory induction mechanism(s) associated with the xenobiotic-induced 43 kDa protein. As stated above, the mEH-related 43 kDa protein might be expressed either through post-translational modification of mEH protein or through activation of the gene closely related with mEH.

In this study, a species difference in mEH induction was examined in rabbits in response to these xenobiotics. Treatment of rabbits with either thiazole or pyrazine failed to increase the levels of mEH in hepatic tissue, as determined by immunoblotting analysis. Interestingly, however, the 43 kDa protein was induced in this species by pyrazine (Fig. 4).

In summary, these results demonstrated that treatment of rats with either thiazole or pyrazine causes elevation in hepatic mEH expression whereas pyrazine treatment results in induction of mEH-related 43 kDa protein, as evidenced by chromatographic profile, immunochemical analysis of N-terminal amino acid analysis, and that a distinct species difference exists between rats and

rabbits in the induction of mEH by these xenobiotics.

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=국문초록=

Thiazole 또는 Pyrazine유도성 Microsomal Epoxide Hydrolase의 순수정제: Epoxide Hydrolase-관련성 43 kDa 단백질의 유도증가

덕성여자대학교 약학대학

김 상 건

Microsomal epoxide hydrolase (mEH)은 epoxide형 중간대사물을 해독화하는 효소이다. 본 실험실에서는 *thiazole* 또는 *pyrazine*을 *rat*에 투여할 때 *mEH* mRNA수준이 증가되고 mEH가 유도증가한다는 것을 밝힌바 있다(Carcinogenesis, Kim et al, 1993). 본 연구에서는 Thiazole처리를 한 rat의 간 microsome 분획으로 부터 DEAE-cellulose column chromatography를 이용하여 mEH를 순수분리하였고, 이를 SDS-PAGE분석 및 N 말단 amino acid 서열분석으로 확인하였다. Pyrazine처리를 한 rat의 간 microsome분획에서는 mEH와 더불어 이와 관련된 43 kDa 단백질이 함께 정제되었다. 정제된 thiazole 유도성 mEH를 토끼에 주사하여 항체를 생산하였고, 이 항체를 이용한 immunoblot 분석을 하였을 때 간 microsome 분획의 mEH가 thiazole투여군에서는 대조군에 비하여 10배, pyrazine 투여군에서 7배 증가하였다. Pyrazine처치한 rat의 간 microsome 분획에서는 mEH 관련성 43 kDa 단백질이 동시 유도증가하는 것을 면역화학적 반응으로도 확인하였다. 이때 Pyrazine으로 유도된 rat의 간 microsome 분획 또는 정제분획에 존재하는 43 kDa 단백질과 mEH의 비율은 1 : 15로 나타났다. 정제된 mEH와 43 kDa 단백질의 N 말단 amino acid 서열을 분석하였을때 43 kDa 단백질의 N 말단이 mEH와 동일하게 나타나 관련 단백질임을 확인하였다. 이러한 mEH 유도현상에 종차가 있는지를 알아보기 위하여 thiazole과 pyrazine을 각각 rabbit에 투여하였을 때 rabbit에서는 mEH의 유도증가가 일어나지 않았으며, pyrazine 투여군에서 43 kDa 단백질의 증가는 관찰되었다. 본 연구는 thiazole 또는 pyrazine 투여후 mEH 발현이 유도증가되며, pyrazine 투여후에는 mEH 및 이와 관련된 43 kDa 단백질이 동시유도되고, 이러한 mEH 유도발현에 rat와 rabbit간에는 종차가 있음을 보여준다.