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DETECTION OF TWO HEME OXYGENASE ISOFORMS IN THE HUMAN TESTIS

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This study shows heme oxygenase multiplicity is common to rat and human tissues. The isozymes in man and rat, however, are heterogenous proteins that share certain characteristics. Two forms of heme oxygenase, HO-1 and HO-2, were identified in human testis. HO-2 form was the prevalent form. Human and rat HO-1 differed in chromatographic behavior and molecular weight; human HO-1 was a larger molecule (35,400 vs 30,000). The two forms, however, were similar in that immunochemically human HO-1 exhibited reactivity toward antibody to rat HO-1. Human and rat HO-2 also were dissimilar in chromatographic behavior and showed only a weak immunological cross-reactivity. Human and rat HO-1 were essentially the same size. As in rat organs, the microsomal cytochrome P-450 content in human testis was reciprocal to heme oxygenase activity.

The oxidative cleavage of heme at the α-meso carbon bridge to form biliverdin IX α is catalyzed by microsomal heme oxygenase in the presence of NADPH-cytochrome P-450 reductase and NADPH. In mammalian systems, the latter is reduced to bilirubin by biliverdin reductase. We have recently characterized two forms of heme oxygenase (1-3) in rat organs. The enzymes, HO-1 and HO-2, differ with respect to molecular properties, tissue expression, regulatory mechanism, and are products of two distinct genes (4). HO-1 is the commonly known form and is inducible by a variety of chemicals (5,6). At this time it is not known whether more than one form of heme oxygenase is also present in other species, including the human.

The purpose of the present study was to examine the possibility of the existence of multiple forms of heme oxygenase in the human tissues; if so, to compare the properties of the human and the rat enzymes.

MATERIALS AND METHODS

Materials: Male Sprague-Dawley rats (180-200 g) were purchased from Harlan Industries, Madison, WI. The animals were treated (sc) with 2 mmol/kg bromobenzene and killed 24 h later. Human testes were obtained from three patients who underwent orchiectomy for treatment of advanced prostatic cancer. Pooled testis microsomes were used for DEAE-Sephacel chromatography. The testis

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were normal in pathology, and were procured from the Department of Urology, Strong Memorial Hospital, Rochester, NY, within 1-2 h after removal. Chemicals were of the highest quality commercially available.

Immunological Procedures and Enzyme Purifications: HO-1 was purified from the liver of bromobenzene-treated rats and HO-2 was purified from the testis of control rats (1,2). The enzymes were used for production of antibodies in New Zealand rabbits as described before (1,2). Western immunoblotting was performed according to the procedure of Towbin et al. (7), as detailed elsewhere (3). NADPH-cytochrome P-450 reductase was purified from rat liver microsomes as described by Yasukochi and Masters (8), and biliverdin reductase was purified from rat liver cytosol (9).

Resolution of Heme Oxygenase Activity: Human and rat testis microsomes were solubilized with sodium cholate and Triton X-100 as described earlier (2). The details of chromatographic procedure are described in the legend to Figure 1.

Assay Procedures: Heme oxygenase activity was determined as detailed previously (10). The assay system contained purified preparations of NADPH-cytochrome P-450 reductase, biliverdin reductase, 15 uM hematin, and 0.5 mM NADPH. NADPH-cytochrome P-450 reductase activity was measured as described by Strobel and Dignam (11) and NADH-cytochrome b_5 reductase activity was measured as described by Mihara and Sato (12). Cytochrome b_5 concentration was measured by the procedure of Estabrook and Werringloer (13), and protein concentration was assessed by method of Lowry et al. (14). Cytochrome P-450 concentration was measured as described by Omura and Sato (15).

RESULTS AND DISCUSSION

A series of experiments were performed to characterize the chromatographic behavior of human heme oxygenase. When solubilized human testis microsomal fraction was subjected to DEAE-Sephacel chromatography, the activity resolved in two peaks (Fig. 1). This observation was similar to that previously made with the rat testis (2) and hence we used the sequence of their elution from the column to designate them as HO-1 and HO-2. As judged by the area under the curve, the human testis contains mainly HO-2 form of the enzyme, with the ratio of HO-2 to HO-1 being approximately 2:1. We have noted before that in the rat testis the most prevalent form is also HO-2; however, in rat the ratio of the 2 isozymes is nearly 8:1 (2). The detection of relatively high levels of HO-1 in the human testis may represent a species difference; alternatively, it could reflect the induction of HO-1 in the testes of patients as the result of exposure to a variety of medication prior to removal of the organ. This possibility is consistent with finding that rat liver HO-1 is the only inducible form of heme oxygenase (1). To date an effective agent to enhance HO-2 activity has not been identified.

Subsequently, the chromatographic behavior of human HO-1 and HO-2 were compared with that of rat enzymes. In this experiment, a mixture of rat liver and testis microsomes were subjected to DEAE-Sephacel chromatography under similar conditions as used with human testis microsomes. The livers were

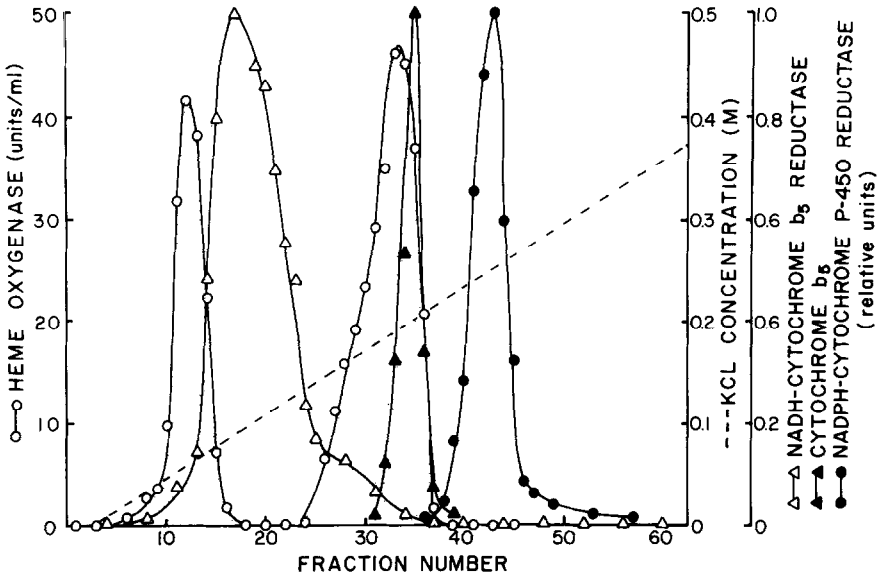

FIGURE 1. DEAE-Sephacel Chromatography of Human Testis Microsomal Heme Oxygenase Activity.
The microsomal preparation was solubilized with Triton X-100 and sodium cholate as described previously (2). The solubilized microsomes (48.0 mg protein) were diluted with 0.5 v of a 20 mM Tris buffer, pH 8.0, that contained 1 mM EDTA and 0.5% (w/v) sodium cholate and loaded onto a DEAE-Sephacel column with a 6.0 ml (1.0 x 7.6 cm) bed volume (V_t) that was previously equilibrated with 6.0 V_t of a 20 mM Tris-buffer, pH 8.0, that contained 0.1 mM EDTA, 0.4% (v/v) Triton X-100, and 0.2% (w/v) sodium cholate. The column was washed with 1.0 V_t of the above buffer but containing 0.1% (w/v) sodium cholate and adjusted to pH 7.7 and eluted with concurrent linear gradients of KCl (0 to 400 mM) and Triton X-100 (0.4 to 0.9% (v/v)) that were prepared in the same buffer. The flow rate, salt gradient slope, detergent gradient slope, and elution fraction volume were adjusted to 0.75 V_t/h, 50.0 mM KCl/V_t, 0.0625% (v/v) Triton X-100/V_t, and 0.125 V_t/fraction, respectively. Elution fractions were assayed for the indicated activities and the results are expressed in absolute units for heme oxygenase and in relative units (i.e., relative to the activity of the peak fraction) for all other activities. Peak fraction activities for NADH-cytochrome b_5 reductase, cytochrome b_5, and NADPH-cytochrome P-450 reductase were 171.95 units/ml, 5.67 nmoles/ml, and 1.48 units/ml, respectively.

enriched with HO-1 by prior treatment of rats with bromobenzene. When the elution profile of human heme oxygenase isozymes (Fig. 1) is compared with that of the rat enzymes (Fig. 2), differences are noted between the two profiles. Specifically, the elution pattern of human testis HO-1 and HO-2 with respect to the marker enzymes, NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase, and cytochrome b_5, differs from that observed with rat HO-1 and HO-2. Human HO-1 elutes from the column before cytochrome b_5 reductase; the reverse is the case with the rat reductase. Furthermore, elution of human HO-2 precedes that of cytochrome b_5, but in the rat they co-elute. The elution of

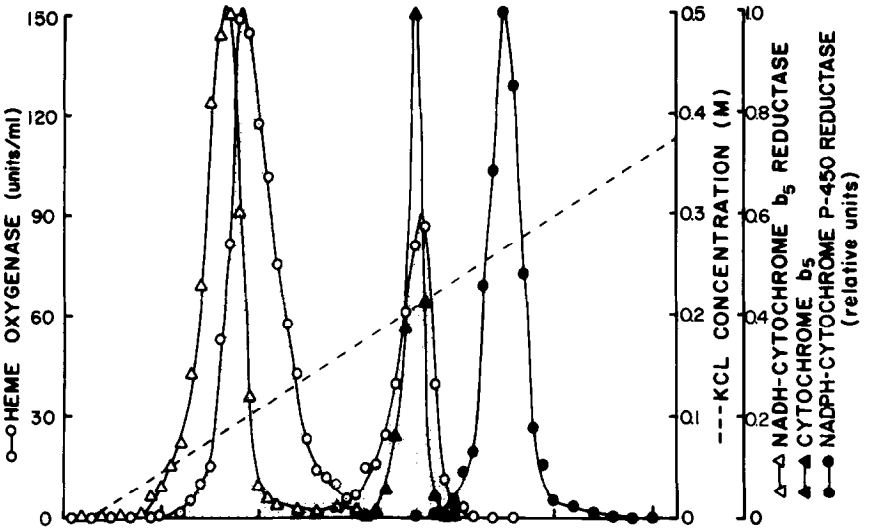

FIGURE 2. DEAE-Sephacel Chromatography of Rat Liver and Rat Testis Heme Oxygenase Activity.
Chromatographic conditions described in the legend to Figure 1 were used. Heme oxygenase source was a mixture of rat liver (bromobenzene-treated) and rat testis microsomal fractions. Peak fraction activities for NADH-cytochrome b_5 reductase, cytochrome b_5, and NADPH-cytochrome P-450 reductase were 55.72 units/ml, 10.98 nmoles/ml, and 4.22 units/ml, respectively.

NADPH-cytochrome P-450 reductase follows HO-2 in both the human and the rat. The differential chromatographic behavior of the human and rat isozymes, however, suggest the likelihood of differential molecular properties of the enzymes in these species. The data discussed in the following is supportive of this possibility.

The human HO-1 and HO-2 were compared with the purified rat liver HO-1 and rat testis HO-2 by Western immunoblotting technique. The peak fractions of DEAE-Sephacel chromatography of human testis were used as the source of HO-1 and HO-2. In one experiment, rat and human HO-1 preparations were tested against antiserum raised against rat HO-1 (Fig. 3, lanes 2 and 3, respectively) and in a second experiment rat and human HO-2 preparations were tested against antiserum to rat HO-2 (Fig. 3, lanes 5 and 6, respectively). Lanes 1 and 4 contain molecular weight markers and were visualized using Amido Black. As shown, human HO-1 exhibited immunological cross-reactivity with antibody to the rat HO-1 preparation. In this experiment similar amounts of HO-1 protein, estimated from the apparent enzyme activity, were added to electrophoretic channels. Lane 3 contained human HO-1 and lane 2 contained the purified rat

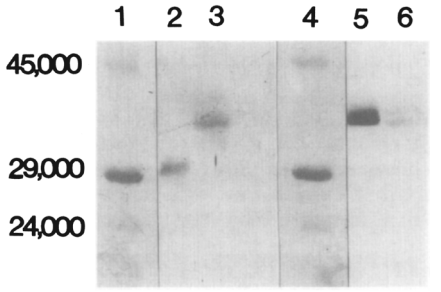

FIGURE 3. Western Immunoblot of Human Testis HO-1 and HO-2 Preparations and Purified Rat Liver and Testis HO-1 and HO-2.
Heme oxygenase preparations were subjected to SDS-polyacrylamide gel electrophoresis, and electroblotted onto a nitrocellulose sheet. The blots were treated with primary antibody, then goat anti-rabbit IgG and finally peroxidase-antiperoxidase complex. Lane 2, purified rat liver HO-1; lane 3, human testis HO-1. In this experiment, antibody raised to rat liver HO-1 was used. Lane 4, purified rat testis HO-2; lane 5, human testis HO-2. In this experiment, antibody raised to rat testis HO-2 was used. Lanes 1 and 4 are molecular markers and were stained with Amido Black.

HO-1. As noted, both rat and human HO-1 preparations exhibited similar intensity of cross-reactivity with the antibody. However, human HO-1 had a significantly larger molecular weight than rat HO-1 (approximately 35,400 vs 30,000). The immunoblot, however, demonstrates an extremely weak reactivity between antibody to rat HO-2 and human HO-2 (lane 6). Furthermore, the human HO-2 and rat HO-2 preparations (lanes 6 and 5, respectively) appeared to have a similar molecular weight (approximately 36,000). The differential intensity of reaction between rat and the human HO-2 with antibody to rat HO-2 was not due to the differential amount of protein used for the Western immunoblot analyses since the samples added to lanes 5 and 6 contained a similar amount of heme oxygenase protein, assessed by the activity of the preparations.

At this time we cannot predict whether the two forms of heme oxygenase are expressed in other human organs or if the human enzymes display the dissimilar regulatory mechanisms identified with the rat HO-1 and HO-2. Furthermore, although the present study clearly identifies 2 forms of the enzyme in the human testis, the possibility that other species of the enzyme may be present but could not be resolved by the presently used protocols cannot be dismissed. It is reasonable, however, to suggest that HO-1 may be also expressed in the human liver, and that in the liver human HO-1 has similar immunochemical property and molecular weight as the human testis HO-1. This suggestion is

based on the recently reported molecular weight of the human "heme oxygenase" obtained from cDNA which was in turn isolated by screening a human cDNA library with a rat cDNA (16). The human heme oxygenase was inducible by heme (15). We have noted earlier that heme induces HO-1 in the rat liver, but does not effect HO-2 (1). Moreover, the present finding that human HO-1 and the rat HO-1 display similar immunoreactivity with antibody to rat HO-1 is consistent with the reported 80% homology between the cDNA sequence-deduced amino acid compositions of rat and human heme oxygenase (16).

At this time, the biological significance for the presence of two forms of heme oxygenase in human testis is not clear. However, our recent findings with reconstituted heme oxygenase systems showing the differential rate of reactivity of HO-1 and HO-2 toward rat cytochrome P-450 isozymes (17) may suggest the differential function of the enzymes in the regulation of cytochrome P-450-dependent biotransformation reactions. In vivo in the rat, a reciprocal relationship between heme oxygenase activity and the concentration of cytochrome P-450 has been observed (6). The data shown in Table 1 demonstrates that such a relationship is also present in the human testis, and may suggest the role of heme oxygenase in regulating cytochrome P-450 level in this organ.

TABLE 1

COMPARATIVE ACTIVITY OF HEME OXYGENASE AND THE CONCENTRATIONS OF CYTOCHROMES P-450 IN HUMAN TESTIS

Sample Number	Heme Oxygenase (nmol/mg/min)	Cytochrome P-450 (pmol/mg)
1	19.2	76
2	24.5	68
3	30.6	39

The testis were obtained from 3 different patients. The organs were placed in ice-cold saline immediately after removal, and were used for preparation of the microsomal fraction. The various assays were performed as detailed in the text. Heme oxygenase activity was measured in the presence of added purified NADPH-cytochrome P-450 reductase and the activity was assessed based on the rate of bilirubin formation.

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REFERENCES

1. Maines, M.D., Trakshel, G.M. and Kutty, R.K. (1986) J. Biol. Chem. 261, 411-419.
2. Trakshel, G.M., Kutty, R.K. and Maines, M.D. (1986) J. Biol. Chem. 261, 11131-11137.
3. Trakshel, G.M., Kutty, R.K. and Maines, M.D. (1988) Arch. Biochem. Biophys. 260, 732-739.
4. Cruse, I. and Maines, M.D. (1988) J. Biol. Chem. 263, 3348-3353.
5. Maines, M.D. and Kappas, A. (1977) Science 198: 1215-1221.
6. Maines, M.D. (1984) CRC Crit. Rev. Tox. 12: 241-315.
7. Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
8. Yasukochi, Y. and Masters, B.S.S. (1976) J. Biol. Chem. 251, 5337-5344.
9. Kutty, R.K. and Maines, M.D. (1981) J. Biol. Chem. 256, 3956-3962.
10. Trakshel, G.M., Kutty, R.K., and Maines, M.D. (1986) Arch. Biochem. Biophys. 251, 175-187.
11. Strobel, H.W. and Dignam, J.D. (1978) Methods Enzymol. 52: 89-96.
12. Mihara, K. and Sato, R. (1978) Methods Enzymol. 52, 102-108.
13. Estabrook, R. and Werringloer, J. (1978) Methods Enzymol. 52, 212-220.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
15. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239: 2379-2385.
16. Yoshida, T., Biro, P., Cohen, T., Muller, R.M. and Shibahara, S. (1988) Eur. J. Biochem. 171, 457-461.
17. Kutty, R.K., Daniel, R.F., Ryan, D.E., Levin, W. and Maines, M.D. (1988) Arch. Biochem. Biophys. 260, 638-644.