Immunochemical studies of haem oxygenase. Preparation and characterization of antibodies to chick liver haem oxygenase and their use in detecting and quantifying amounts of haem oxygenase protein

Yvonne J. Greene

John F. Healey

Herbert L. Bonkovsky

University of Massachusetts Medical School

Follow this and additional works at: http://escholarship.umassmed.edu/oapubs

Part of the Biochemistry Commons

Repository Citation

http://escholarship.umassmed.edu/oapubs/220
Immunochemical studies of haem oxygenase

Preparation and characterization of antibodies to chick liver haem oxygenase and their use in detecting and quantifying amounts of haem oxygenase protein

Yvonne J. GREENE,*† John F. HEALEY,‡ and Herbert L. BONKOVSKY*†
Departments of *Biochemistry and Molecular Biology and †Medicine, University of Massachusetts Medical School, Worcester, MA 01655, and ‡Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

Monospecific polyclonal rabbit antibodies to a purified form of haem oxygenase of chick liver, showing sequence similarity to mammalian haem oxygenase-1, were raised and used to study characteristics of the oxygenase. The antibodies inhibited activity of the purified oxygenase, but not other enzyme components (NADPH:cytochrome reductase and biliverdin reductase) of the standard assay mixture of haem oxygenase. In addition, the antibodies inhibited activity of haem oxygenase in microsomes (microsomal fractions) from Cd²⁺-treated chick liver, spleen, testis and brain. Western (immuno-) blots of microsomal proteins of selected organs from chick, rat and man, and homogenates of chick-embryo liver-cell cultures, probed with the antibodies, showed a major protein with a molecular mass of 33–34 kDa and a lower-molecular-mass protein (28–29 kDa) of variable intensity. Studies with trypsin and selected proteinase inhibitors established that the smaller peptide was a proteolytic product of the larger. Treatment of chick-embryo liver-cell cultures with CdCl₂, a potent inducer of haem oxygenase, increased the degree of proteinase-mediated cleavage of the 33 kDa protein to the lower-molecular-mass form. These results indicate that, under at least some conditions, such cultures should be homogenized in the presence of trypsin inhibitor to prevent proteolytic degradation of the enzyme and allow maximal expression of haem oxygenase activity. The antibodies also reacted with haem oxygenase from spleen, testis and brain of both chicks and rats, and the spleen of humans. A method for quantifying the amount of haem oxygenase protein was developed with use of slot-bLOTS and laser densitometry; linearity was observed from 0 to 5 ng of haem oxygenase protein per slot, and the method was applied to sonicated cultured chick-embryo liver cells treated with Cd²⁺ (0.3 mM) or iron plus glutethimide. In both cases, increases in enzyme activity were of similar magnitude to increases in amounts of enzyme protein. Approximate amounts of haem oxygenase protein in microsomes of several organs from intact animals could also be estimated by the use of slot-blot–laser densitometry, and the amounts measured were increased by the addition of purified haem oxygenase to the microsomal preparations. Results of these studies indicated that haem oxygenase-1 could be detected in microsomes from all chick or rat organs studied, including testis and brain. However, since antibodies to the 1 isoenzyme inhibited total haem oxygenase activity of testis or brain microsomes less than that of liver or spleen of Cd²⁺-treated chickens, a second isoenzyme of the oxygenase (HO-2) appears to occur in testis and brains of chickens, as well as mammals.

INTRODUCTION

Haem oxygenase (EC 1.14.99.3) is the rate-controlling enzyme for haem breakdown [for reviews, see Kikuchi & Yoshida (1983), Abraham et al. (1988), Maines (1988) and Shibahara (1988)]. Breakdown of the haemoglobin haem of senescent erythrocytes has long been attributed principally to the action of haem oxygenase of the reticuloendothelial system (Tehnunen et al., 1968; Pimstone et al., 1971; Bissell et al., 1977). In contrast, in a previous study, only a minor role of haem oxygenase in breakdown of hepatocyte haem was suggested (Bissell & Guzelian, 1980). More recent studies, however, indicated that all or nearly all endogenous hepatic haem is broken down by haem oxygenase (Grandchamp et al., 1981; Lincoln et al., 1989).

Activities of haem oxygenase are subject to a wide array of regulatory factors. The enzyme is induced by a number of chemicals, including haem itself, some metallic ions, most notably Co²⁺ and Cd²⁺, and by phenobarbital-like drugs. We (Lincoln et al., 1988) and others (Kikuchi & Yoshida, 1983) provided evidence for at least two disparate mechanisms for induction of the oxygenase, one dependent upon increased cellular haem, and the other independent of haem. The enzyme is also inhibited markedly by metalloporphyrins, especially tin- and zinc-porphyrins (Drummond & Kappas, 1981; Maines, 1981; Drummond et al., 1987; Martasek et al., 1988; Bonkovsky et al., 1990).

In other studies, evidence has recently been presented for the existence in rats, rabbits and humans of two forms of haem oxygenase (Maines et al., 1986; Trakshel et al., 1986a,b; Cruse & Maines, 1988). Form 1 is that originally described and discussed in the above paragraph. It is the predominant form in rat spleen and is the only inducible form thus far identified. Form 2, thus far described only in mammals, is of higher molecular mass, is not inducible, and is said to be the predominant or only form in brain and testis.

To aid in our ongoing studies on the regulation of haem oxygenase and its role in cellular haem and porphyrin metabolism, we purified the enzyme from chick liver and studied its properties in detail (Bonkovsky et al., 1990). In the present paper we describe the preparation of monospecific polyclonal antibodies to chick liver haem oxygenase and their use for detection and quantification of haem oxygenase protein.

MATERIALS AND METHODS

Preparation of polyclonal monospecific antibodies to haem oxygenase in rabbit

Antibodies against purified haem oxygenase were prepared in female New Zealand White rabbits as described by Thomas et al. (1976) and Kaminsky et al. (1981). Purified haem oxygenase was prepared by the method of Bonkovsky et al. (1990). Polyclonal IgG was prepared from serum by the octanoic acid method of McKinney & Parkinson (1987). The method of Girard et al. (1986) was used to prepare monospecific IgG against chick haem
oxygenase. Briefly, purified haem oxygenase (after passage through Sepharose-CL6B) with a specific activity of 159 nmol of bilirubin min⁻¹ mg⁻¹ of protein⁻¹ was separated by PAGE and transferred electrophoretically to nitrocellulose. The nitrocellulose containing the major band corresponding to purified haem oxygenase (Rf 3.65-3.85) was cut into small pieces, placed in a 5 ml syringe and exposed overnight to polyclonal IgG. Affinity-purified antibody was eluted by 100 mM-glycine, pH 2.8, and expelled into a tube containing 100 μl of 0.5 M-Tris base. The final pH was about 8.0.

The protein concentration of the IgG preparation was estimated from its absorbance at 280 nm (A280 = 10). IgG was diluted in 0.134 M-KCl/0.05 M-potassium phosphate, pH 7.4, containing 1% BSA (w/v) and 0.02% (w/v) Na₂SO₄ and stored at −20 °C.

Patients, animals and microsomal preparations

Samples of normal human liver were obtained at the time of upper abdominal surgery for ulcer or gallstone disease. Samples of congested, but non-neoplastic, human spleen were obtained at the time of splenectomy, done as part of surgery for treatment of portal hypertension and bleeding. Patients had given their informed consent for the surgical procedures, and the protocol had been approved by the Human Investigation Committee of Emory University.

For studies of antibody cross-reactivity in different species and organs, 18 h before being killed, 150 g male rats and 42-day-old male chicks were injected subcutaneously with 75 μmol or 52.5 μmol of CdCl₂/kg (in 0.15 M-NaCl) respectively. Control animals were injected with 0.15 M-NaCl alone. Microsomes were prepared from animal organs (liver, spleen, testis and brain), human liver and human spleen that had been homogenized (20%, w/v) in cold 0.25 M-sucrose/20 mM-Tris/HCl, pH 7.4, with four strokes of a Potter-Elvehjem homogenizer. All buffers contained 200 μg of soybean trypsin inhibitor/ml. Homogenates were centrifuged for 10 min at 10000 g (in 1979) at 4 °C and the resulting supernatant was centrifuged at 105000 g (rmax, 107.9 mm) for 1 h, 4 °C. The pellets were resuspended in 0.134 M-KCl/20 mM-potassium phosphate/1 mM-EDTA, pH 7.4, and centrifuged again for 30 min at 105000 g (rmax, 107.9 mm) at 4 °C. The final microsomal pellets were resuspended in 0.1 M-potassium phosphate/20% (v/v) glycerol/1 mM-EDTA, pH 7.4.

Enzyme activity assays

Effects of IgG on haem oxygenase, NADPH-cytochrome reductase (EC 1.6.2.4) and biliverdin reductase (EC 1.3.1.24) activities were measured in purified reconstituted enzyme systems as described by Bonkovsky et al. (1990). Haem oxygenase activity in cultured chick embryo liver cells and microsomal preparations were measured as described by Lincoln et al. (1988). NADPH:cytochrome reductase (0.6 unit/ml) was added to all assays of isolated microsomes.

Chick-embryo liver-cell cultures

Liver-cell cultures were prepared from 16-18-day-old chick embryos as described by Lincoln et al. (1988), except that newborn-calf serum and Ser-X-Tend were omitted. Cells were harvested in 0.1 M-potassium phosphate/20% (v/v) glycerol/1 mM-EDTA, pH 7.4, and sonicated with a single 6 s burst of a W-225 sonicator equipped with a microtip (Heat Systems Ultrasonics, Farmingdale, NY, U.S.A.) set on power level 4. For Western blotting, a 50 μl aliquot of the sonicated material was added to 25 μl of the buffer used for electrophoresis and the mixture boiled for 5 min to denature proteins.

Quantification by slot-blot

Dilutions of sonicated cultures containing up to 1.5 μg of total protein in a 50 μl volume were applied to wetted nitrocellulose (0.45 μm) filters in 0.75 mm × 8.0 mm rectangular slots (6.0 mm² surface area) under a vacuum using a Minifold-II slot-blot apparatus (Schleicher and Schuell, Keene, NH, U.S.A.). After filtration of the samples, the nitrocellulose was removed from the apparatus, allowed to dry and then heated (1 h, 100 °C) in 0.134 M-KCl/0.05 M-potassium phosphate, pH 7.4 (phosphate-buffered KCl) to destroy any endogenous alkaline phosphatase activity. All subsequent incubations were completed at room temperature with gentle shaking. After blocking overnight in phosphate-buffered KCl/1% BSA, the nitrocellulose was immersed in rabbit anti-(chicken haem oxygenase) antibody (25 μg/ml) for 3 h and subsequently washed three times (15 min each) in phosphate-buffered KCl/0.05% Tween 20. The filters were then rinsed with distilled water and immersed in alkaline phosphatase-conjugated goat anti-rabbit IgG from Bio-Rad Laboratories (Richmond, CA, U.S.A.) diluted 1:2000 (by vol.) in phosphate-buffered KCl for 90 min. After washing, colour was developed with an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories) containing Nitroblue Tetrazolium, 5-bromo-4-chloroindol-3-yl phosphate and 0.1 M-Tris/HC1, pH 9.5. The reaction was permitted to develop up to 10 min, after which the nitrocellulose was washed thoroughly in distilled water and left overnight in phosphate-buffered KCl. The next day, the filter was air-dried and the intensity of colour formation was measured by reflectance densitometry using either an LKB Ultrascan laser densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) or a Zeinin Soft Laser scanning densitometer, Model SLR-1D/2D (Biomed Instruments, Costa Mesa, CA, U.S.A.). Absorbance (arbitrary units) was converted into ng of haem oxygenase protein by direct comparison of unknowns to a standard curve constructed with use of known amounts of purified haem oxygenase protein included on the same nitrocellulose sheet. If necessary, amounts of unknowns to be assayed were adjusted such that their densitometrically estimated values fell within the linear range of the standard curve. Phosphate-buffered KCl/0.002%-BSA was used as a negative control in each blot.

Western-blot analysis

Western blots were prepared from proteins separated by SDS/PAGE as described by Evans et al. (1991).

Protein was measured by the method of Lowry et al. (1951), with BSA as standard.

RESULTS AND DISCUSSION

Inhibition of haem oxygenase by rabbit IgG

Preincubation (10 min, 37 °C) of purified haem oxygenase with increasing amounts of IgG from rabbits immunized with haem oxygenase led to a progressive dose-dependent decrease in activity of haem oxygenase (Fig. 1a). At the highest concentration of IgG tested (150 μg/ml), activity of haem oxygenase was inhibited by 95%. By contrast, this same concentration of preimmune IgG did not inhibit haem oxygenase activity (35.9 ± 4.3 nmol of bilirubin/min per mg, 102% of control). In other studies it was found that preimmune or immune IgG (150 μg/ml) had no effect on NADPH:cytochrome reductase activity and inhibited biliverdin reductase activity by only 20%. Since a 4-fold excess activity of biliverdin reductase over that of haem oxygenase was routinely added to the reaction mixtures, the latter inhibition cannot account for the inhibition of haem oxygenase activity by immune IgG. These results support the notion
of specificity of interaction between immune IgG and haem oxygenase, and suggest either that the immunogenic epitopes include the active centre of the enzyme, or that the antibody alters the enzyme's conformation and diminishes its activity.

Similarly, the antibody inhibited oxygenase activity of liver microsomes from Cd²⁺-treated chickens in a dose-dependent fashion (Fig. 1b). Inhibition by the antibody was also observed in microsomes of other organs of Cd²⁺-treated chickens (Table 1).

The form of haem oxygenase that we previously purified from chicken liver (Bonkovsky et al., 1990) and against which we have now raised antibodies shows sequence identity with the isoform of the oxygenase, called HO-1, purified from various species and organs [for a review, see Maines (1988)]. It is the predominant form in spleen and in liver of animals treated with inducers of haem oxygenase. A second form of enzyme, HO-2, which does not cross-react with antibodies to HO-1, has been described in mammals (Maines et al., 1986; Trakshel et al., 1986a; Maines, 1988; Rotenberg & Maines, 1990; Sun et al., 1990) and is said to be the predominant, if not the sole, form of the oxygenase in mammalian brain and testis.

Since our antibody both inhibited activity of haem oxygenase (Fig. 1, Table 1) and bound specifically to a 33–34 kDa protein from microsomes of chick and rat brain and testis (Fig. 2), we conclude that these organs do, in fact, contain the HO-1 isoenzyme. However, since the maximal degree of inhibition of haem oxygenase activity attained by exposure of our antibody to

<table>
<thead>
<tr>
<th>Table 1. Inhibition of haem oxygenase activity from Cd²⁺ treated chicken liver, spleen, testis and brain by anti-(haem oxygenase) IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organ</strong></td>
</tr>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Spleen</td>
</tr>
<tr>
<td>Testis</td>
</tr>
<tr>
<td>Brain</td>
</tr>
</tbody>
</table>

Pooled microsomes from four untreated chicks and three rats pretreated with CdCl₂ (75 μmol/kg) were prepared in the presence of soybean trypsin inhibitor (200 μg/ml of buffer) as described in the Materials and methods section. Proteins were loaded as follows: (a) chick microsomes: lane 1, 15 ng of haem oxygenase standard; lane 2, liver microsomes (1 μg of protein); lane 3, spleen microsomes (1 μg of protein); lane 4, testis microsomes (3 μg); lane 5, brain microsomes (3 μg); (b) rat microsomes: lane 1, 15 ng of haem oxygenase standard; lane 2, liver microsomes (3 μg); lane 3, spleen microsomes (3 μg); lane 4, testis microsomes (3 μg); lane 5, brain microsomes (3 μg). Western blots were done as described in the Materials and methods section.
microsomes of chick brain or testis was less than that attained in microsomes of spleen (Table 1), our results support the view that there is a second form of the oxygenase in chick brain and testis, which accounts for half or more of the total activity found in these organs.

Other attempts to find evidence for a second isoenzyme of haem oxygenase in chick microsomes, by use of antiserum to rat haem oxygenase-2, were unsuccessful, owing to a large degree of non-specific cross-reactivity between the antiserum and several chick microsomal proteins of diverse molecular masses (results not shown).

**Ability of proteinase inhibitors to prevent haem oxygenase degradation**

Polyclonal monospecific antibody ideally should be of use for both qualitative and quantitative immunochemical techniques, including Western blots and e.l.i.s.a. This requires the antibody preparation to be specific for a single protein with minimal background staining. In initial Western blots, a second lower-molecular-mass (~29 kDa) immunoreactive band was sometimes observed. Although this was thought most likely to represent a cleavage product of the 33.5 kDa parent enzyme (Evans et al., 1991), the recent descriptions of two forms of haem oxygenase in mammalian organs (Maines et al., 1986; Trakshel et al., 1986a,b; Cruse & Maines, 1988; Maines, 1988) made it important to clarify the nature and interrelationships of the two immunoreactive peptides. To this end, the effects of proteinase inhibitors on homogenates of chick-embryo liver-cell culture were studied. In the presence of protein inhibitors, only the 33.5 kDa protein was detected (Fig. 3, lanes 2–5), whereas, in the absence of inhibitors, the second, lower-molecular-mass (29 kDa), peptide appeared (Fig. 3, lanes 6–7). Leupeptin or aprotinin alone was able to inhibit proteolysis of haem oxygenase, whereas N-p-tosyl-lysylchloromethane (TLCK) and phenylmethanesulphonyl fluoride, at the concentrations used, were unable to prevent proteolysis. Addition of soybean trypsin inhibitor or sonication of cultured cells directly in electrophoresis denaturing buffer prevented haem oxygenase degradation (results not shown).

Haem oxygenase was rapidly digested by trypsin; within 5 min at 4 °C, only 21 % of the original enzyme activity remained in the sample. After as little as 5 min of exposure to trypsin, the 33.5 kDa protein was no longer detectable (Fig. 4). With longer periods of trypsin exposure, there was also a gradual loss of the 29 kDa peptide, suggesting the generation of lower-molecular-weight, non-immunoreactive fragments. Soybean trypsin inhibitor (200 µg/ml) prevented proteolysis without inhibiting haem oxygenase activity (results not shown). The above results support the conclusion that the rabbit antibodies detect native haem oxygenase and a smaller digested fragment, the latter formed by action of trypsin or trypsin-like proteinases that are inhibited by soybean trypsin inhibitor or leupeptin.

In a fairly recent paper, Yoshida & Sato (1989) reported that a 28 kDa peptide could be released by mild trypsin digestion of microsomal rat haem oxygenase (32 kDa), leaving the hydrophobic C-terminus of the protein embedded in the microsomal membrane. In addition, Evans et al. (1991) described a trypsin cleavage site between lysine-257 and glutamic acid-380 of chick haem oxygenase. These and other results just described indicate that haem oxygenase is quite susceptible to trypsin cleavage and suggest that a site near the C-terminus may be particularly prone to trypsin proteolysis. This lysine residue is fully conserved in chick, rat and human haem oxygenase (Shihahara et al., 1985; Yoshida et al., 1988; Evans et al., 1991).

In other experiments it was found that the markedly induced activity of haem oxygenase produced by exposure of chick-embryo liver cells to Cd²⁺ (Lincoln et al., 1988) was relatively unstable, with a 20–30% loss of homogenous activity after 30 min at 4 °C. The decrease was prevented by addition of soybean trypsin inhibitor. In contrast, such a loss of haem oxygenase activity was not seen in cell cultures treated with glutethimide and iron, which also induce the enzyme, albeit by a different mechanism (Lincoln et al., 1988; Bonkovsky et al., 1990; Cable et al., 1990), suggesting that Cd²⁺ treatment may selectively augment proteolytic-enzyme activities in cell homogenates. Because of these results, it is suggested that soybean trypsin inhibitor be routinely added to cell homogenates to prevent cleavage of the enzyme and possible loss of haem oxygenase activity, especially if possible effects of treatments on enzyme stability have not been studied.
Induction of haem oxygenase by glutethimide, iron and cadmium

Qualitative differences between haem oxygenase induction by drugs and metals could be delineated with Western blots. The Western blot in Fig. 3 illustrates an increase in the amount of haem oxygenase protein in cultures treated with glutethimide (lane 3) and ferric nitritolriacetate (lane 4), and the marked induction of the protein with the combination of the two (lane 5). The increases in the amount of protein were directly correlated with increases in haem oxygenase activity (see the legend to Fig. 3).

Similarly, enzyme induction by Cd^{2+} produced similar increases in both activities and amounts of protein (Table 2 and Fig. 5). Other recent results (Evans et al., 1991) indicate that Cd^{2+} treatment of chick-embryo liver-cell cultures results in increased levels of mRNA for haem oxygenase. This result extends those obtained in other experimental systems (Ishizawa et al., 1973; Shibahara et al., 1987; Alam et al., 1989). Thus one proximate mechanism for the increase in the amount of the enzyme is induction of message, probably leading to increased translation. The possibility that Fe^{2+}, Cd^{2+} or glutethimide treatment also increases the stability of haem oxygenase mRNA or protein requires investigation.

Quantification of haem oxygenase protein by slot-blot

The demonstration that a single protein was stained by the immunochemical procedures already described permitted development of a similar procedure for quantification of the amount of protein present in selected samples. The method involved use of slot-blots and laser-densitometric scanning. A standard curve was developed for each determination of haem oxygenase protein by slot-blot, and a linear relationship between protein amount (0–5 ng) per slot and area (arbitrary absorbance units) was consistently observed (results not shown). Appropriate dilution of unknown samples, such that their area readings fell within this range, allowed quantification of the amount of haem oxygenase protein in sonicated chick-embryo liver-cell cultures (Table 2).

Cross-reactivity of rabbit anti-(chick liver haem oxygenase) IgG with microsomal protein of other organs and species

The Western blot in Fig. 2(a) illustrates the cross-reaction of rabbit anti-chick haem oxygenase) IgG with haem oxygenase of microsomes from chick liver, spleen and testis (lanes 2, 3 and 4). There was also a slight degree of cross-reaction with haem oxygenase of chick brain (lane 5). In addition, the antibody cross-reacted with a protein in microsomes prepared from rat liver, spleen and testis (Fig. 2b, lanes 2–4). There was faint cross-reactivity with a protein of appropriate molecular mass from rat brain (Fig. 2b, lane 5), although the ability to detect haem oxygenase by Western blotting in this preparation was limited by the amount of protein (3 μg) that could be applied to the gel. However, use of slot-blots, in which more microsomal protein can be loaded, confirmed cross-reactivity of our antibody with haem oxygenase of both rat testis and brain (results not shown).

A strongly positive result was also found between the antibody and a 33 kDa protein from human spleen microsomes, but not from human liver microsomes (results not shown). The cross-reactivities observed are not surprising, considering the high degree of similarity in the primary amino acid sequence between chick and rat (62%), chick and human (62%) and rat and human (79%) haem oxygenase-1 (Evans et al., 1991; Shibahara et al., 1985; Yoshida et al., 1988). The lack of identifiable cross-reactivity with haem oxygenase from human liver is probably due to the small amount of the oxygenase protein present, relative to other proteins in human liver microsomes.

Cd^{2+} treatment led to increased testicular haem oxygenase in chicks (supporting the view that the 1 isoenzyme is present and inducible in chick testis), but to decreased activity in rats (results not shown). The latter has been ascribed to inhibition of NADPH:cytochrome reductase (Trakshel et al., 1986b), however, in our hands, activity did not return to the control level, even with addition of the reductase (results not shown). Cd^{2+} treatment failed to induce activity of the oxygenase (results not shown), extending previous results (Maines, 1988) and supporting the idea that the predominant form of brain haem oxygenase is an isoenzyme different from the inducible form which predominates in spleen and in liver after Cd^{2+} treatment.

In conclusion, we have developed monospecific antibodies in rabbit against a form of haem oxygenase from chicken liver which is similar to the haem oxygenase-1 isoenzyme described in mammals. The antibodies are useful for quantifying the amount of this protein in treated chick-embryo-cell culture, as well as for detecting its presence in the liver, spleen, testis and brain of chick and rat, and in human spleen.

We thank B. Donahue for help in performing laser densitometry and M. D. Maines for performing the Western blots of chick microsomal proteins with antisera against rat haem oxygenase-2. We thank S. Burrage and D. A. Thayer for assistance in typing the manuscript. This work was supported by research grants from the National Institutes of Health (DK 38825), Miles Laboratories, and the American Porphyria Foundation.

REFERENCES
