Determination of Heme Oxygenase Activity in Murine Macrophages for Studying Oxidative Stress Inhibitors

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Heme oxygenase (HO)2 (EC 1.14.99.3) is the microsomal enzyme which degrades heme, producing biliverdin IXα which is further reduced to bilirubin by biliverdin reductase (1). In mammalian cells, HO induction is a universal response to oxidative stress (2) and HO mRNA measurement was established as a sensitive marker of oxidative stress. Since its induction is inhibited by antioxidants, HO induction can also be used to measure a free radical-scavenging activity (3).

HO activity can be quantified spectrophotometrically measuring bilirubin production by tissue or cellular microsomal fractions (4, 5). Such methods are adequate for tissue analysis but they are relatively inconvenient for in vitro work as they require relatively large quantities of cells. A more sensitive assay using gas chromatography was established to quantify the carbon monoxide production due to the HO activity of cellular cytosolic fractions (6), while another recent technique is based on the detection of [14C]bilirubin formation by cellular microsomal fractions, using [14C]heme as a substrate (7). Although both methods are sensitive, they require certain skills and access to equipment that is not standard in many laboratories. This and the necessity of making kinetic assays in order to investigate the effects of different oxidative stress inhibitors upon HO induction made us develop an assay based on the quantitation of the bilirubin excreted into the culture medium. As HO is strongly induced in macrophages after erythrophagocytosis or exposure to hemin which also induces a stress-like effect (5), we chose to use macrophage cultures as an in vitro cell system to establish this new HO assay. The addition of antioxidants to the system should decrease HO induction according to their free radical scavenging effect, so this simple in vitro cellular system should allow the screening of antioxidant agents.

Materials and Methods. Benzene was purchased from Fluka (Buchs, Germany), and BaCl2·2H2O from Prolabo (Paris, France). Protoporphyrin IX zinc (II) was from Aldrich (St. Quentin Fallavier, France) while bilirubin, dimethyl sulfoxide (DMSO), hemin (Hm), and Trizma base were purchased from Sigma (St. Quentin Fallavier, France). Porphyrin solutions were prepared as follows: the porphyrin and Trizma base (in a 1:10 molar ratio) were dissolved in a minimal volume of 1 M NaOH, then neutralized with 1 M HCl; finally, deionized water was added to make a 10 mM final concentration. This stock solution was aliquoted and kept frozen at −20°C. Its addition to the cell culture did not significantly influence the pH of the culture medium.

BALB/c bone marrow-derived murine macrophages (BMMφ) were obtained according to the method established by Munder et al. (8) by the culture of bone marrow cells in hydrophobic Teflon bags (a kind gift of Dr. Manuel Modolell from the Max-Planck Institut für Immunobiologie from Freiburg-im-Breisgau, Germany). After 12 days of culture, BMMφ were collected and distributed in 6-well cell culture plates (purchased from Nunc, Roskilde, Denmark) at a concentration of 2 × 105 cells/well/2 ml culture medium. The culture medium was RPMI 1640 (from Gibco, Life Technologies, Eragny, France) containing 10% fetal calf serum (FCS, decomplemented 30 min at 56°C), 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin.

HO activity was measured as follows: after plating, BMMφ were left overnight to adhere, and then the porphyrins were added. All further manipulations were carried out in dim light and the plates were protected against light by wrapping in aluminium paper. The plates were incubated for different periods of time, then 0.5 ml of each culture supernatant was collected and 250 mg BaCl2·2H2O/probe was added. After vitamin (10–15 s), 0.75 ml benzene was added;

References

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2. Abbreviations used: HO, heme oxygenase; DMSO, dimethyl sulfoxide; Hm, hemin; BMMφ, bone marrow-derived murine macrophages; FCS, fetal calf serum.


then, tubes were vortexed vigorously (50–60 s) leading to the formation of a relatively stable milky-white emulsion. After centrifugation (13,000 g, 30 min, without cooling), the upper benzene layer was collected and the absorbance at 450 nm with reference wavelength at 600 nm was measured using a Shimadzu UV-160A spectrophotometer. In a separate tube, 0.5 ml of fresh culture medium was processed in the same way and the benzene layer was collected and used as a blank. The quantity of bilirubin produced was calculated using a molar extinction coefficient of bilirubin dissolved in benzene \( \varepsilon_{450} = 27.3 \text{ mM}^{-1} \text{ cm}^{-1} \).

Results and discussion. Bilirubin can be completely extracted from cell culture medium with benzene in a single step. Bilirubin was dissolved in DMSO (stock solution, 10 mM), diluted in RPMI 1640 + 10% FCS at concentrations close to those observed in activated macrophage cultures (5 \( \mu \text{M} \)), and then extracted with benzene (Fig. 1). Complete extraction needed supersaturating concentrations of \( \text{BaCl}_2 \) and was optimal, in this range of concentrations, for a ratio of 1:3 for culture supernatant:benzene. The absorbance at 450 nm of the bilirubin dissolved in benzene is proportional to the concentration of bilirubin for the range of concentrations susceptible to be found in cell culture supernatants. We also found that bilirubin concentration is relatively stable in the culture medium and that it is not taken up or further catabolized by BMM\( \phi \). Exogenous bilirubin was added in a BMM\( \phi \) culture; samples from the culture supernatant were collected immediately after addition and after 24 and 48 h of incubation at 37°C in an atmosphere containing 5% \( \text{CO}_2 \). Bilirubin was extracted in benzene and its apparent decrease was calculated, showing that after 24 h of incubation, 87% of the initial quantity could be recovered.

Finally, we tested the HO assay in a BMM\( \phi \) culture. We observed a dose-dependent increase of bilirubin concentration in the culture medium related to the dose of hemin added to the BMM\( \phi \) (Fig. 2). We also measured the bilirubin content of the BMM\( \phi \) and observed that practically all the produced bilirubin was excreted. We also observed that a HO inhibitor, zinc (II) protoporphyrin IX, inhibits the production of bilirubin (Fig. 3) by BMM\( \phi \).

The assay of the HO activity that we describe here could be useful to measure the efficiency of free radical scavenging agents and could also be used for kinetic assays. The main advantage of this assay resides in the small number of cells which are necessary for every assay (allowing complex experiments and the use of slow-proliferating cells). This advantage results from the increase in sensitivity due to the benzene extrac-

![Fig. 1. Bilirubin extraction with benzene in the presence of supersaturating concentrations of \( \text{BaCl}_2 \) (column 1, 5 \( \mu \text{mol/liter} \) bilirubin dissolved in culture medium; column 2, medium alone).](image1)

![Fig. 2. Bilirubin production by BMM\( \phi \) incubated for 24 h in the presence of different concentrations of hemin.](image2)

![Fig. 3. Inhibition of bilirubin production by BMM\( \phi \) cultured in the presence of hemin (100 \( \mu \text{M} \)) by ZPP (for a 24-h incubation time).](image3)
tion and to the higher amount of detectable bilirubin due to the longer incubation time.

Other authors extracted the HO-dependently produced bilirubin in chloroform before spectrophotometry (9) to increase sensitivity, but it seems that sometimes chloroform extraction does not succeed in a quantitative extraction of bilirubin (7, 10). We chose to modify a method used previously to assay nonconjugated bilirubin in the serum (11). That method used nonsaturating barium chloride concentrations and several successive extractions of bilirubin in benzene. We induced the formation of an emulsion by adding much larger quantities of barium chloride, which allowed the use of a single-step extraction.

The linearity of the calibration curve in a region which largely encompasses the interval of cell culture excreted bilirubin values allows the concentration of the produced bilirubin to be calculated according to the absorption at 450 nm. In the case of cells having low HO activity or for shorter incubation periods, the sensitivity of this assay can be further increased by using more cells, by concentrating the supernatant before extraction or by decreasing the ratio between benzene and the culture supernatant.

In the case of very high level induction of HO, the activity of biliverdin reductase might theoretically become limiting for the generation of bilirubin. In vivo, such a situation is not found in rodents or humans which do not excrete biliverdin even when they must degrade high amounts of heme (as in hemolytic anemias). In the case of the BMMφ culture that we use, if some biliverdin were excreted, it would be measured as well given its absorption at 450 nm, which is close to that of bilirubin.

Therefore, this new method of HO activity determination in cell cultures represents a good alternative to other assays due to its simplicity and sensitivity, and the possibility of using it for kinetic experiments.

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