The presence of heme oxygenase and NADPH cytochrome P-450 (c) reductase, the latter an integral component of heme oxygenase and cytochrome P-450-dependent drug metabolizing enzymes, was demonstrated in human corneal epithelium. We reported for the first time that human corneal epithelium contains heme oxygenase activity as high as 20% of that reported for the human liver. Using immunological techniques, we demonstrated that heme oxygenase proteins from human cornea and liver are very similar; both have a molecular weight of 32,000 as demonstrated by Western blot analysis. We also studied the presence of NADPH cytochrome P-450 (c) reductase. The human corneal epithelium contains significant amount of NADPH cytochrome P-450 (c) reductase activity, and this corneal protein is similar to the known liver protein; both have a molecular weight of 71,000 and react with antibodies prepared against purified liver NADPH cytochrome P-450 (c) reductase. As the heme oxygenase system is the rate limiting step in heme degradation, this system plays a pivotal role in regulation of cellular heme in corneal epithelium, thus modulating the activity of hemoproteins such as catalase, tryptophan pyrrolase and thromboxane synthetase. Invest Ophthalmol Vis Sci 28:1464-1472, 1987

Most topically applied drugs are well tolerated by the corneal epithelium despite the locally high concentrations reached by this route of delivery. Systemic drugs such as chloropromazine hydrochloride and chloroquine used chronically are often seen deposited in the corneal epithelium without apparent toxicity to these cells. These observations suggest to us that the corneal epithelium is protected by drug metabolizing systems as are other tissues throughout the body.

The cytochrome P-450 system provides a major pathway for the metabolism of numerous exogenous and endogenous drug substrates. It is composed of the hemoprotein cytochrome P-450, NADPH cytochrome P-450 (c) reductase and a phospholipid component. Cytochrome P-450, as the terminal acceptor in the NADPH-dependent-mixed function oxidase system, exists as a family of isozymes with varying specifications, some of which may be detected only after induction with a specific drug. Although the primary site of drug metabolism is the liver, cytochrome P-450 has been described in extrahepatic tissues such as lung, kidney, heart, bone marrow, blood vessels and intestine. Cytochrome P-450 and its specific activity as aryl hydrocarbon hydroxylase have been demonstrated in bovine and murine cornea, ciliary body, retinal pigment epithelium, lens epithelium and retina.

The level of cytochrome P-450 and other hemoproteins, such as catalase, tryptophan pyrrolase and thromboxane synthetase, are regulated by the availability of microsomal heme. Cellular heme, generally, is regulated by the level of heme oxygenase (EC 1.14.99.3), the rate limiting enzyme in heme degradation. The heme oxygenase system, which consists of heme oxygenase and NADPH cytochrome P-450 (c) reductase, catalyzes the oxidative degradation of protoheme (Fe-protoporphyrin IX) to biliverdin IX. The highest activity of this enzyme is found in the spleen, but it is also present in the kidney, brain, macrophages and heart. Although the enzyme is normally present in liver and other tissues in very low levels, the activity of heme oxygenase can be elevated...
by the actions of various inducers such as chemicals, drugs and heavy metals.\textsuperscript{19-21} Induction of heme oxygenase is accompanied by perturbation of heme metabolism, enhanced degradation of cytochrome P-450 and the associated depression of monooxygenase activities which are dependent on this hemoprotein.\textsuperscript{2,22} Up to date, there is no information available on the presence and role of heme oxygenase in the eye. A knowledge of the regulation of heme catabolism in the ocular tissues is essential to an understanding of the control of important hemoproteins such as the cytochrome P-450 system and, ultimately, understanding of the physiology and pathophysiology of drug metabolism in ocular tissue.

The aim of this study was to identify the presence of heme oxygenase and NADPH cytochrome P-450 (c) reductase (a mutual component of cytochrome P-450 and heme oxygenase systems) in the human corneal epithelium. The significance of the heme oxygenase and cytochrome P-450 systems for the function of the cornea is discussed.

Materials and Methods

Reagents

$\beta$-nicotinamide adenine dinucleotide phosphate (NADP$^+$), glucose 6-phosphate, bovine serum albumin, protein markers for molecular weight determination, phenylmethyl-sulfonyl fluoride (PMSF), and cholic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Cholate was recrystallized in 50% ethanol using activated charcoal and celite (in the first step), dried in vacuo over silica, made up to a 20% solution (w/v), adjusted to pH 7.5 with NaOH, and filtered through Whatman paper. Acrylamide, sodium dodecyl-sulfate (SDS), bisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), Coomassie blue R-250, Biobeeds SM-2, and glycine were obtained from Bio-Rad Labs (Richmond, CA). Glucose 6-phosphate dehydrogenase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). DEAE-cellulose (DE 52) was obtained from Whatman (Clifton, NJ). Sephadex G-100, 2'-AMP and 2',5'-ADP Sepharose were purchased from P.L. Biochemicals (Piscataway, NJ). 125I-protein A was obtained from NEN Research Products (Boston, MA). Hipure liquid gelatin was from Norlan Products, Inc. (New Brunswick, NJ). All other chemicals, of highest quality, were obtained either from Sigma or Fisher Scientific (Springfield, NJ).

Preparation of Corneal Tissues

Human eyes or cornea, unsuitable for transplantation, were obtained from the Eye Bank for Sight Restoration, Inc., New York, after 1–2 days storage in organ culture media that preserved their viability. The eyes or cornea were washed twice with 0.9% saline and the corneal epithelium was scraped off gently with a dull razor blade. The tissues were placed in cold phosphate buffered saline (PBS), pH 7.4, containing 0.4 mM PMSF and then homogenized by using a glass tissue grinder operated at low speed with a Con-Torque power unit (Eberbach, Ann Arbor, MI). The homogenate was from six to eight cornea and was used for the enzyme assays.

Fresh bovine eyes were obtained from a local abattoir. They were collected within 10 min after slaughtering and immediately immersed in an ice-chilled saline solution and brought to the laboratory on ice within 1–2 hr. The eyes were washed twice with 0.9% saline and the corneal epithelium was gently scraped off into PBS and homogenized as described previously. The homogenate was centrifuged at 1500 g for 10 min and the supernate was centrifuged at 10,000 g for 20 min. The 10,000 g supernate was further centrifuged at 105,000 g for 90 min and the resulting microsomal pellet was resuspended in PBS, pH 7.4.

Purification of Human Liver NADPH Cytochrome P-450 (c) Reductase and Heme Oxygenase

Human liver was obtained from the laboratories and research department of the Westchester County Medical Center (Valhalla, NY) by autopsy a few hours after death. Approximately 200–300 g liver was homogenized in 100 mM Tris-HCl buffer, pH 7.6 containing 150 mM NaCl, 0.4 mM PMSF and 250 mM sucrose. Microsomes were prepared by successive centrifugation steps as described previously.\textsuperscript{23} The purification steps used to obtain heme oxygenase and cytochrome P-450 (c) reductase are schematically summarized in Figure 1 and are as follows: the microsomal pellet was washed with 100 mM sodium pyrophosphate, pH 7.5, to eliminate hemoglobin. Washed microsomes were resuspended in 20 mM Tris-HCl, pH 7.7 containing 0.4 mM PMSF and 0.5 mM EDTA to a protein concentration of 2–5 mg/ml. Triton X-100 and sodium cholate were added to the microsomal suspension to a final concentration of 2.0% and 1.0%, respectively. The suspension was gently stirred for 30 min and then centrifuged at 105,000 g for 90 min. The solubilized microsomes were loaded onto a column (0.4 × 50 cm) of DEAE-cellulose pre-equilibrated with 20 mM Tris-HCl, pH 7.7 containing 20% glycerol, 0.5 mM EDTA, 0.5% Triton X-100 and 0.5% sodium cholate. The DEAE-cellulose was washed with 2 liters of this buffer. The enzymes were then eluted with 2 liters of the same buffer in which the KCl concentration was increased.
by a concave gradient (R₁ : R₂ = 1:3), from 0 to 0.5 M KCl at a flow rate of 140 ml/hr. Fractions were collected in 11 ml aliquots. Heme oxygenase activity was eluted at 0.12 M KCl (Fraction A). Fractions between 0.2-0.5 M KCl contained most of the activity of the NADPH cytochrome P-450 (c) reductase (Fraction B). Fraction A was used for further heme oxygenase purification as described previously. Fraction B was concentrated in an Amicon (Danvers, MA) ultrafiltration cell, dialyzed in 20 volumes of 30 mM potassium phosphate buffer, pH 7.7 containing 0.1 mM sodium cholate (buffer C) for 10 hr and used for further purification as described by Yasukochi and Masters. The concentrated fraction B was then applied to a 2',5'-ADP-Sepharose 4B column (1.7 X 13 cm), previously equilibrated with 10 mM potassium phosphate buffer, pH 7.7 containing 0.2% Triton X-100, 0.2% sodium cholate, 0.05 mM EDTA and 0.2 mM dithiothreitol. The column was washed with 250 ml of 200 mM potassium phosphate buffer, pH 7.7 containing 20% glycerol, 0.1% sodium cholate, 0.4 ml EDTA and 0.1 mM dithiothreitol, and then 100 ml of buffer C. The column was then eluted with 50 ml of buffer C without cholate but containing 5 mM 2'-AMP and 0.4 mM PMSF. Those fractions with reductase activity were pooled and concentrated in an Amicon ultrafiltration cell, passed through a Sephadex G-100 column (2.2 X 40 cm) to remove unbound 2'-AMP and stored in small aliquots at -70°C. This fraction had a specific activity of NADPH cytochrome P-450 (c) reductase of 46.6 unit/min as measured by the assay described below.

**Preparation of Antibodies to NADPH Cytochrome P-450 (c) Reductase and Heme Oxygenase**

Antibodies were raised in male New Zealand white rabbits. The rabbits were housed individually and fed regular chow ad libitum. The use of the rabbit conform to the ARVO Resolution on the Use of Animals in Research. Pure antigen was always mixed 1:1 (v/v) with Freund's complete adjuvant before injection. Rabbits were initially injected subcutaneously with 50 µg of antigen. After 3 weeks the rabbits were re-injected with 25 µg of antigen. Five weeks later, animals were given a second booster injection of 12.5 µg of antigen. Seven days after the final booster injection, blood was withdrawn from the marginal ear vein and allowed to clot at room temperature for 30 min. The clot was broken, left at 4°C overnight and then centrifuged at 16,000 g for 15 min at 4°C. Supernatant solution containing the antiserum was de-complemented by heating for 20 min at 56°C. The serum (100 ml) was brought to 40% saturation with solid ammonium sulfate; the precipitate was collected by centrifugation and dissolved in 50 ml of 0.05 M potassium sulfate, pH 7.6. This step was repeated twice as described by Thomas et al. The precipitate was dissolved in 0.02 M potassium sulfate buffer, pH 8.0, and dialyzed overnight against ten volumes of the same buffer. This preparation was applied onto a DEAE-cellulose column and equilibrated with 0.02 M potassium phosphate buffer, pH 8.0; the protein peak eluted in the column volume was collected and concentrated by Amicon ultrafiltration using a PM-50 membrane. Control antibody was purified in an identical manner using sera from preimmunized rabbits. The final protein concentration of the antibody preparation was 6 mg/ml.

**Dot Blotting**

The method described by Jahn et al was used with some modifications. Nitrocellulose membranes (Millipore (Bedford, MA), Type HA, 0.45 µm) were washed for 5 min in PBS, pH 7.4, blotted on filter paper to remove excess PBS and placed in a Bio-Dot microfiltration apparatus (Bio-Rad). Samples were serially diluted in PBS and filtered through the membrane by gravity. Minimal loading volume was 20 µl. Each well was then rinsed with PBS at a volume two-fold that of the corresponding sample. The rinse was filtered through by vacuum. Membranes were re-
moved from the apparatus, air-dried, and fixed for 15 min in 10% acetic acid/25% isopropanol. After several washes in distilled water and one in PBS, the membranes were incubated in the following solutions in order: (1) blocking solution consisting of 3% Hi-pure liquid gelatin in PBS containing 0.02% sodium azide; (2) antibody preparations diluted 1:1000 in the blocking solution; and (3) $^{125}$I-protein A (10$^6$ cpm) resuspended in the blocking solution. Incubation in each solution was for 1 hr at room temperature with shaking (50 rpm). After incubation with both the antibody and protein A, the membranes were washed first in PBS, then in PBS containing 1% Triton X-100, and finally in PBS. Washes were for 10 min each at room temperature with shaking (100 rpm). The membranes were air-dried and subjected to autoradiography at $-80^\circ$C with Cronex intensifying screens (Dupont, Wilmington, DE) for 11 to 18 hr. After autoradiography, each dot was excised and measured for radioactivity in a gamma scintillation counter.

**Western Blotting**

Western blotting was performed according to Burnett. One-dimensional SDS-polyacrylamide gel electrophoresis was performed according to standard procedure. Proteins were transferred to nitrocellulose in a Bio-Rad Trans-Blot cell. The blotting was at 60 v, 0.21-0.23 A for 5 hr in a solution containing 25 mM Tris, 192 mM glycine, and 20% methanol. Immunodetection was identical to that described above for dot blotting.

**Measurement of Heme Oxygenase Activity**

Human and bovine corneal heme oxygenases were determined as described previously in our laboratory. Briefly, the enzyme activity was measured in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.1% Tween 80 (Sigma). Bilirubin formation was determined, in an Aminco (Urbana, IL) DW-2C spectrophotometer, by using the difference in absorption from 460 to 530 nm and an extinction coefficient of 43.5 cm$^{-1}$. Blanks for the assay were prepared by adding HgCl$_2$ to a final concentration of 4 mM in the complete assay mixture.

**Assay of NADPH Cytochrome P-450 (c) Reductase**

The reaction mixture for cytochrome C reduction contained 0.3 M potassium phosphate buffer, pH 7.7, 0.1 $\mu$mol NADPH, purified NADPH cytochrome P-450 (c) reductase (0.5 $\mu$g) and the acceptor cytochrome C in a final volume of 1 ml. The reaction was carried out at 30°C by following the reduction of cytochrome C at 550 nm (extinction coefficient of 21 mM$^{-1}$ cm$^{-1}$) on SLM/Aminco DW-2C dual beam spectrophotometer.

**Protein Determination**

Protein concentration was determined by the method of Lowry using bovine serum albumin as the standard.

**Results**

Heme oxygenase and NADPH cytochrome P-450 (c) reductase activities were measured in human and bovine corneal preparations and compared to the levels found in human liver microsomes. As seen in Table 1, the activity of human corneal heme oxygenase is relatively high, 20% of that found in the human liver. Similarly, the activity of NADPH cytochrome P-450 (c) reductase is detectable in the human corneal epithelium although the basal activity was 16 nmol/mg/min compared to 340 nmol/mg/min observed in human liver microsomes. However, the activities of these two enzymes in the bovine corneal epithelium are low, 40–50% of those found in human corneal epithelium. Thus, the human corneal epithelium possesses significantly high activities of heme oxygenase and NADPH cytochrome P-450 (c) reductase.

In order to confirm that the corneal heme degradation and cytochrome P-450 (c) reduction are catalyzed by heme oxygenase and NADPH cytochrome P-450 (c) reductase, respectively, we used immunological techniques. These techniques allowed us to further characterize the molecular size, homology and cross-reactivity to known enzyme systems such as those of the human liver. Furthermore, the sensitivity and specificity of these immunological approaches appear to be useful for enzyme characteriza-
Fig. 2. Dot-blot assay for heme oxygenase in human and bovine corneal epithelium. Dot blot was done as described in Methods. A: bovine corneal microsomes, 0.31–0.80 μg protein; B: human corneal epithelial homogenate, 0.048–12.2 μg protein; C: bovine corneal homogenate, 0.52–134 μg protein.

When a limited amount of tissue is available, such as in human cornea, Figure 2 shows a dot blotting screen for heme oxygenase proteins in bovine corneal epithelial homogenate and microsomes as compared to the homogenate of human corneal epithelium. It can easily be seen that the human corneal epithelium contains much more heme oxygenase protein than either bovine corneal epithelial homogenate or microsomes. Antibody binding was clearly demonstrated in human corneal homogenate containing as little as 0.19 μg of protein. This level of sensitivity is comparable to that found in human liver microsomes where the minimal protein was 0.16 μg (data not shown). We also included specimens of bovine origin in the test. In dot-blot titration assay, immunoreactive substances could be detected in bovine corneal epithelial homogenate containing 4.19 μg protein. The microsomal fraction, where heme oxygenase activity is located, contained higher amounts of the immunoreactive substances than the homogenate, and antibody binding was detected in 1.25 μg of protein.

To further quantitate the binding of anti-hepatic heme oxygenase to proteins in the corneal epithelial preparations, we measured radioactivity bound to each sample and examined the relationship between the binding and the amount of tissue protein used. As shown in Figure 3, when 5 μg of proteins were tested, the human preparation had 8.9 times as much radioactivity bound as the bovine preparation. When 10 μg of proteins were tested, there was a 7.5-fold difference in the amount of heme oxygenase between the two preparations. The microsomal fraction of bovine corneal epithelium contained, per amount of protein, 1.6 to 1.8 times as much immunoreactive substances as the crude homogenate. In general, radioactivity bound increased with the amount of protein used. The binding in the bovine corneal homogenate reached a plateau at 35 μg of protein.

The immunoreactive proteins in the corneal samples were further characterized by Western analysis. As seen in Figure 4, antibodies to human liver heme oxygenase reacted with both bovine and human corneal epithelial proteins. In all tissues tested a single immunoreactive species was observed which had the same electrophoretic mobility as the purified liver enzyme corresponding to a molecular weight of approximately 32,000; thus a homology exists between hepatic and corneal heme oxygenase.

We also used the same immunodetection systems to assay for another key enzyme in the heme oxygenase system, NADPH cytochrome P-450 (c) reductase. The levels of sensitivity observed were similar to or higher than those seen for heme oxygenase: 0.19 μg of protein for the human preparation, 0.625 μg for the microsomal fraction and 2.1 μg for the homogenate of the bovine corneal epithelium (Fig. 5). Proportionality similar to that described for heme oxygenase also existed between the binding of anti-NADPH cytochrome P-450 (c) reductase antibody and the amount of tissue protein used (Fig. 6). Radioactivity bound was higher in the human preparation than in the bovine preparation for the same amounts of protein: 6.3-fold difference for 8 μg of protein and 4.8-fold difference for 3 μg of protein. The microsomal

Fig. 3. Immunotitration of heme oxygenase in human and bovine corneal epithelium. Radioactivity bound in each dot was measured as described in Methods; the results are from a representative experiment. The same qualitative results were obtained in two more experiments.
fraction of the bovine corneal epithelium, where NADPH cytochrome P-450 (c) reductase is located, contained 1.8 times as much immunoreactive substance per amount of protein as the crude homogenate.

The specificity of the anti-NADPH cytochrome P-450 (c) reductase antibody binding was demonstrated in the Western blotting assay. When the homogenates of both human and bovine cornea were analyzed, only one band appeared on the autoradiogram corresponding to a protein the size of that of NADPH cytochrome P-450 (c) reductase, i.e., 71,000 dalton (Fig. 7). This indicates the presence of a homology between hepatic and corneal enzymes.

Discussion

Our results indicate that heme oxygenase and NADPH cytochrome P-450 (c) reductase are present both in human and bovine corneal epithelium. We demonstrated, using immunochemical quantitations, that the level of heme oxygenase protein is 8–10-fold higher in human corneal epithelium when compared to bovine corneal epithelium; moreover, the human corneal epithelium demonstrated a significant heme oxygenase activity which is one-fifth that of human liver and/or rabbit kidney. Both human and bovine corneal epithelial heme oxygenase showed a homology to human liver heme oxygenase.

Similarly, NADPH cytochrome P-450 (c) reductase levels were found to correlate with heme oxygenase levels. Although human corneal epithelium exhibited a low activity of NADPH cytochrome P-450 (c) reductase (Table 1) and is only 5% of that found in human liver, using immunochemical techniques we were able to demonstrate that human corneal epithelium possesses a level of the reductase 5–6-fold higher than bovine corneal epithelium. The low activity of NADPH cytochrome P-450 (c) reductase in human cornea may be due to its susceptibility to proteases, as has been shown in other human tissues. Bovine corneal epithelial NADPH cytochrome P-450 (c) reductase activity is only 50% lower than that found in bovine ciliary bodies, which have the highest levels of drug metabolizing enzymes. Again, both human
and bovine corneal epithelial NADPH cytochrome P-450 (c) reductase showed a homology with human hepatic NADPH cytochrome P-450 (c) reductase as demonstrated by the Western blot technique (Fig. 7).

These results, and our previous report of cytochrome P-450 and aryl hydrocarbon hydroxylase activity in corneal epithelium and other ocular tissues, demonstrate that the major components of the hemoprotein regulatory heme oxygenase system and the drug metabolizing cytochrome P-450 pathway both exist in human corneal epithelium. The significance of these findings is that they provide a basis for drug detoxification in corneal epithelium. An example of this would be the glucocorticoids. The corneal epithelium is a target tissue for glucocorticoids and their metabolism is known to be cytochrome P-450-dependent.

Our findings may have additional significance. In addition to cytochrome P-450, heme oxygenase also determines the activity of catalase (H₂O₂ → H₂O + O₂). The catalytic activity of catalase is of the utmost importance in protecting cells in which H₂O₂ concentrations exceed physiologic limits. Catalase is found in the corneal epithelium as well as in most other ocular tissues. Hydrogen peroxide accumulates in the humor, causes oxidative damage to cells and contributes to cataract formation. High heme oxygenase activity may reduce catalase activity in corneal epithelium and therefore lessen the possible oxidative damage and cataract formation. Activity of the hepatic oxygenase has been shown to increase with age; ocular tissues may not differ in this respect.

Recently, arachidonic acid has been found to be metabolized via a cytochrome P-450 monoxygenase-dependent pathway to a novel series of metabolites, the biological activities of which have just begun to be understood. This third pathway of arachidonic acid metabolism has been identified in corneal epithelium and we have demonstrated, in preliminary studies, that some of these metabolites are biologically active: one is a potent inhibitor of Na⁺-K⁺-ATPase, another is a vasodilator. As previously noted with catalase activity, levels of cytochrome P-450 are dependent upon heme oxygenase activity.

In view of these recent findings, studies which determine the physiological role and contribution of corneal cytochrome P-450 in the activation of endogenous compounds or drug detoxification may help elucidate the mechanism by which some pharmacological agents may manipulate corneal function. Furthermore, understanding of the role of heme oxygenase in ocular tissue may open a new avenue to the prevention of oxidative damage and cataract formation.

Key words: human cornea, heme oxygenase, cytochrome P-450, NADPH cytochrome P-450 (c) reductase, catalase.

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