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CYTOCHROME P450 DEPENDENT METABOLISM OF ARACHIDONIC ACID IN BOVINE CORNEAL EPITHELIUM

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SUMMARY: Microsomes prepared from bovine corneal epithelium metabolized ^{14}C-arachidonic acid into two unidentified products, separated by thin-layer chromatography and called Peaks I and II. Each peak was further separated by high performance liquid chromatography into two metabolites. The formation of these metabolites was dependent on the addition of NADPH and inhibited by carbon monoxide and SKF-525A, suggesting a cytochrome P450-dependent mechanism. The presence of cytochrome P450 in the corneal epithelium was assessed directly by measurement of the carbon monoxide reduced spectrum and indirectly by measuring aryl hydrocarbon hydroxylase activity. The activity of aryl hydrocarbon hydroxylase was protein-and NADPH-dependent and was inhibited by SKF-525A. © 1985 Academic Press, Inc.

Cytochrome P450 consists of a family of isozymes which are active in the oxidation of numerous drugs, xenobiotics and endogenous compounds (1). Among the latter group cytochrome P450(s) are involved in both the synthesis and metabolisms of various substrates including fatty acids, sex steroids, glucocorticoids, vitamin D, leukotrienes and prostaglandins.

Cytochrome P450-dependent monooxygenase represents the third pathway by which arachidonic acid (AA) can be metabolized in animal tissues (2). This pathway converts AA to several oxygenated metabolites: monohydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids which can undergo hydrolysis by epoxide

Abbreviations Used: AA, Arachidonic acid; HETE, Hydroxyeicosatetraenoic acid; TALH, Thick ascending limb of Henle's loop; HPLC, High performance liquid chromatography; TLC, Thin layer chromatography; AHH, Aryl hydrocarbon hydroxylase; BP, Benzo(a)pyrene.

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hydrolase to form the diol derivatives, and w, w-1 and w-2 hydroxylation products (3-6). The formation of these metabolites is strictly dependent on NADPH and molecular oxygen and is inhibited by carbon monoxide and SKF-525A (2). Capdevila et al. (7) have shown that the epoxides of AA are potent stimuli of peptide hormone release. The 5,6 epoxyeicosatrienoic acid has been shown to inhibit chloride transport in the collecting tubules (8). Recently, we have reported that in epithelial cells isolated from the thick ascending limb of Henle's loop (TALH) of rabbit kidney, the nephron segment that is important for establishing the solute gradient for urinary concentration, the major pathway by which AA is metabolized, is via an NADPH cytochrome P450-dependent enzyme (9). TALH cells convert AA to two biologically active compounds: one inhibits Na^+-K^+-ATPase activity and the other relaxes blood vessels (10).

AA Metabolism via the cyclooxygenase and lipoxygenase pathways has been demonstrated in ocular tissues (11,12,13). Shaeffer et al. (14) have shown that corneal homogenates are capable of producing both cycloxygenase and lipoxygenase metabolites. Bazan et al. (15) have shown that these two enzyme systems are present in all layers of the cornea and that the formation of prostaglandins and HETEs are stimulated after corneal injury. AA metabolites of lipoxygenase and cyclooxygenase have been implicated in ion transport mechanisms and inflammatory process in the eye (16,17).

The epithelial cell layers of the cornea can be characterized as a "tight" ion transporting functional syncytium. Transport properties of the corneal epithelium are similar to those of the epithelial cells of the TALH as both transporting epithelia possess active chloride transport coupled to Na^+-K^+-activated-ATPase (18). The observations that cytochrome P450-dependent AA metabolites may act as a modulator(s) of transport processes, and the similarity of the corneal epithelium to TALH as regards ion transport mechanism, led us to investigate the

possibility that cytochrome P450-dependent AA metabolism exists in the corneal epithelium. To our knowledge, the retina is the only ocular tissue that has been reported to possess cytochrome P450 monooxygenase system (19). Therefore, our present experiments were designed to study the presence of cytochrome P450 in the cornea and the metabolism of AA via this system.

METHODS AND MATERIALS

Preparation of corneal microsome

The corneal epithelium of enucleated calf eyes was scraped off with a razor blade. The tissues were placed in cold phosphate buffered saline (PBS), pH 7.4, and homogenized. The homogenate was centrifuged at 1500 x g for 10 minutes and the supernate was centrifuged at 10,000 x g for 20 minutes. The 10,000 x g supernate was further centrifuged at 100,000 x g for 60 minutes and the resulting microsomal pellet was resuspended in PBS, pH 7.4.

Arachidonic Acid Metabolism

Microsomal suspensions (1-3 mg protein) were incubated with ^{14}C-AA (7 μM) with or without NADPH-generating system which is composed of: glucose 6-phosphate, 0.1 mM; NADP+ 0.4 μM; glucose 6-phosphate dehydrogenase, 1 unit, in a total volume of 1 ml for 30 minutes at 37 C. In experiments using inhibitors, agents were preincubated with microsomes for 10 minutes before the addition of NADPH-generating system and ^{14}C-AA. ETYA was dissolved in ethanol (final ethanol concentration was less than 0.1%). Indomethacin and BW-755C were dissolved with sodium bicarbonate. SKF-525A was dissolved in water and diluted with PBS. Carbon monoxide was bubbled into the microsomal suspension for 1 minute before additions of NADPH-generating system and ^{14}C-AA. The reaction was terminated by acidification to pH 3.5-4.0, and the AA metabolites were extracted with ethylacetate. Extraction efficiency was 60-70%. The final extracts were subjected to thin layer chromatography (TLC) for separation of AA and oxygenated metabolites using the upper phase of ethylacetate: iso-octane: acetic acid: water (110:50:20:100). The TLC plates were scanned on a Packard model 7230 radiochromatogram scanner. Radioactive zones were visualized by autoradiography, cut and counted in a Searle model 6880 Mark III liquid scintillation counter.

High performance liquid chromatography (HPLC)

HPLC was used to separate oxygenated metabolites of ^{14}C-AA. Reverse-phase HPLC was performed on C_{18} microsorb column (250 x 4.6 mm, Rainin Instrument Co. Inc. MA) using linear gradient of 1.25%/min from acetonitrile: water: acetic acid (50:50:0.1) to acetonitrile: acetic acid (100:0.1) at a flow rate of 1 ml/min. Radioactivity was monitored by a flow detector (Radiomatic Instrument and Chemical Co. Inc., Tampa, FL).

Protein determination

Protein concentration was determined by the method of Lowry et al. (20) using bovine serum albumin as the standard.

Cytochrome P450 content

Cytochrome P450 was determined by dithionite difference carbon monoxide spectra (21) after addition of freshly prepared NADH at a final concentration of 1 mM to both sample and reference cuvettes. The method was chosen in order to correct for hemoglobin and mitochondrial cytochrome aa_3 contamination. Both cytochrome aa_3 and hemoglobin bound to carbon monoxide are present in the same amounts in both sample and reference cuvettes. When the difference spectrum is taken, there is no contribution from cytochrome aa_3 - carbon monoxide or hemoglobin. Treatment with sodium dithionite required for reducing cytochrome P450 was carefully controlled since excess dithionite appears to cause microsomal degradation with consequent loss of spectrally detectable cytochrome. Although all these precautions were taken, a large peak at 427 nm still appeared. The absorbance difference between 450 and 490 nm was used to calculate the cytochrome P450 content using a molar extinction coefficient of 91 $\mu M^{-1} cm^{-1}$ (21).

Aryl Hydrocarbon hydroxylase (AHH) activity

The AHH assay was determined by using benzo(a)pyrene (BP) as the substrate according to the method of Nebert and Gelboin (22) as modified by Abraham et al. (23). The fluorescence was quantitated by comparison with the standard curve obtained with various dilutions of 3-OH BP (kindly provided as a gift of Dr. Croci Tiziano of the American Health Foundation, Valhalla, NY).

Materials

Bovine serum albumin (fraction V) glucose 6-phosphate, NADPH, NADP, NADH and BP were obtained from Sigma Chemical Co., St. Louis, MO. Glucose 6-phosphate dehyrogenase was purchased from Boehringer Biochemicals. Metyrapone was from Aldrich Chemical Co. Inc. (Milwaukee, WI), SKF-525A was a gift from Smith Kline and French (Philadelphia, PA). [1 $^{-14}$C] - AA (specific activity of 56 mci/mmol) was obtained from Amersham. Arachidonic acid was from Nu-Chek Prep, Inc.

RESULTS

Incubation of the microsomes (1-3 mg protein, about 5 cornea) with ^{14}C-AA (7 μM) for 30 min resulted in the formation of several radioactive metabolities; 5-7% of the total radioactivity was present as prostaglandins (6K-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGE_2), and about 7-10% of the total radioactivity was present in a non-polar peak having an Rf value similar to 5-HETE standard (Fig. 1). Addition of NADPH or an NADPH-generating system to the incubation medium increased the conversion of ^{14}C-AA by more than 4-fold without affecting prostaglandin formation. ^{14}C-AA was converted in approximately 60% yield to two radioactive peaks, I and II (Fig. 1). The formation of peaks I and II was not affected by indomethacin (10-20 μM), a

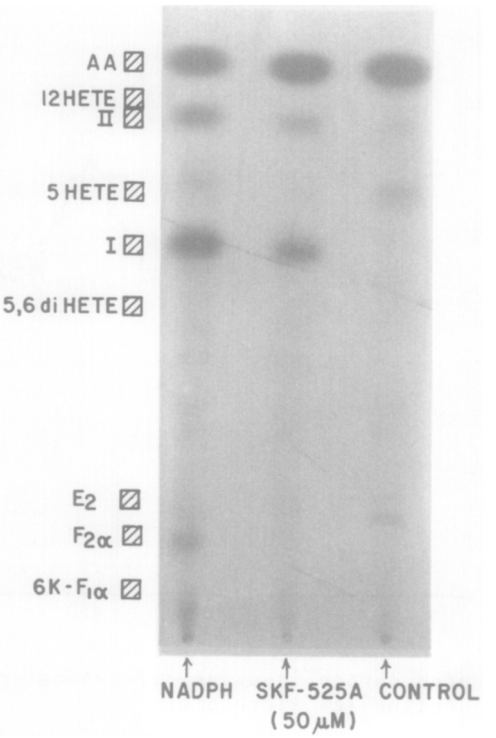


Fig. 1. Autoradiography of ^{14}C-arachidonic acid oxygenated metabolites formed by corneal epithelium and separated by thin-layer chromatography as described in Methods.

cyclooxygenase inhibitor, nor by BW755C (100 μM), a dual inhibitor of cyclooxygenase and lipoxygenase. ETYA, the acetylenic analogue of arachidonic acid, inhibited the formation of peaks I and II by more than 50%. SKF-525A, which inhibits cytochrome P450-dependent enzymes by type I binding to the hemoprotein (1), inhibited formation of both peaks by 40% (Fig. 1). Preincubation of the microsomal preparation with carbon monoxide resulted in about 50% inhibition of peaks I and II formation (data not shown).

The dependency of peaks I and II formation on NADPH, and the inhibitory effect of SKF-525A and carbon monoxide on their formation, raised the question whether a cytochrome P450 system exists in the corneal epithelium. Although there is a high concentration of heme and other hemoproteins which have an absorbance around 420 nm, we were able

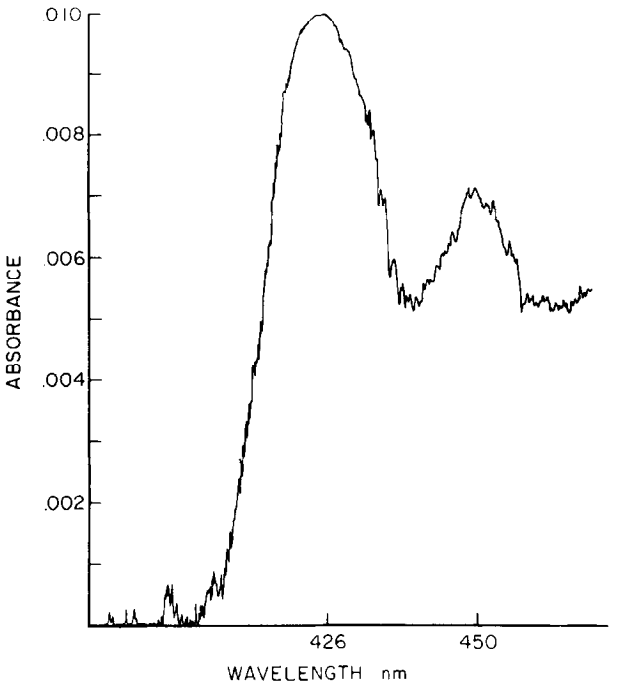
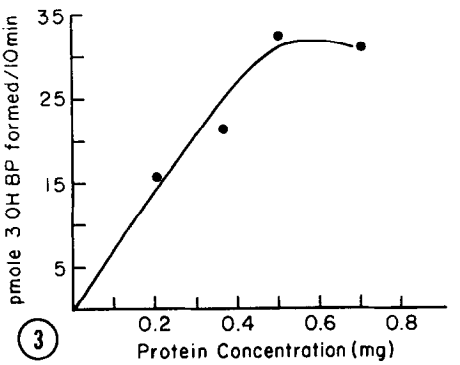


Fig. 2. The reduced carbon monoxide difference spectrum of the corneal epithelial microsomes.

to measure cytochrome P450 from the reduced carbon monoxide difference spectrum of the microsomes of the corneal epithelium as shown in Fig. 2. The cytochrome P450 content of this preparation was found to be 161 pmol/10 mg protein. We also measured the activity of AHH, a cytochrome P450-dependent monooxygenase, using BP as the substrate. In the five batches of corneal microsomes, each prepared from 50 eyes, AHH activity ranged from 11-39 pmol 3-OH-BP/mg protein/10 min. Figure 3 shows that AHH activity was protein dependent with a maximum activity at 0.8 mg protein/ml incubation mixture.

Using reverse-phase HPLC, we were able to separate each TLC peak into two radioactive metabolites (Fig. 4). Peak I of the TLC separation was resolved into metabolites A and B which had retention times of 4 and 6 min, respectively. Metabolites C and D are associated with TLC peak II and had retention times of 18.5 and 20 min, respectively, similar to the retention times of TALH cells AA metabolites (10).



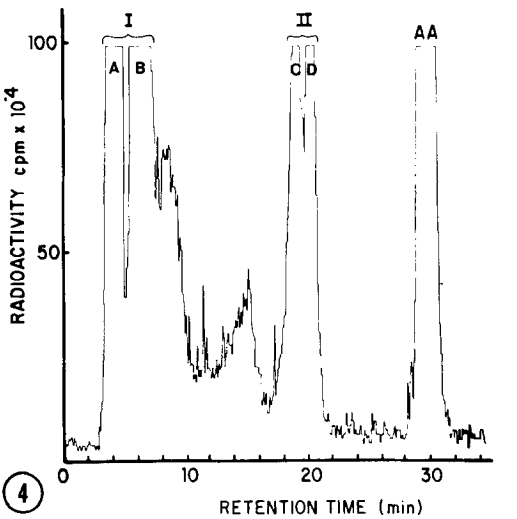


Fig. 3. Effect of protein concentration on the activity of aryl hydrocarbon hydroxylase in microsomes of the corneal epithelium. Activity is measured as the rate of conversion of benzoapyrene to its 3-OH metabolite.

Fig. 4. Reverse-phase HPLC separation of ^{14}C-arachidonic acid metabolites formed by corneal epithelial microsomes.

DISCUSSION

In this study, we demonstrated for the first time the presence of a cytochrome P450 system in the bovine corneal epithelium capable of metabolizing AA. This tissue exhibits a basal level of cytochrome P450, lower than that found in the kidney (9,24) but similar to that in blood vessels (25). The presence of cytochrome P450 was further characterized by measuring the activity of AHH. The activity of this cytochrome P450-dependent enzyme was about 11-39 pmol 3-OH-BP/mg protein/10 min. It is clear that corneal epithelia possess a higher activity of AHH than that reported for bovine retinal pigmented epithelium choroid (19).

The metabolism of AA in cornea has been studied extensively. It has been shown that both cyclooxygenase and lipoxygenase can convert AA in the corneal epithelium, stroma and endothelium to several products (15). In this study we have demonstrated the existence of the third pathway of AA metabolism - the cytochrome P450 pathway-in the cornea. Microsomes of the corneal epithelium convert AA to four different

oxygenated metabolites, the formation of which is dependent on NADPH and inhibited by carbon monoxide and SKF-525A, both are specific inhibitors of cytochrome P450-dependent enzymes. These features together with the presence of a cytochrome P450 and its drug metabolizing enzyme, aryl hydrocarbon hydroxylase, lead us to conclude that the cytochrome P450-dependent monooxygenase in the corneal epithelium is the system by which AA is transformed to these four metabolites.

In view of the recent reports that some AA metabolites of cytochrome P450 are biologically active compounds (7,8,10), the discovery of this pathway in the cornea is important. Epithelial cells of the TALH segment generate a cytochrome P450-dependent AA metabolite which inhibits Na^+- K^+ ATPase (10). As the corneal epithelium possesses similar transport properties to those of the TALH, AA metabolites of the cytochrome P450 in this tissue may have an important role in the modulation of ion transport mechanisms which rely on the pump function. The structure of these metabolites, together with their biological activities, remains to be determined.

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