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IMMUNOCHEMICAL LOCALIZATION OF EPOXIDE HYDRATASE IN RAT LIVER: EFFECTS OF 2-ACETYLAMINOFLUORENE

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SUMMARY. The distribution of epoxide hydratase was studied immunohistologically in paraffin sections of p-benzoquinone-fixed livers obtained from normal and 2-acetylaminofluorene-treated rats. In controls the enzyme was localized preferentially in the centrilobular hepatocytes. After the administration of 2-acetylaminofluorene, the staining was evenly distributed within the lobules suggesting the possibility that this hepatocarcinogen preferentially induced epoxide hydratase in perilobular parenchymal cells. Nonhepatocytic cells were considerably less extensively stained than hepatocytes.

Many aromatic and olefinic compounds including endogenous steroids, pesticides, drugs and polycyclic hydrocarbons (1-4), are metabolized by microsomal monooxygenases to epoxides which are often very electrophilic and covalently bind to cellular macromolecules such as DNA. The microsomal enzyme epoxide hydratase, also called epoxide hydrase [EC 4.2.1.63] catalyzes the conversion of many epoxides to the corresponding dihydrodiols and thereby plays a major role in the inactivation of these reactive intermediates (5-8). However, there is a more complex interrelationship between epoxide hydratase and monooxygenases in the activation and inactivation of polycyclic hydrocarbons, since the hydratase provides the precursors of highly mutagenic and carcinogenic diol epoxides (9-11).

Epoxide hydratase is widely distributed in rat organs (12) and is found in the same subcellular compartment as the monooxygenases (5). However, the localization of epoxide hydratase has only been studied biochemically. Immunohistochemical studies have shown that components of the microsomal electron transport

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chain are inhomogeneously distributed throughout the liver lobule (13,14). In this report the distribution of epoxide hydratase has been studied using an immunohistochemical procedure.

METHODS

Preparation of monospecific antibodies. Epoxide hydratase was purified from livers of untreated rats to apparent homogeneity as described (15). An adult female goat received two subcutaneous injections of 0.5 mg enzyme protein each in complete Freund's adjuvant, separated by a two-month interval. One week after the second injection, serum was collected and the immunoglobulin fraction (IgG) was isolated by means of DEAE-cellulose chromatography (16).

For the purification of monospecific antibodies (IgG), an affinity chromatography column was prepared as follows: Epoxide hydratase was coupled to divinylsulphone-activated sepharose as described by Porath (17). The activated sepharose (5 ml) was suspended in an equal volume of 0.3 M Na_2CO_3, pH 10.0 and 7 mg epoxide hydratase was added in 0.3 ml 10 mM sodium phosphate buffer, pH 7.0. The reaction was allowed to proceed for 2 h at room temperature and was stopped by washing with 0.3 M Na_2CO_3, pH 10.0 (20 ml). Finally, the sepharose was washed with 200 ml each of 0.3 M Na_2CO_3, pH 10.0 containing 1 M NaCl; 0.3 M glycine, pH 3.0 containing 1 M NaCl; 50 mM Tris-HCl, pH 7.5 containing 0.5 M NaCl and 10 mM potassium phosphate, pH 8.0. Measurement of the protein content of the initial 20 ml wash showed that over 85 % of the epoxide hydratase was bound to the sepharose. The IgG fraction (20-25 mg in 1 ml 10 mM potassium phosphate buffer, pH 8.0) was applied to columns which contained 5 ml sepharose-bound epoxide hydratase. After the solution had run into the columns, the flow was stopped for 30 min before eluting with 10 mM potassium phosphate, pH 8.0. Elution was continued until no more protein was washed from the column (E_{280}). The anti-epoxide hydratase IgG was then eluted by 10 mM potassium phosphate, pH 8.0 containing 3 M KSCN. Fractions containing protein were combined, dialysed immediately against 10 mM potassium phosphate, pH 8.0 to remove the thiocyanate and lyophilized. Specific anti-epoxide hydratase amounted to between 15 and 20 % of the protein in the IgG fraction. The epoxide hydratase sepharose could be used several times provided that the time for which it was in contact with 3 M KSCN was kept at a minimum.

Immunohistochemical procedures. Adult male Sprague-Dawley rats weighing 180-200 g were fed standardized laboratory chow (Nafag No. 890), for 6 days; one group of animals received a diet containing 0.05 % 2-acetylaminofluorene (Fluka AG) for the same duration. Food was withdrawn 24 h prior to sacrifice. Small pieces of liver were treated essentially according to Baron et al. (13), including fixation of the tissue with 0.05 % (w/v) p-benzoquinone (Polysciences, Inc.), dehydration, paraffin embedding, sectioning, deparaffination of sections in xylene, and suppression of endogenous peroxidase activity with 0.01 % H_2O_2 in 95 % methanol. The sections were washed with 0.05 M Tris-HCl buffer, pH 7.75, containing 0.154 M NaCl, treated with 10 % ov-

albumin (Fisher Scientific) in the same buffer solution for 30 min (18) and covered with a few drops of monospecific goat anti-rat epoxide hydratase antibodies (0.13 mg/ml) in 0.05 M Tris-HCl buffer, pH 7.75, containing 0.154 M NaCl for 60 min at room temperature in a moist chamber. Following a wash with the same buffer solution, the sections were exposed to rabbit anti-goat immunoglobulin (Miles Laboratory) in 0.05 M Tris-HCl buffer, pH 7.75, containing 0.154 M NaCl (0.07 mg/ml) for 30 min in a moist box. The preparations were washed and incubated for 30 min at ambient temperature with a soluble goat horseradish peroxidase antiperoxidase complex (Miles Laboratory) diluted 1:20 in the same buffer solution (19). The sections were transferred into a buffer solution and histochemical peroxidase reaction performed by their incubation in 0.05 M Tris-HCl buffer, pH 7.75, containing 0.154 NaCl, 0.005 % H_2O_2 and 0.012 % 3,3'-diaminobenzidine tetrahydrochloride (Fluka AG) for 20 min at room temperature (13). Following a wash with several changes of distilled water, the sections were post-fixed with an aqueous 2 % OsO_4 solution for 3 min, washed in distilled water, dehydrated in graded ethanol solutions and mounted in Fluormount (Gurr Ltd.).

To assess the specificity of the procedures, the following immunological and histochemical controls were performed: a) exposure to goat anti-rat epoxide hydratase antibodies was replaced by an incubation with 0.05 M Tris-HCl buffer, pH 7.75, which contained 0.154 M NaCl; b) the same buffer was used instead of the bridging rabbit anti-goat immunoglobulin; c) incubation with the soluble goat horseradish peroxidase-antiperoxidase complex was substituted by a buffer treatment; d) the rabbit anti-goat immunoglobulin was replaced by a preimmune rabbit immunoglobulin (Miles Laboratory); e) H_2O_2 was omitted from the incubation medium used to develop the histochemical peroxidase reaction.

RESULTS AND DISCUSSION

The control incubations outlined above resulted in virtually unstained preparations, which could only be visualized after marked reduction of the microscope diaphragm. Incubations containing the full system resulted in dark brown preparations which were readily visualized. In preparations from untreated rats the centrilobular zones of the liver lobules stained darker for horseradish peroxidase than the perilobular parts (Fig. 1). This suggests that epoxide hydratase is located mainly in the centrilobular hepatocytes. A similar distribution has been reported for other components of the microsomal toxifying-detoxifying system, e.g. cytochrome b_5 (20), NADPH cytochrome c reductase (13), benzo(a)pyrene metabolizing enzymes (21), and NADH-, NADPH-nitrobluetetrazolium reductase (22). These findings could be explained by a higher volumetric share of the endoplas-

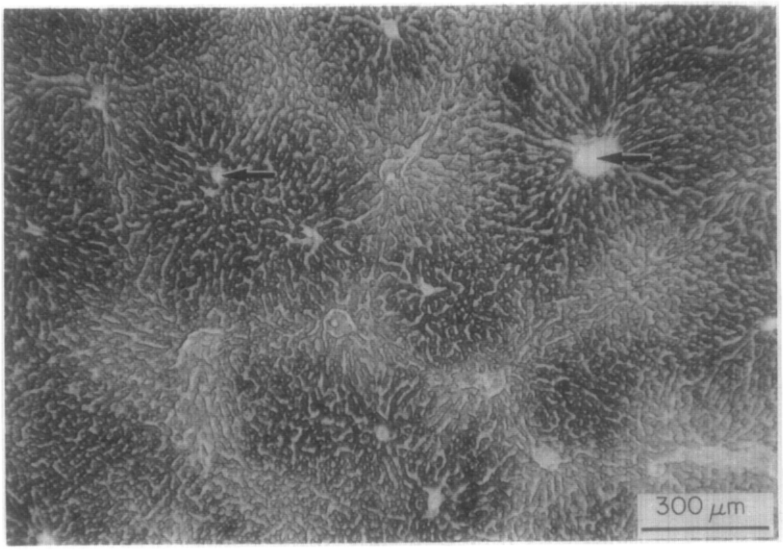

Fig. 1. Immunohistochemical demonstration of epoxide hydratase in liver sections from control rats. Note that the immunostain appears to be more concentrated in centrilobular hepatocytes. Arrows point to central veins.

mic reticulum in centrilobular than in more peripherally located hepatocytes (23,24). Furthermore, the smooth membranes of centrilobular hepatocytes seem to proliferate more extensively in response to phenobarbital treatment than their periportal counterparts (14,24,25), apparently in parallel with an increase in cytochrome P-450 content (14).

It has been previously shown that the administration of multiple doses of 2-acetylaminofluorene to rats caused a considerable increase in activity of hepatic epoxide hydratase (26-28) which was also considered as a preneoplastic antigen (29). After treatment of rats with this hepatocarcinogenic agent, the intralobular distribution of epoxide hydratase, as revealed immunohistochemically, was different from that observed with controls (Fig. 2). The immunostaining was more regularly distributed throughout the lobules and the centrilobular enrichment seen in preparations from control animals was no longer apparent. This suggests that the observed increment in epoxide hydratase activity might be, at least in part, due to a preferential induction of this enzyme in perilobular hepatocytes. This is in

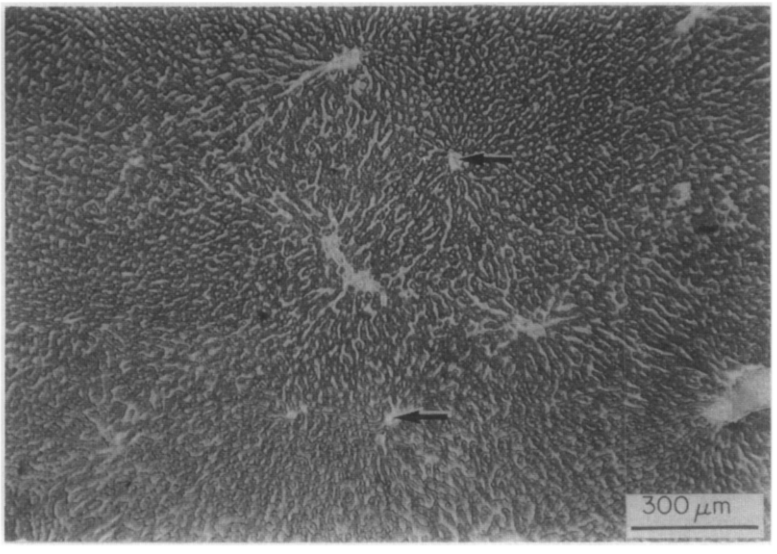

Fig. 2. Immunohistochemical demonstration of epoxide hydratase in liver sections from rats fed 2-acetylaminofluorene. The reaction product is evenly distributed throughout the lobules. Central veins are labeled with arrows.

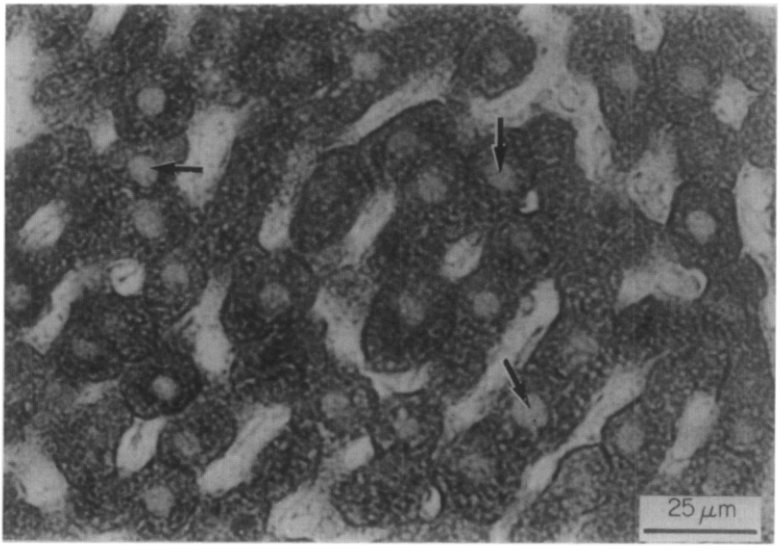

Fig. 3. Immunohistochemical demonstration of epoxide hydratase in liver sections from control animals. The cytoplasm of hepatocytes is homogeneously stained, whereas the nuclei (arrows) remain pale. The cells occupying the nonhepatocytic space appear unstained.

contrast to the situation after the induction of monooxygenase components by 3-methylcholanthrene (21) and phenobarbital (14,25).

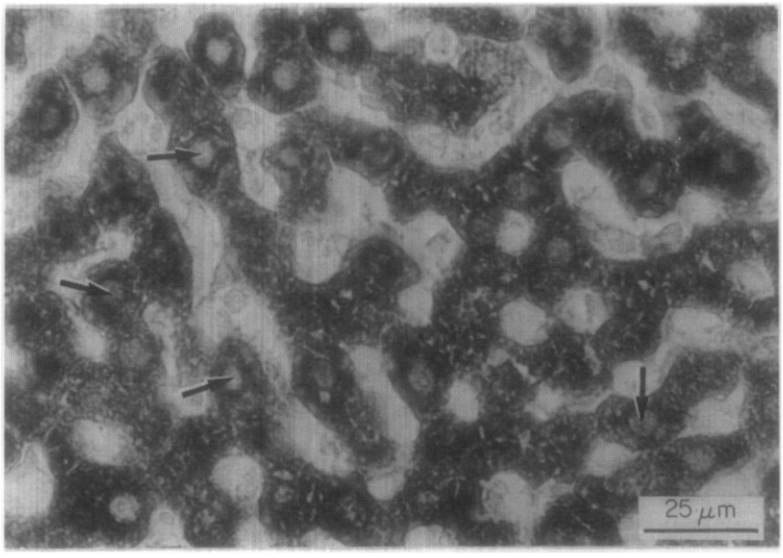

Fig. 4. Immunohistochemical demonstration of epoxide hydratase in liver sections of animals fed 2-acetylaminofluorene. Many of the nuclei (arrows) are surrounded by a dense perinuclear cytoplasm. Again, nonhepatocytic cells appear unstained.

Higher magnifications of preparations from control and 2-acetylaminofluorene pretreated rats are shown in Figs. 3 and 4 respectively. In both cases Kupffer cells and endothelial cells are much less densly stained than hepatocytes. Thus epoxide hydratase would seem to be located mainly in the hepatocytes as are NADPH cytochrome c reductase (13) and cytochrome b_5 (20).

In liver sections from control animals the reaction product was homogeneously distributed throughout the hepatic cytoplasm. The cell nuclei were devoid of immunostain but were in some cases delimited by a dark ring, suggesting the presence of epoxide hydratase in the nuclear membrane (29-32). In many hepatocytes of rats fed 2-acetylaminofluorene the immunostain appeared in patchy condensations within the perinuclear cytoplasm, whereas the nuclei, with the exception of their envelope, were not appreciably stained (Fig. 4). It is possible that these 2-acetylaminofluorene-induced changes in the distribution of immunohistochemically reactive sites are related to the proliferative response of the smooth endoplasmic reticulum which occurs in hepatocytes as an early event during intoxication of rats with this agent (34).

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