IMMUNOCYTOCHEMICAL LOCALIZATION OF CATECHOLESTROGEN IN THE RAT LIVER AFTER USING VARIOUS FIXATIVES

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Catecholestrogen was localized in rat liver by immunocytochemical methods to study optimal fixation conditions in paraffin-embedded tissue. Rabbit anti-2-hydroxyestrone antiserum was obtained using 2-hydroxy estrone coupled with bovine serum albumin as the antigen. The most constantly positive staining for catecholestrogen was obtained with periodate-lysine paraformaldehyde (PLP) and buffered picric acid-paraformaldehyde (PAF), although other fixatives also showed staining results.

Catecholestrogen is the major product of biotransformation of estrogen in man. The possible significance of catecholestrogens as potential mediators of estrogen action and a direct biochemical link between estrogen and catecholaminergic function has been considered (2, 4, 7–10, 16, 20).

Numerous studies have shown that catecholestrogen is widely distributed in animal tissue with the highest levels present in the liver (2, 3, 5–8, 11, 16, 20, 21).

Despite much speculation, the specific localization of catecholestrogen has remained uncertain since no direct method for determining its localization within cells and tissues has been available. Ball et al. (1), Yoshizawa and Fishman (25) have reported the radioimmunoassay of 2-hydroxyestrone, one of the catecholestrogens. A specific antibody to catecholestrogen has permitted us to localize catecholestrogen immunocytochemically. In this paper we present studies on the immunocytochemical localization of catecholestrogen in the liver after using various fixatives.

MATERIALS AND METHODS

1. Preparation of antisera

The antigen, 2-hydroxyestrone-17-(O-carboxymethyl)-oxime bovine serum albumin conjugate (2-OHE-BSA), was prepared using the method of Yoshizawa and Fishman (25). One milligram of the lyophilized steroid-protein was dissolved in 1 ml of 0.1 M phosphate-buffer saline (PBS) and emulsified in 1 ml of Freund’s complete adjuvant. This material was administered subcutaneously to a domestic rabbit to obtain the antiserum.
The schedule of injections was 2 ml once a week for 8 weeks. Intravenous injections of 0.5 mg of antigen in 1 ml of PBS were then continued at monthly intervals. Blood was obtained from the ear vein prior to and 2, 5, 8, 11 and 12 weeks following the first injection of antigen, and the plasma was separated and frozen. The antiserum obtained was tested for cross-reactions with antigen or BSA by the Ouchterlony technique. The specificity of the antiserum was evaluated for immunoperoxidase staining of affinity gel coupled with 2-OHE-BSA or BSA.

After 12 weeks the rabbits were anaesthetized with Nembutal and bled after fixing a cannula in the carotid artery. Sera were stored at −20°C or lyophilized.

2. Preparation of affinity gel for immunoperoxidase staining

The affinity gel was prepared by coupling 2-OHE-BSA or BSA with Sepharose 4B (Pharmacia P-L Biochemicals, Sweden) treated with NaIO₄. The gel in 0.1 M PBS was poured into a Pasteur capillary pipette for immunoperoxidase staining at 4°C (Fig. 2a).

3. Preparation of tissue

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Liver samples were obtained from Wistar male rats (4 weeks old) under anaesthetization with Nembutal. Tissues were immediately immersed in fixative.

Fixation

The following fixation fluids were used:

- b. Buffered picric acid—paraformaldehyde (PAF) (22).
- c. Sublimate—formaldehyde prepared as follows:
  - HgCl₂ 6 g
  - glacial acetic acid 5 ml
  - paraformaldehyde 20% 20 ml
  - distilled water 75 ml
- d. Paraformaldehyde 4%—glutaraldehyde 2.5%
- e. Acetic acid 1%—ethanol 95%

Tissues were fixed over a 3 hour period at 4°C. Tissues were dehydrated through a graded alcohol series and embedded in paraffin. Sections 5 μm in thickness were cut, mounted on glass slides with egg albumin and glycerine, and dried overnight at 37°C.

4. Immunocytochemical staining

After deparaffinization, slides were washed with distilled water and then treated with 0.005 M unbuffered periodic acid and 0.003 M unbuffered sodium borohydride to inhibit endogenous peroxidase. Sections were rinsed with two changes of distilled water for 10 min and PBS for 5 min. Slides were incubated with a 1 : 50 dilution (in PBS) of normal goat serum for 30 min at room temperature.

Slides were incubated for 24 hours at 4°C in a 1 : 1000 dilution (in PBS) of either rabbit antiserum to 2-OHE-BSA which was absorbed with BSA, or, as a control, in normal rabbit serum, and rabbit antiserum to 2-OHE-BSA absorbed with 2-OHE-BSA similarly diluted; then in a 1 : 10 or 1 : 50 dilution (in PBS) of goat anti-rabbit serum (Polyscience Inc., Warrington, Pa. or Cappel Lab., USA) for 30 min at room
temperature; and finally in a 1:50 (in PBS) dilution of peroxidase-antiperoxidase complex (PAP) (23) (Polyscience Inc., Warrington, USA) for 30 min at room temperature. All slides were washed for 15 min each in three changes of PBS between each step. Peroxidase reaction product was developed by incubation of sections for 5 min in 3, 3'-diaminobenzidine (0.05%) in 0.05 M Tris buffer, pH 7.6, containing 0.01% \( \text{H}_2\text{O}_2 \). After a final wash in distilled water the sections were dehydrated through a graded alcohol series, cleared with xylene, and mounted in Canada Balsam.

Affinity gels coupled with 2-OHE-BSA or BSA were also stained with either rabbit antiserum to 2-OHE-BSA or antiserum which was absorbed with BSA by a similar procedure to that used for the slides.

**RESULTS**

Specificity of antiserum to 2-OH-estrone-BSA (2-OHE-BSA)

Double-diffusion tests

The serum of rabbits immunized with 2-OHE-BSA reacted with both the 2-OHE-BSA or BSA (Fig. 1). After absorption with BSA, the antiserum did not react with BSA as shown in Fig. 1.

Immunoperoxidase staining of affinity gel coupled with 2-OHE-BSA

The affinity gel coupled with 2-OHE-BSA (Fig. 2b-1) or BSA (Fig. 2b-2) respectively was stained with anti 2-OHE-BSA by the PAP method (23). However, after absorption with BSA, the antiserum stained the affinity gel coupled with 2-OHE-BSA (Fig. 2b-4), but not the affinity gel coupled with BSA (Fig. 2b-3). Therefore the antisera raised seem to be specific to 2-OH-estrone (2-OHE).

Immunocytochemical results

The results of immunoperoxidase staining of rat liver that had been fixed in different types of fixative are presented in Figs. 3–8.

PLP fixative (Figs. 3 and 4)

2-OHE-positive reaction product in the hepatic cells was diffusely present throughout the cytoplasm, but was absent from the nucleus (Fig. 3). The cytoplasm of nearly all hepatic cells contained dark granular deposits (Fig. 3). No specific staining occurred in control sections (Fig. 4).

PAF fixative (Fig. 5)

The cytoplasm of almost all hepatic cells was weakly stained. Many of the hepatic cells contained dark granular deposits, frequently presented close to the nucleus.

Sublimate-formaldehyde fixative (Fig. 6)

2-OHE-positive reaction product was diffusely localized throughout isolated hepatic cells.

Formalin 4%-glutaraldehyde 2.5% (Fig. 7)

Some hepatic cells contained 2-OHE in the form of dark brown deposits present throughout the cytoplasm.

Acetic acid 1%-ethanol 95% (Fig. 8)

Most hepatic cells showed 2-OHE-positive reaction in the peripheral cytoplasm. The best immunostaining was observed after fixation of the tissue in PLP fixative. PAF fixative permitted adequate immunocytochemistry. The form-
aldehyde-glutaraldehyde mixture altered the antigenicity of 2-OHE so that no specific staining occurred in hepatic cells lying superficially in the tissue.

**DISCUSSION**

In this study, the anti 2-hydroxyestrone-bovine serum albumin (2-OHE-BSA) rabbit antiserum used was obtained by the same method as that described in the paper of Yoshizawa and Fishman (25). The results of the double-diffusion studies and immunoperoxidase staining of affinity gel coupled with 2-OHE-BSA or BSA indicated that the antiserum used in this study possessed high specificity for 2-OHE. However, for immunostaining it is necessary for anti-2-OHE-BSA to be absorbed with BSA. This is because of the fact that during immunization of the rabbits by 2-OHE-BSA, formation of BSA antibodies occurred, as well as that of antibodies specific for 2-OHE. When the sections of rat liver were stained with unabsorbed 2-OHE-BSA antiserum immunocytochemically, all tissues in the liver were diffusely stained. This staining feature was distinguishable from the appearance of sections stained with anti-2-OHE-BSA antiserum absorbed with BSA immunocytochemically.

The above results lead us to believe that immunocytochemical staining can be used to study the localization of 2-OHE in the rat liver with high specificity and sensitivity. However, we cannot exclude the possibility that our results also show the presence of 2-hydroxyestradiol and 2-methoxyestrone in the liver, although Yoshizawa and Fishman (25) reported that their anti-2-OHE produced only minor cross-reactions with 2-hydroxyestradiol and 2-methoxyestrone. We can therefore safely state that highly specific antibodies were obtained in the present study which allowed immunocytochemical localization of catecholestrogen in the tissues examined.

Another problem exists in immunocytochemical staining for steroid hormone like catecholestrogen in that such compound have a very low molecular weight and are both soluble and diffusible, making it easy to err when attempting to localize them. In the literature various immunocytochemical methods have been reported (14, 18, 24). However, as yet, no adequate immunocytochemical technique has been reported. In the present study, however, we have found that the antigenicity
of catecholestrogen can be preserved in paraffin-embedded specimens fixed with various fixatives. It therefore appears to be possible to demonstrate catecholestrogen in routinely fixed paraffin-embedded specimens. Although a number of other fixatives may be used with success, PLP and PAF fixatives appeared to give the most consistent results for catecholestrogen.

Finally, in the liver, catecholestrogen-positive reaction was evenly distributed throughout the cytoplasm of the hepatic cells. This observation is in agreement with biochemical evidence that liver is the richest source of catecholestrogen (6, 11, 16, 20, 21). It is very interesting to note that catechol-O-methyltransferase (COMT) has been shown to be present in rat liver immunocytochemically (13, 15) and that the liver also contains estrogen-2-hydroxylase, the biosynthetic enzymes of catecholestrogen (5, 11, 19). Catecholestrogen is rapidly O-methylated to 2-methoxyestrogen by COMT (8). Furthermore, Hoffman et al. (12) have reported that the enzymatic formation of catecholestrogen from 2-methoxyestrogen occurs in rat liver. It seems that COMT, estrogen-2-hydroxylase, and other enzymes by which catecholestrogen is formed from 2-methoxyestrogen in the liver may play an important role in modulating the release of biologically active circulating catecholestrogen.

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REFERENCES


