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EFFECT OF DIFFERENT DOSES OF 3-METHYLCHOLANTHRENE ON THE LOCALIZATION OF THE 3-METHYLCHOLANTHRENE-INDUCIBLE ISOENZYMES OF CYTOCHROME P450 WITHIN THE CENTRILOBULAR AND PERIPORTAL ZONES OF THE RAT LIVER

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Abstract—Immunohistochemical staining techniques were used to investigate the localization of the 3-methylcholanthrene inducible isoenzymes (P450 IA1 and IA2) in the rat liver. The rats were induced with different doses of 3-methylcholanthrene, ranging from 2.5 to 25 mg/kg body weight. A heterogeneous induction pattern was observed with induction doses of 2.5; 5; 7.5 and 10 mg/kg body weight with the highest concentration of the isoenzymes around the central vein. With a dose of 25 mg/kg body weight, a homogeneous pattern was found. Induction with a dose of 15 mg/kg body weight resulted in an intermediate situation.

The cytochrome P450 mixed function oxidase system consists of multiple forms [1] which are mainly located in the smooth endoplasmic reticulum of the parenchymal cells in the liver. The content of the different isoenzymes in the parenchymal cells can be induced selectively using chemicals such as phenobarbital and 3-methylcholanthrene [1–5]. Only a few immunohistochemical studies have been performed to investigate the localization of these isoenzymes in the liver lobule with or without induction [2–4, 6–10]. Induction of the PB inducible isoenzymes results generally in a heterogeneous induction pattern with the highest content in the parenchymal cells around the central vein [2–4, 9, 11]. Induction of the 3-MC inducible isoenzymes results generally in a homogeneous induction pattern with a slightly greater content in the pericentral parenchymal cells [2, 4]. In contradiction to the previous studies, it was found that using a low dose of 3-MC for induction, the localization of the 3-MC inducible isoenzymes was heterogeneous in the liver lobule. In the present study, doses from 2.5 to 25 mg/kg body weight were used to investigate the lobular localization of the 3-MC inducible isoenzymes.

MATERIALS AND METHODS

Liver tissue was obtained from 3-month-old male rats (230–280 g) of the Brown Norway (BN/BiRij) strain, T.N.O. Rijswijk. The animals were kept on a standard diet (AM II, Hope Farms, Woerden, the Netherlands) and water *ad lib.*, under clean conventional conditions as described by Hollander [12].

The rats received different doses of 3-methylcholanthrene, obtained from the Sigma Chemical Co. (St Louis, MO) dissolved in corn oil i.p. once daily for three consecutive days, the last dose being given 24 hr prior to being killed. The six different doses used for induction were 2.5, 5, 7.5, 10, 15 and 25 mg 3-MC/kg body weight. The dose groups 2.5, 5 and 7.5 mg each contained four animals, the dose groups 10 and 15 mg each contained three animals and two animals received 25 mg. Stock solutions of 3-MC were adjusted in such a way that treated and control rats were administered equal volumes (about 1 mL) of vehicle. All rats were starved overnight before being killed. The rats were anesthetized under light ether and their livers were perfused through the portal vein *in situ* for 3 min with ice-cold 0.9% NaCl and 5 min with phosphate buffered (28.9 mM $NaH_2PO_4.H_2O$ and 45.7 mM $Na_2HPO_4.2H_2O$, obtained from Merck, Darmstadt, F.R.G.) 3.7% formaldehyde (formaldehyde was obtained from Baker chemicals, Deventer, Netherlands). The fixative was prepared just before use. After the perfusion, the livers were removed and kept in the same fixative for 24 hr at room temperature. The median lobes of the fixed livers were embedded in paraffin the next day. From the embedded median lobe, 3 μm thick slices were mounted on alcohol cleaned slides and air dried (37°) overnight. These slices were deparaffinated the next morning followed by an indirect immunoperoxidase staining. In order to quench endogeneous peroxidases, the slices were incubated in 0.3% H_2O_2 in methanol for 30 min. Then the slices were washed three times in phosphate buffered saline (PBS) (5.4 mM $Na_2HPO_4.2H_2O$, 1.29 mM KH_2PO_4, 0.154 M NaCl, pH 7.4, obtained from Merck) for 20 min. The following steps were performed in a humidified box. The slices were incu-

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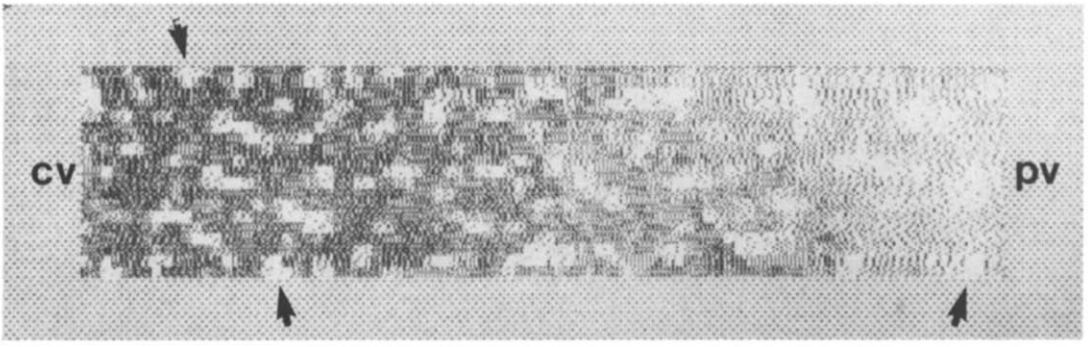

Fig. 1. Absorbance measurement (related to the amount of the cytochromes P450 IA1 and IA2) of a rectangle enclosing an area between central and portal vein. CV = central vein, PV = portal vein, arrow = sinusoid area. For further details see text.

bated with 1% normal goat serum for 20 min to block nonspecific binding sites, followed by an incubation with anti-βNF, a rabbit serum containing polyclonal antibodies against cytochrome P450-IA1 and IA2 (this polyclonal, originally prepared by Guengerich *et al.* [13], was a gift from Dr P. Kremers, Liège) in a 1:600 dilution in 1% bovine serum albumin in PBS for 30 min. After the incubation with the polyclonal antibodies, the slices were washed three times in PBS for 15 min and incubated with a biotinylated goat anti-rabbit antibody (obtained from Vector Laboratories, Burlingame, U.S.A.) for 30 min. After three washes with PBS for 15 min, the slices were incubated with an Avidin Biotinylated peroxidase Complex (ABC, obtained from Vector Laboratories) for 60 min. Then the slices were washed three times in PBS for 15 min. The peroxidase activity was revealed by an incubation in 3.3′-diaminobenzidine (obtained from Sigma), and H_2O_2 for 10 min. Finally, the slices were washed in aqua dest., dehydrated and mounted under cover glass. Negative control studies were performed with normal rabbit serum and with the staining procedure in which the polyclonal antibodies were deleted.

The localization and the distribution of the 3-MC inducible cytochrome P450 isoenzymes after immunohistochemical staining were investigated by absorbance measurements of the staining in different lobules of each slice. In a liver lobule, the distance from the central vein to the surrounding portal veins is variable. However, the staining distribution seems to be independent of the absolute distance. In short, the decrease in staining (when extant) is correlated with the percentage of the absolute distance. This assumption made it possible to compare the staining distribution and staining intensities of different lobules. At random, six different lobules of the median lobe of each rat were chosen for further investigation. For this purpose, absorbance measurements of the amount of the reaction product of peroxidase and DAB were measured at a wavelength of 466 nm. These absorbance measurements were performed with a microscope photometer (Zeiss, Oberkochen, F.R.G.) interfaced to an Atari 520 ST computer. The ARRAYSCAN program of the HIDACSYS package [14] was used for computation of the absorbance measurements. With this program, one rectangular part of one lobule, enclosing an area between a central and a portal vein was selected. The system scanned across the rectangle and each 2 μm a measurement was made. A grey-scale is used to represent different absorbance values (Fig. 1 is an example of the digitized image of the rectangle). The lowest grey value was based on the absorbance measurements at the sinusoids. To obtain an impression of the organization of the staining from the central to the portal vein, the rectangle was divided in successive sections of the same size. The number of successive sections ranged from 8 to 15, circumscribed to the possibilities of the computer program. Because of the variation in number of sections, they were scaled to a fixed distance representing 100% of the distance from the central vein to the portal vein. Visualized in bar charts, an impression of the relative distribution of the staining for a rectangle was obtained (Fig. 2).

To obtain an absolute value for the total absorbance, the area under the curve (AUC) was calculated for each bar chart. To obtain an impression of the relative distribution of the staining in the periportal cells and in the pericentral cells, absorbance measurement values close to the central vein were divided by absorbance measurement values close to the portal vein. To obtain this ratio, the first and the last value of the successive sections were used. A mean value of the first two successive sections was calculated when the value closest to the central vein was lower than the second one.

Comparison of the area under curve values was performed using the Student's *t*-test with Bonferroni correction, because the Bartlett test showed no differences in the standard deviations. The Wilcoxon test was used for comparison of the ratios.

RESULTS

The induction of cytochrome P450 IA1 and IA2 isoenzymes with 3-MC was determined by immunohistochemical staining methods on liver sections of rats injected with 0, 2.5, 5, 7.5, 10, 15 or 25 mg 3-MC/kg body weight. After injection of the doses

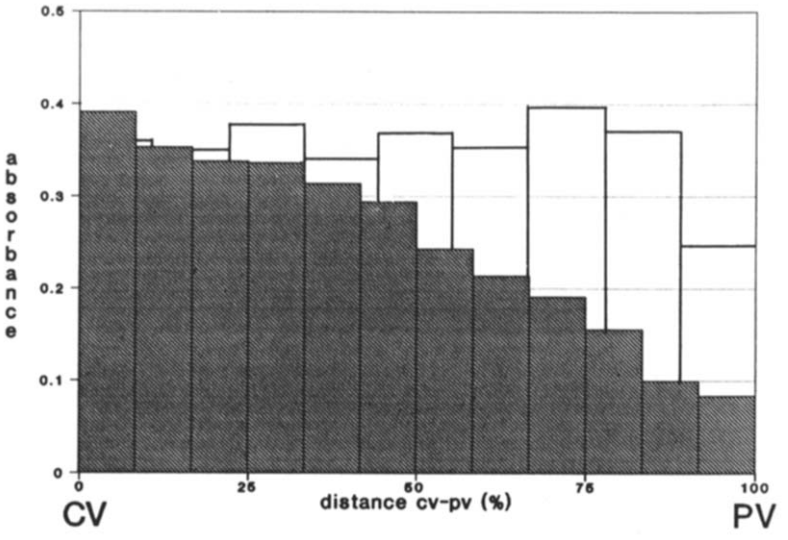

Fig. 2. The absorbance values (related to the amount of the cytochromes P450 IA1 and IA2) of successive sections between central and portal vein. CV = central vein, PV = portal vein. Typical bar charts of rats treated with 7.5 (closed bars) and 25 (open bars) mg 3-MC/kg body weight.

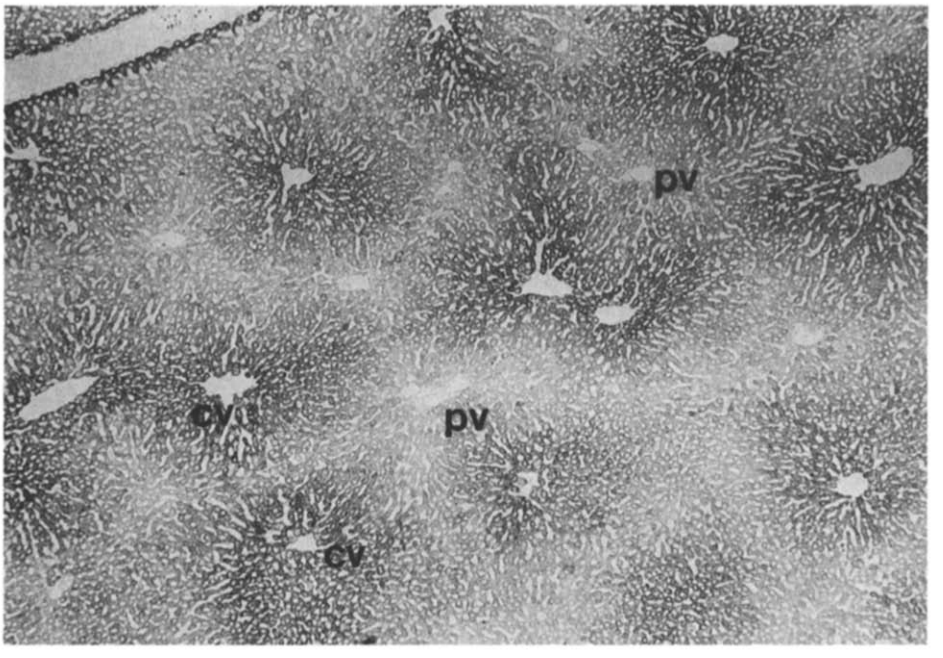

Fig. 3. Heterogeneous lobular distribution pattern of the cytochromes P450 IA1 and IA2. The rat was treated with 5 mg 3-MC/kg body weight. CV = central vein, PV = portal vein. Magnification 57×.

2.5, 5, 7.5 and 10 mg, a heterogeneous staining pattern with the highest content of staining in cells around the central vein was observed (Fig. 3), whereas an injection with 25 mg resulted in a homogeneous staining pattern (Fig. 4). The liver sections of rats injected with 15 mg showed an increased area of stained cells around the central vein in comparison with rats injected with lower doses. The liver sections of the control rats showed no staining at all.

To obtain an impression of the penetration of the final staining product, 10 μm thick slices were immunohistochemically stained. A cross-section of the stained slice shows a penetration of ± 1 μm (Fig. 5). The localization and the distribution of the 3-MC inducible isoenzymes were investigated by absorbance measurements as described in Materials and Methods. There was a decrease in absorbance values, from central- to portal vein, for rats injected with

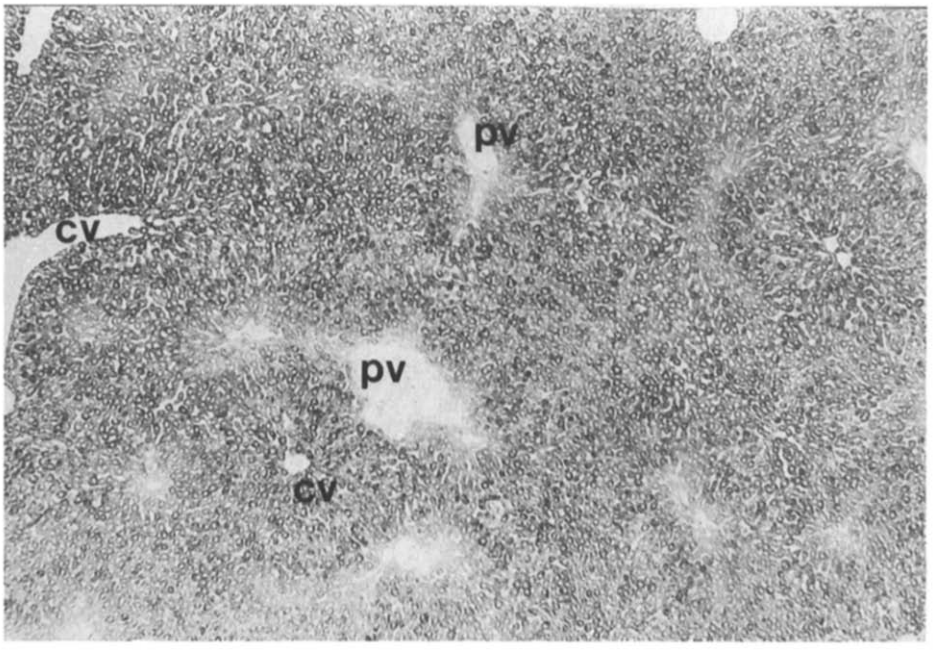

Fig. 4. Homogeneous lobular distribution pattern of the cytochromes P450 IA1 and IA2. The rat was treated with 25 mg 3-MC/kg body weight. CV = central vein, PV = portal vein. Magnification 57×.

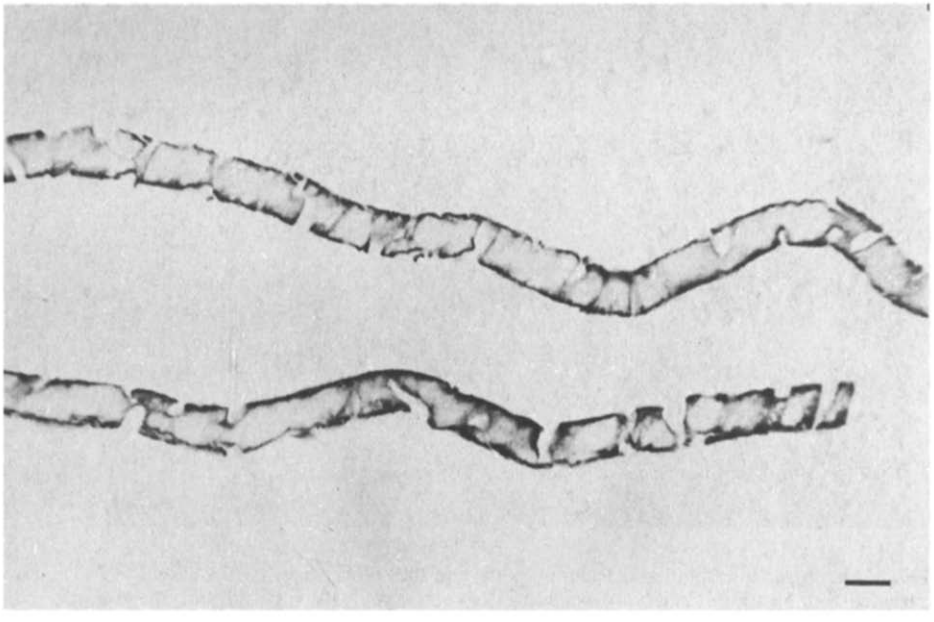

Fig. 5. An immunohistochemical staining of a cross section of a 10 μm thick slice stained with antibodies specific for the cytochrome P450 isoenzymes IA1 and IA2. Bar represents 10 μm.

2.5, 5, 7.5 or 10 mg (an example of a rat injected with 7.5 mg 3-MC/kg body weight is shown in Fig. 2, closed bars). In rats injected with 15 mg, there was a less dramatic decrease. When there was a decrease, it started either directly after the first section or after a plateau starting at the first section. In addition to the trend described above, the proportional decrease in staining relative to the distance from the central to the portal vein varied independent of the dose. Rats injected with 25 mg (Fig. 2, open bars) showed no decrease or a very slight one.

Table 1. The effect of the induction dose on the AUC's and on the ratios of absorbance values from the areas close to the central and portal vein

3-MC dose (mg/kg)	N	AUC (a.u.)	CV/PV ratio (a.u.)
2.5	4	1.42 ± 0.27	2.50 ± 0.53
5.0	4	1.84 ± 0.61	2.88 ± 0.68
7.5	4	2.14 ± 0.63	2.83 ± 0.73
10	3	1.76 ± 0.42	3.17 ± 0.95
15	3	2.34 ± 0.99	2.27 ± 0.43
25	2	3.14 ± 0.17	1.79 ± 0.42

Area under the curve (AUC) is calculated from the bar chart in which the absorbance values (related to the amount of cytochrome P450 IA1 and IA2 isoenzymes) are plotted against the distance between central and portal vein (see Fig. 2). Values are expressed as arbitrary units (a.u.) and presented as mean ± SD. Six measurements per rat were performed.

To obtain a quantitative assessment of the staining, six different rectangles from a central vein to a portal vein were scanned to obtain a mean AUC-value for each rat. The values of the individual rats per dose group were averaged to obtain a final mean AUC. The relationship between the induction dose and the averaged AUC value is shown in Table 1. The values of rats injected with 2.5, 5 and 10 mg were significantly ($P < 0.05$) lower than the value of rats injected with 25 mg. To obtain an objective impression of the heterogeneity, ratios of absorbance values from sections close to the central and portal veins were calculated. The relationship between the induction dose and these ratios is shown in Table 1. The combined ratios of rats injected with 2.5, 5, 7.5 or 10 mg are significantly ($P < 0.05$) higher (according to the Wilcoxon test) than the combined ratios obtained from rats injected with 15 or 25 mg.

DISCUSSION

The staining intensities of the cytochrome P450 isoenzymes IA1 and IA2 in the periportal and pericentral regions of the liver lobule after 3-MC induction were evaluated by absorbance measurements of the reaction product of DAB and H_2O_2. The absorbance measurements were integrated not to define the total amount of enzymes present, but to obtain a value with which the lobular distribution of the enzymes in the different induced rats could be compared. The finding that no staining was observed in control rats was caused by the high dilution factor of the antibody. To exclude variation in the formation of the reaction product of DAB and H_2O_2, all the slices used for the absorbance measurements were stained "en masse" in one solution of the reagents.

With the light micrographs, a homogeneous pattern was observed with 25 mg 3-MC. However, quantitative absorbance measurements of the staining revealed a slight decrease close to the portal vein (Fig. 2, open bars), resulting in a CV/PV ratio higher than 1 (Table 1).

It might be argued that the induction pattern is dependent on the time elapsed after the first administration of a dose and not just on the magnitude of the dose. However, studies of Boobis *et al.* [15] revealed that multiple injections of 3-MC, administered at 24 hr intervals, resulted in cytochrome P450 enzyme activities which reached a plateau level already after one day and were maintained for up to five days.

The homogeneous induction pattern after administration of 25 mg 3-MC/kg body weight was in agreement with the results of Baron *et al.* [4] and Wolf *et al.* [2] who induced with 25 and 20 mg 3-MC/kg body weight for 3 consecutive days, respectively. In the present study, however, next to this homogeneous pattern, a heterogeneous distribution of the 3-MC inducible isoenzymes in the liver lobules was found after induction with low doses of 3-MC. This finding that the induction pattern is heterogeneous at low doses and homogeneous at high might be explained by the hypothesis that in rats treated with a low dose still a high reserve capacity to metabolize xenobiotics has been available, whereas at high doses a great part of the reserve capacity has been used up.

Beside the dose dependent trend, there was a variability independent of the dose regarding to the greatest decrease that occurs relative to the distance from the central vein to the portal vein. This dose-independent variability could be caused by the morphological section through the liver lobules. The liver lobule has the shape of a barrel and a section right through the middle of this barrel could differ from a section sloping through the barrel. Consequently, the cytochrome P450 isoenzyme distribution could be dependent on the section through the barrel.

Summarizing, the induction pattern of cytochrome P450 IA1 and IA2 isoenzymes within the centrilobular and periportal zones of the rat liver depends on the induction dose of 3-MC. A heterogeneous distribution pattern was observed with induction doses of 2.5; 5; 7.5 and 10 mg/kg body weight with the highest concentration of the isoenzymes around the central vein. With a dose of 25 mg/kg body weight, a homogeneous pattern was found. Induction with a dose of 15 mg/kg body weight resulted in an intermediate situation. Therefore, to make meaningful comparisons of induction studies it is useful to pay attention to the injected dose.

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