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Androgen-Linked Control of Carbonic Anhydrase III Expression Occurs in Rat Perivenous Hepatocytes; an Immunocytochemical Study

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ABSTRACT

Carbonic anhydrase (CA) isozymes CAII and CAIII were assayed by a radioimmunosorbent technique in liver cytosolic fractions and in isolated hepatocytes of adult male and female rats. Male livers contained 0.16 mg of CAII and 57 mg of CAIII per g cytosolic protein. Corresponding values for female livers were 0.34 mg CAII and 4 mg CAIII. Similar values and differences between CAII and III were found in isolated hepatocytes.

Neonatal and adult castration of males reduced the CAIII levels to those of the females. Treatment with testosterone for three weeks restored the copulatory behaviour in the males castrated at adult age, but restored only partially the levels of CAIII. No significant effects of the endocrine manipulations were seen on CAII.

Oophorectomy, with or without testosterone substitution, had no significant effect on CAII and CAIII levels in female rats.

Immunohistochemistry and histochemistry showed that the regulation of CAIII is confined to perivenous hepatocytes. CAIII can therefore serve as a useful marker in the separation of these cells.

CAIII appears to belong to the proteins and enzymes of the rat liver, known to be regulated via the hypothalamo-pituitary-liver axis. It may be used as a model of gene regulation in perivenous hepatocytes.

INTRODUCTION

The rodent liver contains the cytosolic carbonic anhydrase (CA; EC 4.2.1.1) isozymes CAI (4), CAII (8) and CAIII (10,21). In addition, there is a mitochondrial isozyme, CAV (16), a membrane-bound form, probably CAXIV (15) and a lysosomal isozyme, related to CAII (19).

Of these isozymes, CAIII and CAII exhibit sexual dimorphism in rat liver with CAIII present at 20-30 times higher concentration in the male liver (21) and with CAII at 3-4 times higher concentrations in the female liver (8). We have shown (4) that these isozymes and trace amounts of CAI

are located in hepatocytes around central veins in liver. Castration of male rats drastically reduces the concentration of CAIII (5), and substitution of testosterone to these males partially restores the levels (22). Also CAII has been shown to be hormonally regulated in the rat liver (6).

It is not known whether the regulation of CAIII and CAII occurs in the perivenous hepatocytes, or whether it involves also other liver cells. The aim of the present work was to quantitate, and locate at the cellular level, the effects of neonatal and adult castration, with or without a subsequent treatment with testosterone, on CAIII and CAII levels in the livers of adult male and female rats; hence extending previous studies based on homogenates.

The amounts of CAII and CAIII in liver homogenates were measured by a radioimmuno-assay, and the distribution of the isozymes in liver sections was studied by immunofluorescence and histochemistry.

METHODS

Animals

The experiments were approved by the local Ethical Committee on Animal Experimentation. The animals were born in the laboratory; Sprague-Dawley females, were 15-17 days pregnant upon arrival to the laboratory from a commercial breeder (ALAB, Sollentuna, Sweden). The environmental conditions implied: temperature 21°C, commercial food pellets, tap water ad libitum and a reversed light cycle from day 25 after birth (light on between 21 and 09 h).

Surgery and treatment

Castration in neonatal males was made at 24-36 h after birth, and in adult males at about 90 days of age. Females were ovariectomized at 75 days of age. The females were given either oil (controls) or 0.1 mg of testosterone propionate (TP) subcutaneously (s.c.), on the fourth day after birth.

All animals were used at an age of about 270 days, and were given oil or 0.5 mg x kg^{-1} body wt of TP in oil, s.c., three times a week for 3 weeks, before they were sacrificed.

Confirmation of endocrine effects

The effects of the early endocrine manipulation were studied according to Meyerson & Eliasson (14). Female controls had a regular 4-day oestrous cycle as estimated by vaginal smears, and all neonatally TP treated females had irregular cycles or were in constant oestrous. Vaginal opening was shown at day 33 both for the neonatal control group, and for the neonatally TP treated females.

The copulatory behaviour was recorded in the ovariectomized females after administration of estradiol benzoate (10 μg x kg^{-1}, s.c.) and progesterone (0.4 mg, given 48 h after estradiol).

All controls, and four out of six early TP treated females, displayed a full lordosis response to the mounting of a male.

The neonatally castrated males were smaller and had a softer fur, and were not willing to copulate even after three weeks of treatment with TP. The males castrated at adult age did not copulate, but all did so after TP treatment.

Tissue Preparation

Before sacrifice the rats were anaesthetised with 75 mg x kg^{-1} of pentobarbital sodium, intraperitoneally (i.p.). Rat livers were perfused with saline before removal. One part was immediately frozen and stored at -70° C for future biochemical assay. The remaining part was cut into slices. Some slices were immersed in Bouin's fluid for 6 h, and then dehydrated and embedded in paraffin for immunofluorescent staining. Other slices were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, and then embedded in the water soluble resin JB for histochemical studies.

After thawing, the livers were homogenised in three parts of 0.04 M-Tris buffer (pH 8.5). The homogenate was centrifuged at 100,000 x g for 60 min and the supernatant was removed and used for analysis. Protein was determined by the Lowry method.

To allow correction for contamination of CAII from blood, known to contain 850 μg CAII x mL^{-1} whole blood (12), haemoglobin was determined in the supernatants by a cyanmethaemoglobin method. No correction for contamination of CAIII was needed since only trace amounts of CAIII are found in rat erythrocytes.

Isolation of hepatocytes

Adult female and male Sprague-Dawley rats of 250 g were anaesthetised with sodium pentobarbital, i.p. The livers were perfused with a collagenase containing buffer through the portal vein, with the inferior caval vein cut open. The suspension of liver cells was then filtered and the cells separated by isopycnic centrifugation in Percoll™ (Pharmacia, Uppsala, Sweden) as described by Pertoft & Smedsrød (17).

Biochemical assay

CAII and CAIII proteins were assayed by a radioimmunosorbent technique (25). The antisera were tested for specificity and affinity by immunodiffusion and radiosorbent techniques. The sensitivity of this radioimmunoassay is 0.2 ng enzyme protein x mL^{-1} tissue fluid, and the precision is 5% in duplicate determinations.

Immunohistochemical demonstration of CAII and CAIII

This was performed as described by Lönnerholm et al. (12). Sections 3-6 μm thick from the liver were put onto gelatine-coated glass slides, deparaffinized and rehydrated through xylol and graded ethanols, and exposed to specific and non-specific (control) rabbit antiserum for 30 min at room temperature. The antisera were tested as serial dilutions from 1:10 to 1: 2560. After incubation the sections were rinsed in phosphate buffered saline (PBS) for 10 min and then exposed to a 1:10 dilution of goat anti-rabbit immunoglobin, labelled with fluorescein isothiocyanate (FITC) (Behring Institut) for 30 min. After a final wash in PBS for 30 min, followed by rinsing in distilled water the sections were mounted under glass cover slips in PBS-glycerol (one part PBS, nine parts glycerol). No counter-staining was used.

Histochemical demonstration of CA activity

The histochemical cobalt-phosphate method was used, as described by Lönnerholm et al. (1986).

CAIII is 10000 times more resistant to inhibition by acetazolamide (American Cyanamid Company) than CAII (11). Incubation of the slices with 10 μM of this inhibitor can therefore be used to differentiate the catalytic activity of CAIII from that of CAII.

RESULTS

Quantitative immuno-assay

Capital letters within parenthesis refer to the different groups of animals in Table1.

CAII. The amount of CAII in the livers of females (F) was about twice that of males (A). Oophorectomy, with (H) or without (G) treatment with testosterone, had no significant effect on the levels of CAII in the female liver.

In the males, castration at neonatal (D) or at adult (B) age, and administration of testosterone (TP) (C and E) to these castrated males had no certain effect on liver CAII.

CAIII. The males (A) had 14 times more CAIII in the liver than the females (F). Castration of the neonatal (D) and adult (B) males, lowered the levels of CAIII to those of normal females. When these castrated males were substituted with TP (C and E) the levels of CAIII tended to increase but were still much lower than those in untreated males (A). In females, oophorectomy with (H) or without (G) addition of TP gave slightly higher levels of CAIII, when compared to the normal untreated animals (F).

The values for CAII and CAIII in isolated hepatocytes of male (I) and female (J) rats corresponded to those of the whole liver homogenates (A and F).

Table 1. Immunoassayable levels of CAII and CAIII in livers of nine months old Sprague-Dawley rats. Figures are means ± SE. TP is testosterone propionate given 3 weeks before sacrifice.

Type of rats and treatment	Number of rats	CAII	CAIII
		mg x g^{-1} protein	
A. Untreated males	11	0.16 ± 0.05	57 ± 6
B. Castrated males	7	0.08 ± 0.02	5 ± 2
C. Castrated males + TP	7	0.11 ± 0.01	8 ± 1
D. Neonatally castrated males	5	0.18 ± 0.02	3 ± 1
E. Neonatally castrated males + TP	6	0.15 ± 0.02	6 ± 2
F. Untreated females	8	0.34 ± 0.08	4 ± 0.4
G. Oophorectomized females	6	0.20 ± 0.03	6 ± 0.8
H. Oophorectomized females + TP	6	0.18 ± 0.02	7 ± 1
I. Isolated hepatocytes from males	2	0.05	90
J. Isolated hepatocytes from females	2	0.07	7

Immunohistochemistry of CAII and CAIII proteins, and histochemistry of CA activity

CAII. Immunofluorescent staining with anti-CAII demonstrated presence of CAII in all groups of animals (not shown). The staining was mainly found in hepatocytes surrounding central venules, but also in the epithelium of bile ducts. The perivenous staining tended to be weaker and varied more between individuals and groups in the male rats than in the female rats. There was no clear correlation between the degree of staining and the amounts of CAII found in the different groups of males (Table 1). This is understandable since the assayable amounts of CAII only varied two-fold.

An acetazolamide-sensitive histochemical staining of CAII was seen only in the perivenous hepatocytes where CAII was fluorescing.

CAIII. The sexual dimorphism, as found by the quantitative immunoassay (Table 1), was clearly reflected in the immunofluorescence staining with anti-CAIII antiserum. The CAIII staining varied markedly between the groups. All untreated males showed intense, specific fluorescence (Fig. 1). Hepatocytes surrounding central venules were most intensely stained, with a gradual decrease towards the periphery.

Among castrated males treated with TP (C), weak positive CAIII staining was seen in a few perivenous hepatocytes (Fig. 2).

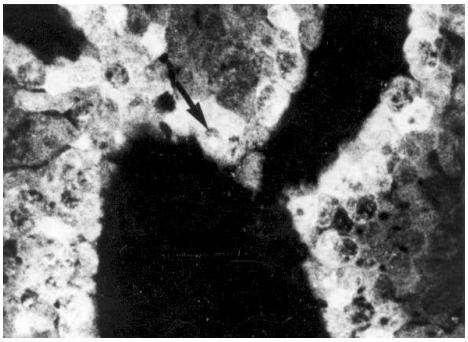

Fig. 1. Adult untreated male (group A). CAIII immunostaining. Note strong fluorescence in the hepatocytes surrounding central venules. In some otherwise stained cells the unstained nucleus stands out as a dark spot. In other cells (arrow) the nucleus is clearly stained. 330x.

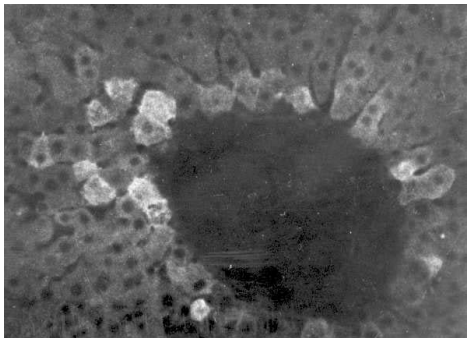

Fig. 2. Adult castrated male treated with testosterone (group C). CAIII immunostaining. A few stained hepatocytes are seen around a central venule. 330x.

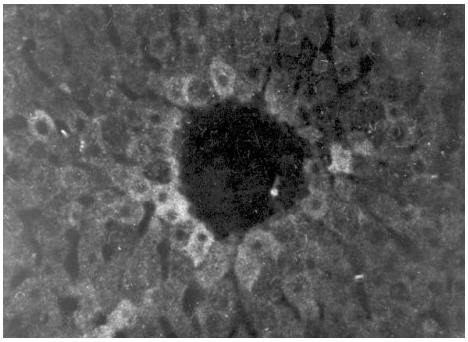

Fig. 3. Adult castrated male (group B). CAIII immunostaining. Very weak staining of a few perivenous hepatocytes. 330x.

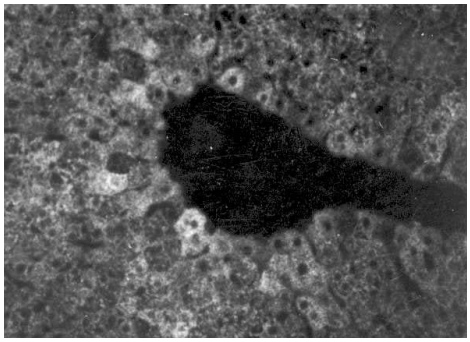

Fig. 4. Adult oophorectomized female treated with testosterone (group H). CAIII immunostaining. Weak staining of a few perivenous hepatocytes. 330x.

In the other groups of males (B, D, E) there was no, or occasional CAIII faint staining of a few cells (Fig. 3).

Untreated females showed no CAIII staining (not shown). In the oophorectomized females treated with TP (H), some weak staining of perivenous hepatocytes was found (Fig.4).

Acetazolamide-resistant weak histochemical staining, indicating the presence of CAIII, was only found in perivenous hepatocytes of some untreated males.

DISCUSSION

Location and regulation of CAII and CAIII in perivenous hepatocytes

The present results and previous findings (see Introduction) show that CAIII is much more abundant in the liver and isolated hepatocytes of male rats than in female rats.

Castration of adult and neonatal male rats resulted in low concentrations of CAIII in the livers, that could not be detected by the immunofluorescent and histochemical methods. Treatment of the adult castrated males with testosterone induced copulatory behaviour and slightly raised the amount of CAIII in the livers, but not to the levels of adult untreated males. The most plausible explanation is that the amounts of testosterone used were inadequate to compensate for the effect of castration. Indeed, it has been shown by Shiels et al. (22) that the levels of CAIII in castrated males are dependent on the dose of testosterone, and how it is administered These authors also could show that testosterone promotes, whereas oestrogen inhibits the synthesis of CAIII.

The levels of CAII were 12 and 356 times lower than those of CAIII in female and male livers, respectively. The levels were not significantly changed by castration or substitution with testosterone in either sex. However, Jeffery et al (8), showed that oestrogen treatment of castrated males gives values of CAII higher than those of females. Also, Garg (6) reported that CAII can be induced by oestrogen in the livers of both male and female rats. Thus, it appears that CAII can be influenced by endocrine manipulation but to a much smaller extent than CAIII.

Both immunofluorescence and histochemistry localised the regulation of CAIII and CAII to the perivenous hepatocytes. This specific localisation could indicate that there are different types of hepatocytes, perhaps with different functions. These hepatocytes may now be separated, using CAIII as a marker. However, one difficulty with such a separation is that isolated hepatocytes have been found (2) to rapidly lose their CAIII content, and addition of epidermal growth factor, insulin-like growth factor, or insulin to the culture medium, fails to change this reduction.

The levels of CAIII in male rat livers are age-dependent, which would suggest that they reflect changes in levels of testosterone and/or androgen receptors during development (22). CAIII may therefore be seen also as a senescence marker protein (20).

Jeffery et al. (9) have shown that the testosterone control of liver CAIII is an indirect one, insofar as testosterone is acting on the pituitary rather than directly on the liver. They could further show that the secretion of growth hormone regulates the sexual dimorphism of liver CAIII and CAII. The cytoplasmic isozymes of CA therefore may be included among the liver proteins and enzymes, which are regulated by the so-called hypothalamic-pituitary-liver axis (7). However, the role of growth hormone as a physiological regulator of sexual differentiation of liver function is still obscure.

It is also intriguing that CAIII in livers of other species, such as mice, is not sex-dependent (23,24). The androgen-linked control of CAIII in the rat liver is still therefore a mystery. However, it should be possible to use CAIII as a tool in the study of gene regulation in perivenous hepatocytes.

CAIII is also abundant in skeletal red muscle fibres and in adipocytes, but there is no sex-dependent expression of CAIII in these tissues (3,13). However, after denervation of the skeletal muscle CAIII increases several-fold in the white muscle fibres (3). This is different from the liver where the regulation of CAIII occurs within the perivenous hepatocytes, where CAIII is found normally.

The function of CAII and CAIII in the liver

CA isozymes are catalysts of the forward and reverse reaction $CO_2 + H_2O \leftrightarrow H_2CO_3$. The location of CAII in hepatocytes of both sexes is compatible with a role in the secretion of bicarbonate ions into the canalicular bile (1,4).

However, the function of the large, hormone-regulated amounts of CAIII in liver, remains an enigma. Since CAIII is a much slower catalyst in the CO_2 - H_2CO_3 system than the other CA isozymes, there has been speculation about roles for CAIII outside this system (13). Recently, CAIII was found to protect cells from oxidative damage (18). Why this seemingly important property should be regulated in male liver is difficult to understand, however. There is an urgent need for a selective inhibitor, which would enable further studies of the function of CAIII.

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