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SERUM AND LIVER ALKALINE PHOSPHATASE IN ETHINYLESTRADIOL-INDUCED CHOLESTASIS (A PARALLEL BIOCHEMICAL AND MORPHOLOGICAL STUDY)

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The purpose of this paper was to investigate the effect of higher estrogenizing EE doses on serum APase and to compare these results with the histochemical and ultrastructural assessment of the enzyme in the liver under the same conditions. We induced cholestasis with EE in doses of 3 and 6 mg/kg b. m. in 93 female rats. The effect of EE on the activity of both serum and tissue APase on the 24th hour as well as after a 3, 5, and 7-day-long treatment was analyzed. The influence of EE on the hepatic APase activity was semiquantitatively read (-, ±, +, ++, +++) on tissue sections after demonstration accorrding to Gomori's method and ultrastructurally, after identification according to the method of Hugon and Borgers. There was an enhancement of both serum and hepatic APase activity by 100 per cent established already on the 24th hour when EE in a dosis of 6 mg/kg b.m. was applied but hardly after the third day when EE in a dosis of 3 mg/kg b. m. was administered. The maximal effect of increasing enzyme activity in the superficial hepatocytic membranes was achieved on the third day with the dosis of 6 mg/kg b. m. but on the fifth day with the dosis of 3 mg/kg b. m.; in the serum, these time intervals were 7 and 3 days, respectively. There were great differences concerning the intensity and distribution in the hepatic lobulus - from a completely absent enzyme induction to a disseminated enzyme reaction in all the areas of the hepatic lobules. Electron microscopically, a progressive damage of the canalicular villi manifested by a vacuolization, reduction and even loss of APase activity as well as by an appearance of enzyme reaction in the lateral and sinusoidal membranes of hepatocytes was observed. APase secretion through the lateral and sinusoidal membranes of hepatocytes could explain the increased enzyme values in the serum.Our investigation demonstrated that higher estrogenizing doses caused a rapider and more intensive enzyme induction in the hepatocytic membranes in 80-100 per cent of the animals. Serum enzyme elevation was detected in all the animals which was probably due to the enhancement of the osseous isoenzyme, too.

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Alkaline phosphatase (APase) is one of the enzymes which increases first in the serum with cholestasis (1,4,7,9,10). It is considered an indicator of hepatocellular damage prior to the changes of ALAT, ASAT, and serum bilirubin (5). Ethinylestradiol (EE) causes a reversible cholestasis in humans and animals which mechanism remains unclarified yet (2,6,8). According to the investigation by using an experimental model on rats reported by Simon and Irvin, serum APase is not elevated in EE-induced cholestasis. In our previous study on rats, we established that serum APase increased in 54,5 per cent of the animals after a 7-day EE treatment with a dosis of 1 mg/kg b. m. (3).

The purpose of the present paper is to assess the effect of the higher estrogenizing EE doses on serum APase level as well as to compare these results with data from the histochemical and ultrastructural investigation of this enzyme in the liver under these conditions.

MATERIAL AND METHODS

The experiment covered 103 female rats divided in 7 groups of 12 animals each but group eight given 6 mg/kg b. m. ethinylestradiol on the 7th day consisted of 19 animals. The rats were treated with EE (17-α-Ethinylestradiol from Organon, the Netherlands) diluted in sunflower oil in two different daily doses, i. e., of 3 and 6 mg/kg b. m. EE was subcutaneously introduced for 1, 3, 5 and 7 days. Blood for examination was obtained from the sublingual vein immediately prior to decapitation after ethereal narcosis. APase was determined after serum separation using Farb-test (Boehringer, Germany). The results from the biochemical examination were statistically processed by the variation analysis. We made use of Student-Fisher's t-distribution when estimating p-values.

Gomori's method (Pierce, 1969) was applied to demonstrate APase in tissue sections. Enzyme activity was semiquantitatively read according to the following degrees: (-) - absent activity; (±) - weak and discontinued activity; (+) - regular and moderately expressed activity; (++) - regular and well-expressed activity, and (+++) - regular and strongly expressed activity.

Electron-microscopically, we identified APase activity after the method of Hugon and Borgers (Gayer, 1979). Incubation was carried out in an medium with sodium-β-glycerophosphate as a substrate at 37^{0}C for one hour.

RESULTS AND DISCUSSION

1. Biochemical examination

Table 1
Serum AP ase in EE-induced experimental cholestasis of intact female rats

EE dosages per day	24th hour	3rd day	5th day	7th day
3 mg/kg b. m.	47,3 ± 3,92 (12)	169 ± 11,97 (12)*	154 ± 18,5 (12)*	151 ± 16,9 (12)*
6 mg/kg b. m.	101 ± 10,39 (12)*	169 ± 8,4 (12)*	68 ± 5,12 (12)	215 ± 19,8 (6)*
Controls	46 ± 14 (6)			
Controls (Organon)				69,5 ± 12,3 (6)

* $p < 0,001$
Digits in brackets indicate the number of animals

No changes were observed 24 hours after the first application of EE in a dosis of 3 mg/kg b.m. However, we found out a statistically significant increase of APase activity ($p < 0,001$) after the third EE application. This enhancement was greatest after the fifth injection. After a 7-day long treatment, APase level decreased statistically insignificantly (Table 1).

We established a statistically reliable increase of serum APase level already on the 24th hour when EE was applied in a dosis of 6 mg/kg b. m. ($p < 0,001$) while APase values of 3-day-long treated animals exceeded by three times the control ones. However, after a 5-day-long treatment, there was a statistically significant reduction of the enzyme activity followed by its sharp enhancement after the 7th day (Table 1).

2. Histochemical investigation

In the control animals, there was no enzyme activity in hepatocytic membranes or only a weak reaction was detected in periportal hepatocytes. APase was established in the adventitia of arterial blood vessels within the large portal spaces and in the lymph vessels surrounding the branches of portal veins within the small portal spaces.

EE treatment induced APase increasing in the superficial hepatocytic membranes in both animal groups. The higher dosis caused an earlier and better expressed, more long-lasting and disseminated APase activity in the superficial hepatocytic membranes (Table 2). EE in a dosis of 6 mg/kg b. m. reached its maximal effect on the third day while EE in a dosis of 3 mg/kg b. m. did so on the fifth one (Fig. 1). Enzyme activity varried according to

Table 2
Liver APase distribution after EE treatment

EE	24th hr		3rd day		5th day		7th day		C
Parameters	n=12 3 mg/ kg	n=12 6 mg/ kg	n=12 3 mg/ kg	n=12 6 mg/ kg	n=12 3 mg/ kg	n=12 6 mg/ kg	n=12 3 mg/ kg	n=19 6 mg/ kg	
Biliary canaliculi									
zone 1	12-	12±	12-	9- 3±	1+	3- 3±	12-	17- 2±	
zone 2	12-	12±	12-	1-3±1+ 3++ 4+++	2- 1± 4+ 5++	1- 5+ 4+ 2++	12±	11-5± 3+	
zone 3	12-	12±	12-	2± 2+ 3++ 5+++	2- 1± 4+ 5++	3- 5+ 2++ 2+++	6- 6±	3- 10± 2+ 3++	
Vascular branches									
v. portae	3- 9±	9±	12±	12-	12-	12-	12-	11- 6±	±
a. hepatica	12++	12+++	4++	1+ 11++	6- 6+	5++ 7+++	12++	10+ 8++ 1+++	++
vv. centrales	-	-	-	-	-	-	-	-	-
Lymph network around biliary canaliculi	5++ 7+++	12+++	12++	10+ 12++	12++	12++	12+	18+ 1++	++

C - control rats treated with sunflower oil only

zone 1 - a centroacinous zone of the hepatic lobule (around *v. centralis*)
zone 2 - an intermediary zone of the hepatic lobule
zone 3 - a periportal zone of the hepatic lobule

(-) - absent activity; **(±)** - weak and discontinued activity; **(+)** - regular and moderately expressed activity; **(++)** - regular and well-expressed activity, and **(+++)** - regular and strongly expressed activity

distribution and intensity from degree one to degree four. In both groups, we established an intensified enzyme reaction in the adventitia of arterial vessels as well as in the lymphatic network around the bile ducts.

3. Electron-microscopic and enzymohistochemical investigation

We observed an enzyme reaction at the apical part of canalicular microvilli in the control rats (Fig. 2).

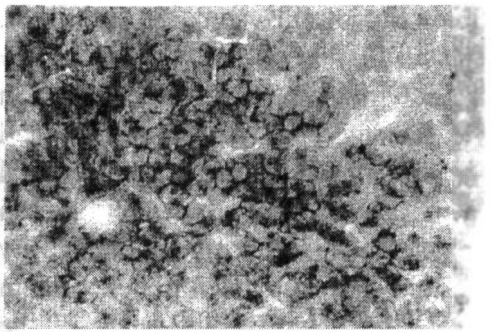

Fig. 1. *Hepatic APase activity in rats treated with EE in a dosis of 6 mg/kg b. m. for 7 days. An outlined reaction in the periportal and intermediate zones is observed. There is no activity in the portal space. Magn. x 160*

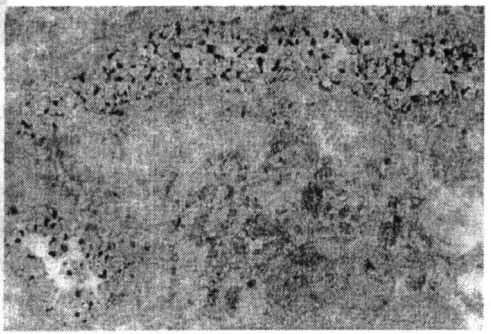

Fig. 2. *APase activity in the canalicular microvilli in the liver of intact female rats. Magn. x 11000*

EE treatment induced a progressive damage of the canalicular microvilli manifested by their vacuolization and reduction. This process was accompanied by the loss of APase activity (1). The disappearance of APase activity of the canalicular microvilli was combined with the appearance of an enzyme reaction on the lateral and sinusoidal

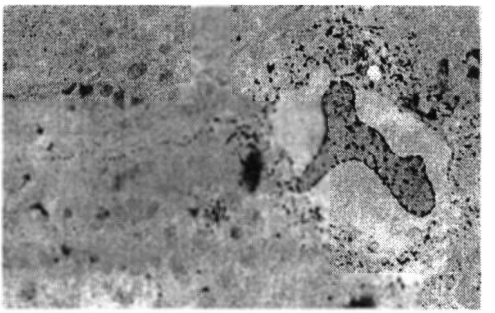

Fig. 3. *An extremely reduced APase activity in the canalicular microvilli is found out. The enzyme activity in the lateral cellular membranes, sinusoidal microvilli, endothelial cell processi and erythrocytic membranes is well-expressed. EE treatment in a dosis of 1 mg/kg b. m. for 5 days. Magn. x 5000*

membranes of hepatocytes after a 5-day-long EE treatment (Fig. 3).

The comparison of the results from the biochemical, enzymo-histochemical and ultrastructural investigations of APase in both serum and tissue sections reveals a stressing parallel increase of APase activity in the serum and liver. The reduction of the enzyme in the serum is accompanied by its diminution in the tissue sections. The serum enzyme elevation can be explained by the increased enzyme reaction in the lateral and sinusoidal membranes. Analogous changes of the enzyme activity have been observed in an experimental model of mechanical cholestasis in rats (Macoto et al., 1980).

According to the data of Ohno et al. (1994), the enhanced serum

APase activity under the influence of EE is related not only with the increase of the hepatic isoenzyme but also with that of the osseous one. The hepatic isoenzyme but not the osseous one is elevated in adrenalectomized rats given corticosterone or aldosterone (Tojo et al., 1994).

The increase of APase in the liver can be explained by the ability of bile acids to induce an intensive APase synthesis by the hepatocyte (Hatoff and Hardison, 1981; Secratam et al., 1986) while its redistribution in the lateral and sinusoidal membranes is probably due to the reversibility of the secretory polarity of the hepatocyte which has been proved for the carriers of bile acids in mechanical cholestasis (Friker et al., 1985). Under conditions similar to our own ones, i. e. with EE treatment in a dosis of 5 mg/kg b. m. for 5 days, Bossard et al. (1993) establish an elevation of serum APase by 2,5 times but of bile acids by 11 times. Our results are partially confirmed by the data of Accatino et al. (1995). On the isolated and perfused liver and inoculated canalicular and sinusoidal membranes, these authors observe an enhancement and considerable lability of this intrinsic membrane protein under the detergent influence of the bile acids secreted.

The individual semiquantitative reading of the enzyme reaction demonstrates great differences concerning the intensity and distribution of the enzyme reaction in the hepatic lobulus.These individual differences range from a complete absence of enzymic induction to a disseminated enzymic reaction in all the areas of the hepatic lobuli. Besides these individual differences in EE-sensitive animals, the dependence on the dosage and duration of treatment stresses, too.

REFERENCES

1. Accatino, L., C. Figueroa, M. Pizarro, N. Salis. *J. Hepatol.*, **22**, 1995, No 6, 658-670.- 2. Bossard, R., B. Stieger, B. O'Neill, G. Fricker, et al. *J. Clin. Invest.*, **91**, 1993, 2714-2720.- 3. Bratchkova, J., P. Bozhidarov, T. Yankova.- In: Third Congr. Bulg. Pathol. Diseases of Modern Society. Tolbukhin, 9-11.X.1982. Abstracts, p. 94.- 4. Desmet, V. J. *Progr. Liver Dis.*, 1973, 97-129.- 5. Hatoff, D. E., W. Hardison. *Gastroenterology*, **80**, 1981, 666-672.- 6. Lorenz, R. Z. *Allgemeinmed.*, **56**, 1980, No 32, 2237-2240.- 7. Makoto, K., T. Gotaro, M. Tori, et al. *Gastroenterol Jap.*, **15**, 1980, No 6, 600-605.- 8. Ohmo, H., H. Tojo, T. Kajimura, M. Nomura. *J. Toxicol. Sci.*, **3**, 1994, Suppl. 19, 507-518.- 9. Sectaram, S., N. L. Sussman, T. Komoda,

D. H. Alpert. *Hepatology*, **6**, 1986, No 3, 374-380.- 10. Simon, R. F., A. M. Irvin. *J. Clin. Invest.*, **52**, 1973, 765-775.- 11. Tojo, H., H. Ohno, M. Nomura. *J. Toxicol. Sci.*, **3**, 1994, Suppl. 19, 519-530.

Алкална фосфатаза в серума и черния дроб при холестаза, предизвикана с Ethinylestradiol (паралелно биохимично и морфологично изследване)

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Резюме: Целта на настоящата работа е да се изследва ефектът на високите естрогенизиращи дози Ethinylestradiol (ЕЕ) върху алкалната фосфатаза (АФ) в серума и се съпоставят резултатите с хистохимичното и ултраструктурно изследване на ензима в черния дроб при тези условия. Холестазата е предизвикана при 103 женски плъха с ЕЕ в дози от 3 и 6 mg/kg тегло. Ефектът на ЕЕ върху активността на серумната и тъканна АФ се изследва на 24. час и след 3, 5 и 7-дневно въздействие. Ефектът на ЕЕ върху чернодробната АФ се отчита полуколичествено (-, ±, +, ++ и +++) на тъканни срези след изявяване по метода на Gomori и ултраструктурно - след изявяване по метода на Hugon and Borgens. Покачване на нивото на серумната и тъканна АФ се наблюдава в 100 % от случаите още на 24. час при въздействие с 6 mg/kg ЕЕ, а при третиране с 3 mg/kg ЕЕ - едва на третия ден. Максимален ефект на нарастване на ензимната активност в повърхностните хепатоцитни мембрани при дози от 6 mg/kg се постига на третия и с 3 mg/kg - на петия ден, а в серума - съответно на седмия и третия ден. Има големи различия по отношение на силата и разпространение на ензимната реакция в чернодробното делче - от пълна липса на ензимна индукция до разпространена ензимна реакция във всички зони на чернодробните делчета. Електронно-микроскопски се установява прогресивно увреждане на каналикуларните микровили, изразяващо се с вакуолизиране, редуциране и загубване активността на АФ и с поява на ензимна реакция на латералните и синусоидални мембрани на хепатоцитите. Секрецията на АФ през латералните и синусоидални мембрани на хепатоцитите може да обясни повишението на ензима в серума. Изследването показва, че по-високите естрогенизиращи дози водят до по-бърза и по-силна ензимна индукция в хепатоцитните мембрани в 80-100 % от животните. Покачването на ензима в серума се открива при всички животни, което вероятно се дължи на повишението и на костния изоензим.