EFFECT OF ESTROGEN ON LIVER PLASMA MEMBRANE IN RATS

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Abstract.....The effect of estrogen on plasma membrane was investigated using the primary cultured rat hepatocytes treated with carbon tetrachloride (CCl_4) and the isolated plasma membrane of rat liver. 17β -Estradiol (E_2), at concentrations of 10^{-10} M to 10^{-4} M, 10^{-8} M to 10^{-6} M and 10^{-12} M to 10^{-4} M, had an inhibitory effect on the CCl_4 -induced leakage of glutamic oxaloacetic transaminase, glutamic pyruvic transaminase and lactate dehydrogenase, respectively from primary cultured rat hepatocytes. Diethylstilbestrol, which caused inhibition at a dose of 10^{-4} M, did not inhibit any enzyme leakage at any further concentrations of 10^{-12} M to 10^{-6} M.

In the isolated plasma membrane of rat liver, Mg^{2+} - and Na^+ , K^+ -adenosine triphosphatase activity was increased by E_2 treatment at concentrations of 10^{-6} M and 10^{-4} M.

Key words : Estrogen, liver, plasma membrane, enzyme leakage, carbon tetrachloride, adenosine triphosphatase.

INTRODUCTION

The effects of estrogen on liver function have been long reported, but the results often differ. Some reports have indicated that estrogen induces the leakage of liver enzymes into serum (Ishibe, 1975 ; Leis et al., 1978 ; Saoshiro, 1980 ; Shinkawa et al., 1985 ; Smith and Sizto, 1983 ; Wadsworth et al., 1977), and this is disputed by others (Cowan et al., 1982 ; Gopinath et al., 1978 ; Heywood and Wadsworth, 1980 ; Leis et al., 1978). In the previous study (Nagae et al., 1991) we had examined the effect of 17β -estradiol (E_2) on the liver function in ovariectomized rats, and have shown that the elevated serum transaminase levels by E_2 are not coincident with histological changes suggestive of liver dysfunction and are attributed to the accelerated synthesis

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of the enzymes. There has been also a possible effect suggested on plasma membrane permeability. The present study investigated the effect of E_2 on leakage of enzymes from primary cultured rat hepatocytes treated with carbon tetrachloride (CCl_4) which is known to injure plasma membrane (Dolak et al., 1988 ; Zimmerman, 1978). A non-steroidal estrogen, diethylstilbestrol (DES) was compared with E_2 in the examination, considering that DES was reported to have the dysfunctional effect on the liver (Ishibe, 1975 ; Shinkawa et al., 1985), and a non-estrogenic steroid, progesterone done as same. The effect of E_2 on adenosine triphosphatase (ATPase) of rat liver plasma membrane was also examined.

MATERIALS AND METHODS

Chemicals :

The following chemicals were used : collagenase type I, trypsin inhibitor type II-S from soybean, DES, bovine pancreatic insulin and adenosine 5'-triphosphate tris [hydroxymethyl]-aminomethane salt (Tris ATP) (Sigma Chemicals, St Louis, MO) ; 17β -estradiol (E_2), progesterone, dexamethasone and CCl_4 (Wako Pure Chemical Industries, Osaka, Japan) ; Williams medium E and calf serum (GIBCO Laboratories, Grand Island, NY) ; penicillin-streptomycin-fungizone mixture (Whittaker M. A. Bioproducts, Walkersville, MD).

Experimental animals :

Crj : CD(SD) male rats (Charles River Japan), 4–6 months of age with a body weight of 400–500 g were used in all experiments. The rats were maintained at a constant temperature and humidity ($23 \pm 3^\circ\text{C}$, $50 \pm 20\%$) with a 12-hour light-dark cycle and were given water and a CE-2 diet (CLEA Japan) ad libitum.

Effect on CCl_4 -induced enzyme leakage from primary cultured rat hepatocytes :

Hepatocytes were isolated by the *in-situ* collagenase perfusion method described by Seglen (1976), the cells were suspended in Williams medium E supplemented with 10% calf serum, insulin (1×10^{-9} M), dexamethasone (1×10^{-6} M), penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$) and fungizone (0.25 $\mu\text{g/ml}$), seeded at a density of 0.4×10^5 cells/cm² on 60-mm diameter collagen-coated plastic dishes (CORNING/IWAKI GLASS, Tokyo, Japan), and cultured as monolayers in 4 ml of medium at 37°C in a humidified atmosphere containing 5% CO_2 in air. The medium was replaced 3 and 18 hours after seeding. After a 40-hour incubation period, the medium was changed to one without serum and hormones. The experiment was initiated 6 hours thereafter. Viability of the hepatocyte preparation was shown to be 90% or higher at initiation of the experiment. 17β -estradiol (E_2) was dissolved in propylene glycol and 50 μl of the E_2 solution added to the media to make final concentrations of 10^{-12} M to 10^{-4} M. Immediately after treatment with E_2 , hepatocytes were exposed to CCl_4 dissolved in ethanol at the final concentration of 2.6 mM, 200 μl of the CCl_4 solution being added to each medium. Control hepatocytes were treated with a vehicle for E_2 and with CCl_4 at the final

concentration of 2.6 mM. Media, which were not changed through the experiment, were sampled 1, 2 and 3 hours after treatment with CCl_4 and their activity analyzed for glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and lactate dehydrogenase (LDH) by the UV-method. A non-estrogenic steroid, progesterone and a non-steroidal estrogen, DES were also estimated for their possible effects on CCl_4 -induced enzyme leakage using primary cultured rat hepatocytes treated in the same manner as with E_2 . Six experiments were conducted with each hormone.

Effect on Mg^{2+} - and Na^+ , K^+ -ATPase of rat liver plasma membrane :

The preparation of liver plasma membranes from rats was obtained by the method of Emmelot et al. (1964) modified by Koizumi et al. (1976). After homogenization of liver in 0.5 mM CaCl_2 and 1 mM NaHCO_3 (pH 7.5) with a Dounce homogenizer and centrifugation (2000 g for 15 minutes), the pellet was suspended in 68 w/v% sucrose. The same volume of 42 w/v% sucrose was layered on the suspension, and the crude plasma membranes were obtained by ultracentrifugation (77000 g for 60 minutes). The crude membranes were suspended in 68% sucrose, and a discontinuous gradient of 42 w/v% and 48 w/v% sucrose was layered on it. After a second ultracentrifugation (77000 g for 60 minutes) the purified plasma membranes were taken from the interface between the zones of 42 w/v% and 48 w/v% sucrose. Electron micrographs showed that the preparation was rich in plasma membranes and was free from other components. Experiments were done with freshly prepared plasma membranes which were suspended in ice-cold distilled water.

ATPase activity was determined at 37°C in the medium of Quigley and Gotterer (1969). One set of tubes contained tris ATP (5 mM), Na^+ (129 mM), K^+ (20 mM), Mg^{2+} (7.5 mM) and tris (30 mM) buffer (pH 7.1) for assay of Na^+ , K^+ , Mg^{2+} -ATPase. In the second set Na^+ and K^+ were omitted, for Mg^{2+} -ATPase. E_2 was dissolved in propylene glycol, and added in the medium at the final concentrations of 10^{-8} M to 10^{-4} M. An appropriate amount of propylene glycol was added for the control. After the reaction had been started by addition of plasma membrane suspensions, tubes were incubated for 15 minutes, after which they were plunged into ice, and ice-cold trichloroacetic acid was added to 10 w/v%. After centrifugation (2000 g for 10 minutes at 4°C) the supernatant was analyzed for liberated inorganic phosphorus (Pi) (Fiske and Subbarow, 1925). Allowance was made for Pi present in membranes and that derived from non-enzymic hydrolysis of ATP. The protein in a sample of membranes was determined (Lowry et al., 1951) with bovine serum albumin as a standard. Experiments were done duplicated with each of the three plasma membrane preparations.

Statistical analysis :

The enzyme activity was statistically analyzed by the F-t test.

RESULTS

Effect on CCl₄-induced enzyme leakage from primary cultured rat hepatocytes :

Leakage of GOT, GPT and LDH from the primary cultured hepatocytes into the media was apparent one hour after treatment with CCl₄ in the control dishes. The GOT and GPT activity did not so largely alter from one hour to three hours after the treatment with CCl₄, while the LDH values showed the highest activity one hour after the treatment, and gradually decreased thereafter. Simultaneous treatment of E₂ had significant inhibitory effects on the CCl₄-induced enzyme leakage from the primary cultured hepatocytes at concentrations of 10⁻¹⁰ M to 10⁻⁴ M for GOT, 10⁻⁸ M to 10⁻⁶ M for GPT and 10⁻¹² M to 10⁻⁴ M for LDH (Table 1). Progesterone also significantly inhibited the CCl₄-induced enzyme leakage from the hepatocytes at concentrations of 10⁻¹⁰ M to 10⁻⁴ M for GOT, 10⁻¹⁰ M to 10⁻⁴ M for GPT and 10⁻⁸ M to 10⁻⁴ M for LDH (Table 2). DES, which caused inhibition at 10⁻⁴ M, did not inhibit the enzyme leakage at any further concentrations of 10⁻¹² M to 10⁻⁶ M (Table 3).

The effects of E₂, progesterone and DES on enzyme leakage from non-treated primary cultured hepatocytes were also individually estimated at concentrations of 10⁻¹² M to 10⁻⁴ M. Any concentration of E₂ or progesterone did not induce significant changes in the enzyme concentration, while DES caused enzyme leakage (124.3±5.7 IU/l, 46.0±1.6 IU/l and 67.3±14.9 IU/l, mean ± standard deviation of GOT, GPT and LDH, respectively at 3 hours) from the non-treated hepatocytes into the media at a concentration of 10⁻⁴ M, compared to the vehicle control (6.3±3.0 IU/l, 3.0±1.6 IU/l and 12.0±24.0 IU/l for GOT, GPT and LDH, respectively).

Effect on Mg²⁺- and Na⁺, K⁺-ATPase of rat liver plasma membrane :

Enzyme activity of non-treated plasma membranes isolated from rat liver was 49.2±12.8 and 8.4±3.5 μmol Pi/mg protein/hour for Mg²⁺-ATPase and Na⁺, K⁺-ATPase, respectively (mean ± standard error of the three preparations). Both Mg²⁺-ATPase and Na⁺, K⁺-ATPase activity was increased by E₂ treatment at concentrations of 10⁻⁶ M and 10⁻⁴ M (Fig. 1).

DISCUSSION

GOT, GPT and LDH leaked from the primary cultured rat hepatocytes into the media apparently one hour after treatment with CCl₄. The GOT and GPT activity did not so largely alter in the media from one hour to three hours after the CCl₄-treatment, suggesting that the enzyme leakage occurs mostly within one hour after the treatment under this experimental condition. The LDH values showed the highest activity one hour after the treatment, and gradually decreased thereafter. In the experiment we did not add calf serum to the media in order to eliminate any effects of steroids present in the serum. The media without albumin might affect the stability of released LDH (Eisenmann et al., 1984).

Table 1. Effect of 17 β -estradiol on CCl₄-induced enzyme leakage from cultured rat hepatocytes.

		Concentration of 17 β -estradiol (M)					
		0	10 ⁻¹²	10 ⁻¹⁰	10 ⁻⁸	10 ⁻⁶	10 ⁻⁴
GOT	1 hr (n=6)	145.2 $\pm$ 20.6	107.5 $\pm$ 37.8	88.7 $\pm$ 39.8*	87.2 $\pm$ 33.6**	82.8 $\pm$ 28.9**	92.8 $\pm$ 42.8*
	2 hr (n=6)	158.2 $\pm$ 45.2	105.7 $\pm$ 66.1	90.3 $\pm$ 58.0*	80.2 $\pm$ 55.2*	77.5 $\pm$ 57.6*	84.7 $\pm$ 65.5*
	3 hr (n=6)	147.5 $\pm$ 24.7	131.7 $\pm$ 27.4	124.8 $\pm$ 26.6	121.7 $\pm$ 25.6	118.8 $\pm$ 23.7	123.0 $\pm$ 29.1
GPT	1 hr (n=6)	55.3 $\pm$ 3.3	49.8 $\pm$ 7.5	46.7 $\pm$ 8.9	46.0 $\pm$ 8.4*	43.8 $\pm$ 7.4**	46.5 $\pm$ 9.4
	2 hr (n=6)	56.0 $\pm$ 5.3	47.3 $\pm$ 12.1	44.8 $\pm$ 13.2	42.7 $\pm$ 13.1*	41.0 $\pm$ 12.8*	42.5 $\pm$ 15.7
	3 hr (n=6)	54.8 $\pm$ 5.1	56.0 $\pm$ 5.2	55.3 $\pm$ 4.7	55.7 $\pm$ 6.3	53.3 $\pm$ 5.1	54.3 $\pm$ 5.7
LDH	1 hr (n=6)	105.7 $\pm$ 22.0	70.7 $\pm$ 26.9*	56.8 $\pm$ 25.7**	52.2 $\pm$ 14.2**	53.7 $\pm$ 14.7**	59.3 $\pm$ 15.5**
	2 hr (n=6)	44.0 $\pm$ 9.1	41.0 $\pm$ 9.6	28.5 $\pm$ 10.1*	38.7 $\pm$ 9.3	35.0 $\pm$ 9.3	33.5 $\pm$ 15.7
	3 hr (n=6)	29.3 $\pm$ 11.3	37.3 $\pm$ 9.1	37.5 $\pm$ 10.2	34.5 $\pm$ 13.8	24.8 $\pm$ 3.6	32.3 $\pm$ 10.1

Primary cultured hepatocytes were exposed to CCl₄ at the concentration of 2.6 mM immediately after treated with 17 β -estradiol.

Each value represents group mean $\pm$ standard deviation (IU/l).

* and ** Significantly different from 0 M (Control), P<0.05 and 0.01, respectively.

Table 2. Effect of progesterone on CCl_4 -induced enzyme leakage from cultured rat hepatocytes.

		Concentration of progesterone (M)					
		0	10^{-12}	10^{-10}	10^{-8}	10^{-6}	10^{-4}
GOT	1 hr (n=6)	166.2±23.8	147.0±30.1	123.3±12.5**	127.5±42.2	110.8±42.4*	109.3±43.5*
	2 hr (n=6)	242.8±23.5	196.2±45.9	156.7±50.6**	150.5±46.1**	156.3±39.8**	144.8±36.2**
	3 hr (n=6)	261.8±23.0	258.5±18.8	248.0±18.9	258.0±29.2	269.8±51.5	245.5±21.8
GPT	1 hr (n=6)	71.8±3.5	71.2±4.4	68.0±5.6	67.0±8.8	64.8±7.1	65.3±8.5
	2 hr (n=6)	76.5±4.3	73.7±5.4	67.5±6.2*	69.3±7.4	68.0±5.9*	68.0±6.0*
	3 hr (n=6)	75.7±7.7	75.2±4.4	74.7±5.9	77.3±6.5	75.8±4.5	78.0±5.8
LDH	1 hr (n=6)	181.0±11.0	164.2±28.7	167.7±84.1	122.3±40.7*	111.3±24.7**	126.8±32.5**
	2 hr (n=6)	146.2±25.5	115.0±30.1	116.8±88.8	79.0±22.2**	71.5±19.5**	89.0±17.3**
	3 hr (n=6)	135.0±18.2	125.5±25.6	140.2±82.0	117.3±23.0	104.0±12.4**	109.2±28.5

Primary cultured hepatocytes were exposed to CCl_4 at the concentration of 2.6 mM immediately after treated with progesterone.

Each value represents group mean ± standard deviation (IU/l).

* and ** Significantly different from 0 M (Control), $P < 0.05$ and 0.01, respectively.

Table 3. Effect of diethylstilbestrol on CCl_4 -induced enzyme leakage from cultured rat hepatocytes.

		Concentration of diethylstilbestrol (M)					
		0	10^{-12}	10^{-10}	10^{-8}	10^{-6}	10^{-4}
GOT	1 hr (n=6)	137.0±25.7	144.5±26.7	141.5±19.2	133.8±22.3	132.3±23.8	122.0±10.8
	2 hr (n=6)	135.7±15.6	135.7±10.2	133.0±12.4	130.2±8.2	124.0±2.8	110.7±11.0**
	3 hr (n=6)	179.5±11.8	178.2±5.3	180.7±4.9	174.8±7.1	175.8±7.7	171.3±6.7
GPT	1 hr (n=6)	45.0±3.0	46.0±3.9	46.0±3.1	44.2±2.6	43.5±3.2	42.5±2.4
	2 hr (n=6)	42.8±3.2	42.7±2.5	43.2±3.4	42.3±2.9	41.3±2.6	40.7±2.4
	3 hr (n=6)	47.5±2.4	47.5±3.3	49.5±3.5	47.8±2.6	47.0±2.1	46.3±2.0
LDH	1 hr (n=6)	144.0±17.6	145.0±20.6	145.3±27.6	140.2±13.7	132.3±17.5	93.0±18.6**
	2 hr (n=6)	76.5±28.1	72.8±22.0	81.8±20.5	65.5±13.7	70.0±12.6	50.7±16.6
	3 hr (n=6)	81.2±27.1	76.0±13.2	76.7±20.9	76.7±13.5	67.8±6.8	38.0±10.5**

Primary cultured hepatocytes were exposed to CCl_4 at the concentration of 2.6 mM immediately after treated with diethylstilbestrol.

Each value represents group mean ± standard deviation (IU/l).

** Significantly different from 0 M (Control), $P < 0.01$.

Fig. 1. Effect of 17 β -estradiol on Mg²⁺- and Na⁺, K⁺-ATPase of rat liver plasma membrane. The plasma membrane isolated from rat liver was treated with 17 β -estradiol, and Na⁺, K⁺, Mg²⁺-ATPase and Mg²⁺-ATPase were assayed. Na⁺, K⁺-ATPase was obtained by subtracting Mg²⁺-ATPase from Na⁺, K⁺, Mg²⁺-ATPase. Data are expressed as group mean $\pm$ standard deviation of percentage of Mg²⁺- and Na⁺, K⁺-ATPase to Na⁺, K⁺, Mg²⁺-ATPase activity in each non-treated membrane.

The etiology of CCl₄-induced hepatotoxicity, which has been reported by many authors, is still under discussion (Bridges et al., 1983 ; Dolak et al., 1988 ; Masuda and Nakamura, 1990 ; Stacey et al., 1982). Plasma membrane has been long proposed as a critical site for the hepatotoxicity of CCl₄, and its permeability change has been quoted as an early appearance after treatment with CCl₄ (Dolak et al., 1988 ; Masuda and Nakamura, 1990 ; Tsokos-Kuhn et al., 1985 ; Zimmerman, 1978). In the present study E₂, at concentrations of 10⁻¹⁰ M to 10⁻⁴ M, 10⁻⁸ M to 10⁻⁶ M and 10⁻¹² M to 10⁻⁴ M, had an inhibitory effect on the CCl₄-induced leakage of GOT, GPT and LDH, respectively from the primary cultured rat hepatocytes. A non-steroidal estrogen, DES, which caused inhibition at a dose of 10⁻⁴ M, did not inhibit any enzyme leakage at any further concentrations of 10⁻¹² M to 10⁻⁶ M.

As one of early liver plasma membrane changes caused by CCl₄-treatment, decreases of Na⁺, K⁺-ATPase and Mg²⁺-ATPase in the membrane have been reported (Kuchil et al., 1973 ; Le Page and Dorling, 1971), and K⁺ leakage, indicating

a decrease of Na^+ , K^+ -ATPase activity, precedes enzyme leakage in CCl_4 -induced hepatotoxicity (Masuda and Nakamura, 1990). Both Na^+ , K^+ - and Mg^{2+} -ATPase activity in the isolated liver plasma membrane was increased by 15-minute treatment with E_2 at concentrations of 10^{-6} M and 10^{-4} M. The increases might have counteracted the CCl_4 -induced decreases of ATPase activity in the early stage, and the counteraction might have inhibited the subsequent enzyme leakage from the CCl_4 -treated cultured hepatocytes. The different experimental conditions, however, should be noticed between the cultured hepatocytes and the isolated plasma membrane, such as that the hepatocytes consisted of plasma membrane and other cellular components which could be affected by E_2 treatment. The effect of DES on ATPase of liver plasma membrane has been investigated in view of the observation of cholestasis, and DES decreases ATPase activity in the isolated liver plasma membrane of rats (Heikel and Lathe, 1970). The different action on ATPase activity in plasma membrane might have given rise to the different effect on the CCl_4 -induced enzyme leakage from the primary cultured hepatocytes.

A different mode of action on the plasma membrane between E_2 and DES was observed also in each effect on the non-treated cultured hepatocytes. Any concentration of E_2 did not induce significant changes in the enzyme concentration in the media, while DES caused leakage of enzymes from hepatocytes into the media at a concentration of 10^{-4} M. It is still unclear why DES has an inhibitory effect on CCl_4 -induced enzyme leakage at a concentration of 10^{-4} M at which it caused leakage of enzymes from the non-treated hepatocytes into the media. The toxic effect of a high concentration of DES on the non-treated cultured hepatocytes might be related to the observations that massive doses of DES caused transient increases in serum GOT and GPT levels (Ishibe, 1975 ; Shinkawa et al., 1985). Progesterone showed similar effects as E_2 on both the non-treated and CCl_4 -treated primary cultured hepatocytes. A possible mechanism for these phenomena on plasma membrane of the hepatocytes might be derived not from the estrogenic potency of E_2 but from the structural specificity of steroid chemistry.

CCl_4 is reductively metabolized to free radicals by the microsomal cytochrome P450-dependent monooxygenase system (Recknagel, 1983 ; Slater, 1984 ; Zimmerman, 1978), and E_2 is known to counteract the stimulatory action of androgens on P450-dependent metabolism (Kato and Onoda, 1970). Further study is needed to examine the possible effect of E_2 on the metabolism under this experimental condition.

In addition to the data of the previous study suggesting the accelerated synthesis of transaminase by E_2 in the liver, the effects on plasma membranes, which include the different mode of action between E_2 and DES, might give rise to the not-uniform observations about the effects of estrogen on liver function.

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