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IN VIVO AND *IN VITRO* ESTIMATIONS OF THE DIRECT EFFECT OF ESTROGEN ON RAT HEPATOCYTES TESTED BY THE CHANGES IN THE UNUSUAL ESTROGEN-BINDING PROTEIN CONTENT

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Summary—The direct effect of estradiol (E_2) on the hepatocytes of mature male rats has been examined by measuring the changes in the unusual estrogen-binding protein (UEBP) content and parallel measuring the level of liver estrogen receptors (ER). The content of UEBP (N_{UEBP}) and ER (N_{ER}) in the liver were determined using the quantitative methods for differential specific determination of the E_2-binding sites of these proteins. It has been shown that the administration of E_2 *in vivo* induced a considerable decrease in hepatic N_{UEBP} not only in intact males, but also in hypophysectomized males during the initial period after the operation (when the content of hepatic ER was still high) and produced no effect in hypohysectomized males during the later period (when liver ER were depleted). Repeated administration of human growth hormone (hGH) (twice a day) resulted in a considerable increase in N_{ER} in hypophysectomized males and restored the sensitivity to the subsequent inhibitory effect of E_2 on UEBP.

We also used rat hepatocytes after a 4-day primary culturing. These cells had a stable morpho-functional status, high ER level, and sex-differentiated UEBP content. Culturing of mature male rat hepatocytes in the medium containing E_2 at concentrations close to physiological levels (10^{-10}–10^{-7} M) decreased N_{UEBP} in a dose-dependent manner. Hexestrol (10^{-7} M) but not cholesterol (10^{-5} M) also exhibited a direct effect on N_{UEBP} in cultured rat hepatocytes. The effect of E_2 was reversible: statistically significant increase in N_{UEBP} was observed 3 days after 10^{-9} M E_2 had been removed from the culturing medium.

It was concluded that hepatocytes may be a primary target for E_2 under physiological conditions and that GH may modulate the direct effect of E_2 at the hepatic level by modifying the content of liver ER.

INTRODUCTION

The sex-dependent pattern of liver metabolism is well recognized [1–4]. Consequently, the liver has a key role in the coordination and integration of adaptive and reproductive processes at the organism level. The investigation of the hormonal mechanisms responsible for the maintenance of the sex-related functions of the liver seems quite reasonable. It has been shown that the main role in these processes is played by the action of sex steroids and growth hormone (GH) or its homologs [1–7]. It is generally accepted that the hepatic sex-differentiating function of sex steroids is performed via their effect on the hypothalamo–hypophysial system, which lead to the development of the sex-dependent pattern of GH release by the the hypophysis. This sex-specific pattern of GH secretion mediates the action of sex hormones on liver functions [4–7].

At the same time there is evidence that such a mechanism of the interrelated action of sex steroids (in particular estrogens) and the hypophysial hormones may not be the unique. One fact is the identification of estrogen receptors (ER) in the cytosol and nuclei of hepatocytes. Another fact is a prominent correlation between the content of liver ER and their redistribution between the cytoplasm and nuclei of hepatocytes on the one hand and the efficiency of estrogen action on certain liver sex-dependent functions, on the other. In addition, a strong dependence of the liver ER content on hypophysis and on the GH level has been shown [1, 2, 8, 9]. Based on these data, we have suggested that under physiological conditions estrogens produce not only indirect but also direct effects on hepatocytes, and that GH can potentiate the primary estrogen action on the hepatic level. To test this suggestion we examined the direct effect of estrogens

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on hepatocytes both *in vivo* and *in vitro* by evaluating the changes in the unusual estrogen-binding protein (UEBP) content.

We have shown that UEBP is a sex-specific intracellular protein which is abundant only in male rat liver and is capable of specific and labile binding with a high affinity ($K_a \sim 10^8\ M^{-1}$) to estrogens and, in a less degree, to androgens [10, 11]. The investigated binding and molecular properties of rat liver UEBP make it different from estrogen receptors and other sex steroid-binding proteins, such as the rat androgen-binding protein (ABP) released by Sertoli cells of testis or the homological to ABP sex hormone-binding protein (SHBP) secreted into the blood stream by human and primate liver [10–13]. SHBP can also be related to the group of hepatic sex-dependent proteins; however its circulating concentrations predominate in women and are up-regulated by estrogens [12]. Presumably, the main function of the rat liver UEBP is the sex specific regulation of the intensity of action and metabolism of either estrogens or androgens within a hepatocyte [14, 15].

The sex-dependence of the UEBP content in the liver is developed under the action of androgens, estrogens, and hypophysial hormones [1, 2, 11]. The administration of physiological concentrations of estrogens reversibly reduces UEBP level in intact male liver, but induces no response after a prolonged absence of the hypophysis [11]. Therefore, the existence of the direct effect of exogenous estradiol (E_2) was examined during the initial period after hypophysectomy. A parallel determination of both the changes in the efficiency of the inhibitory action of E_2 on UEBP and the changes in the liver ER content were also performed depending on the period after hypophysectomy and on the administrationn of GH to hypophysectomized rats.

A primary culture of mature male rat hepatocytes was used as another model to investigate the direct effect of E_2 on the liver cells.

EXPERIMENTAL

Animals

Mature male mongrel albino rats or Wistar rats either intact, 1 or 21–32 days after transauricular hypophysectomy were used in the experiments. The efficiency of hypophysectomy was estimated as described elsewhere [11].

Hormone administration

Estradiol (E_2) was injected intramuscularly at a dose of 10 μg in 0.4 ml of propylen glycole for 6 days. This produced a more pronounced inhibitory effect than a dose of 0.4 μg E_2 [11]. Human GH (hGH) (Sigma, U.S.A. or Kaunas Plant of Endocrine Preparations, U.S.S.R.) was injected subcutaneously at a dose of 60 μg in 0.2 ml of normal saline twice a day for 6 days. In some experiments E_2 was injected after hGH. The levels of UEBP and ER were determined 1 day after the last injection of the corresponding hormone.

Isolation and culturing of hepatocytes

Livers obtained from mature male rats were perfused according to [16], washed *in situ* with Hanks solution (w/o Ca^{2+}, Mg^{2+}) at 37°C under sterile conditions and reperfused with 0.05% collagenase (Sigma, type IY, U.S.A.) in Hanks solution containing 5 mM $CaCl_2$ and 50 mM HEPES (pH 7.5) for 20–25 min at 37°C. The cells thus obtained were centrifuged 3 times at 50 *g* for 3 min. Morphologically, the cell suspension contained not less than 95% hepatocytes. By Trypan Blue staining (0.2%), the viability of the cells was 85–90%. The cell suspension was diluted RPMI-1640 supplemented with 10^{-5} M dexamethazone and 8 μg/ml insulin. In preliminary experiments, it was shown that the *in vivo* administration of these hormones into male rats induced no appreciable changes in the liver UEBP content. Hepatocytes were cultured at 37°C in 100 mm Petri dishes (Leningrad Plant of Biopolymers, U.S.S.R.) precoated with collagen from rat tail tendons [17]. Non-adherent cells (30–50%) were washed with the basal medium. 24 h after plating, the medium was substituted by a new medium with or without estrogens. The culturing medium was changed every day.

The cells were cultured for 4 days. E_2 was added to concentrations ranging from 10^{-11} to 10^{-7} M during 1–3 days. In some experiments, hexestrol (10^{-7} M) or cholesterol (10^{-5} M) (Sigma, U.S.A.) was used.

Morphological and functional characteristics of the hepatocyte culture

By phase-contrast microscopy, the hepatocytes began to flatten 4 h after plating and confluency was reached in 18–20 h. The epithelial morphology was retained for 7–10 days of culturing.

The synthetic activity of the hepatocytes was estimated by their ability to secrete plasma proteins (albumin, fibrinogen, and fibronectin). The content of each protein in the culturing medium was determined by immuno-enzyme methods. Antibodies to these proteins were generated in rabbits immunized with purified rat albumin [18], fibrinogen [19], or fibronectin [20]. Purified antibodies were conjugated with horse-radish peroxidase (Sigma Cat. No. P-8375) according to Wilson and Nakane [21] and used in ELISA. The secretion of these proteins became steady on the 2nd day and was retained at the same level up to the 6th day of culturing. After 6 days in culture, the hepatocytes exhibited certain degradation: the secretion of the acute phase proteins (fibrinogen and fibronectin) increased. It can be seen from Fig. 1, that the UEBP content in cultured male rat hepatocytes is steady and can be compared to that occurring in the initial liver tissue [11]. No UEBP was detected in cultured mature female rat hepatocytes. So, sex dimorphism in respect of hepatic UEBP

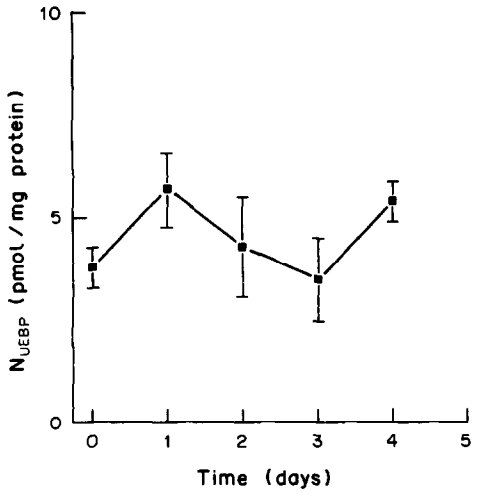

Fig. 1. The UEBP content in the cytosol prepared from the cultured hepatocytes of mature male rats during various periods of culturing, (M ± m). Day 0—indicates N_{UEBP} in hepatocyte cytosol immediately after their isolation.

content, which was observed *in vivo* [11] is also revealed in cultured hepatocytes.

Preparation of liver cytosol

Male rat liver cytosol (protein content 10–20 mg/ml) was prepared as described elsewhere [11]. For the preparation of cytosol of cultured hepatocytes the cells were washed with 40 ml of 10 mM Tris–HCl (pH 7.5) containing 1.5 mM EDTA, 10 mM KCl, 6 mM dithiothreitol (buffer A) or with 20 mM Tris–HCl (pH 7.5) containing 0.5 mM EDTA, 10 mM Na_2MoO_4, 0.1 mM phenylmethylsulphonylfluoride (PMSF), 0.1 mM dithiothreitol, 0.25 mM sucrose, and 10% glycerol (buffer B). The cells were homogenized in a Teflon-glass homogenizer in corresponding buffer. The homogenate was centrifuged at 50,000 *g* for 1 h at 0–4°C. The protein content in the cytosol obtained (0.5–3.0 mg/ml) was determined according to Lowry *et al.* [22] or Bradford [23].

Differential determination of UEBP and ER

This was performed based on different binding properties of UEBP and ER (1,2,10,24). In both cases specific binding was examined using either [2,4,6,7-^{3}H]estradiol ([^{3}H]E_2) or [2,4,6,7,16,17-^{3}H]E_2 (Amersham) with specific radioactivity 98–100 or 140 Ci/mmol, respectively. Specific binding was determined as described previously [10].

The presence of the excess of unlabeled hexestrol, which completely inhibits the binding of [^{3}H]E_2 to ER and has no effect on the binding of [^{3}H]E_2 to UEBP at the concentrations applied [10], was necessary for differential determination of the UEBP content. The evaluation of the UEBP content (N_{UEBP}) was performed based on the technique described previously [11]. The cytosol (50 μl) in buffer A was incubated for 15 min at 4°C with 1–2 nM [^{3}H]E_2 without (total binding) or with 5.0 μM of unlabeled E_2 (non-specific binding). 2.5 μM hexestrol was added to each sample. Unbound hormone was removed by a 1-min incubation at 4°C with 25 μl of the 2% suspension of charcoal coated with 0.4% Dextran. In a separate set of experiments it was shown that the specific binding revealed under the above conditions in cultured hepatocyte cytosol is due to UEBP [10, 11, 24]: this binding was highly labile, considerably decreased by unlabeled 5α-androstan-3α,17β-diol, and was not detected in the presence of 0.15 M sodium thiocyonate.

The differential estimation of the ER content (N_{ER}) was performed in the presence of the excess of 5α-androstan-3α,17β-diol and 0.15 M sodium thiocyonate. N_{ER} in liver cytosol was determined by the binding of the saturating amounts of the hormone (50–60 nM). Cytosol in buffer A was incubated for 3 h at 0–4°C with 50–60 nM [^{3}H]E_2 with or without 6 μM E_2. In hepatocyte cytosol, N_{ER} and the value of the association constant (K_a) were calculated from Scatchard plots [25]. For this hepatocyte cytosol in buffer B was incubated for 20–22 h at 0–4°C with varied amounts of [^{3}H]E_2 (0.02–2.0 nM) with or without 5.0 μM unlabeled E_2. 15 μM 5α-androstan-3α,17β-diol and 0.15 M sodium thiocyonate were added to each sample. Free and protein-bound hormone were separated by a 5-min incubation at 4°C with 4% of charcoal coated with 0.8% Dextran.

RESULTS

The in vivo investigation of the direct effect of E_2 on the liver

It can be seen from Table 1 that the inhibitory effect of exogeneous E_2 on the UEBP level in male rat

Table 1. The inhibitory effect of E_2 on the hepatic UEBP content in mature male rats at various periods after hypophysectomy (M ± m)

Experimental group	N_{UEBP} (pmol/mg of protein) Initial level	After E_2 injections[a]	P[b]
Intact	6.83 ± 0.49 (48)	1.71 ± 1.03 (6)	<0.001
Hypophysectomized			
The early period	7.89 ± 1.12 (12)	3.43 ± 0.33 (9)	<0.01
The late period	3.20 ± 0.33 (6)	3.44 ± 0.33 (8)	>0.1

[a]E_2 (10 μg) was injected during day 2–7 (early period) or during day 21–26 (late period) after hypophysectomy. The UEBP content was determined 1 day after the last injection of E_2.

[b]Significance of differences is given in relation to initial UEBP content.

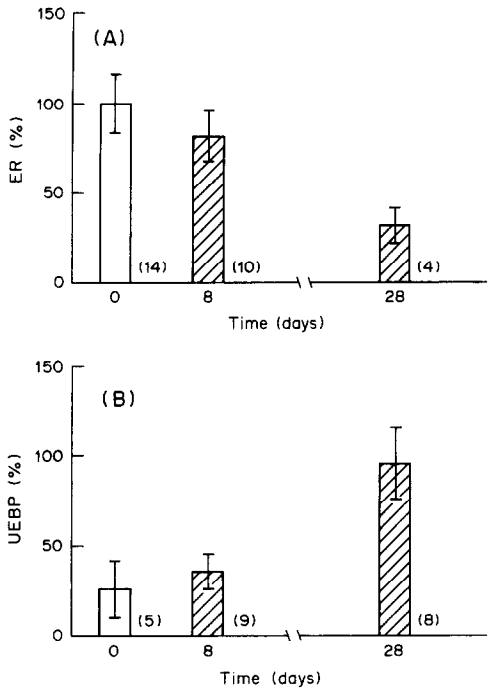

Fig. 2. The comparison of the changes in the ER content in liver cytosol (A) and in the efficiency of the inhibitory effect of E_2 on the liver UEBP content (B) depending on the period after hypophysectomy (M ± m). (A) N_{ER} in liver cytosol at various periods after hypophysectomy shown as the percentage of N_{ER} in liver of intact males. (B) N_{UEBP} in liver cytosol of estrogenized (10 μg E_2, 6 days) intact or hypophysectomized males shown as the percentage of N_{UEBP} in liver of correspondent animals which received no E_2. The broken line shows the variations in the control UEBP level (M ± m).

liver is exhibited at the early period after hypophysectomy. Thus, the administration of E_2 resulted in a 2,3-fold decrease in the liver UEBP content. As known, the hypophysial hormones are virtually absent from the blood stream in 24 h after hypophysectomy. Therefore it can be suggested that this effect of estrogens on the liver is not mediated by the hypophysis.

On the other hand, our results show that the injections of E_2 21–25 days after hypophysectomy produce no statistically significant changes in the UEBP content (Table 1). Presumably, this may be caused by the changes in the liver sensitivity to E_2 during a prolonged absence of the hypophysis. To test this suggestion we determined the ER content in liver cytosol. Here and previously, we and others demonstrated a decrease in liver ER during prolonged hypophysectomy [1–4, 9, 11]. At the same time, at the early period after hypophysectomy ER level is essentially high (Fig. 2A). The comparison of the liver ER content and the effect of E_2 on the UEBP level (Fig. 2A, B) reveals a strong positive correlation between the changes of these parameters with time after hypophysectomy ($r = 0.989$, $n = 6$, $P < 0.001$). During the early period, a high ER level correlates with a prominent ability of E_2 to decrase the UEBP level and during the later period the decrease in ER coincides with the absence of E_2 effect on the UEBP content in the liver. Taken together, these facts suggest that the absence of the E_2 effect on the liver UEBP content in hypophysectomized animals is due to lowered sensitivity of the liver to the direct effect of this hormone. It is unlikely that the absence of this effect is due to the disturbancies in the estrogens–hypothalamo–hypophysial-system liver axis. As it has been shown, the efficiency of E_2 action on the UEBP content in rats at the early period after hypophysectomy was lower as compared with intact animals. Thus, E_2 did not bring down UEBP to below 2 pmol/mg of protein in the first case but only in intact rats (Table 1) although the ER level in liver cytosol was nearly the same in both cases (Table 2). This may be due to the influence of hypophysectomy on the efficiency of nuclear–cytoplasmic redistribution of ER which may take place in the earlier period than its inhibitory effect on the liver ER content.

It was shown that the intermittent administration of hGH to hypophysectomised male rats with liver ER deficiency and without reaction of UEBP to E_2 considerably increased the ER content in the liver. Subsequent injections of E_2 to these animals resulted in a restoration of negative effect of E_2 on the liver UEBP content (Table 2). These results show that GH can not be the mediator of the inhibitory effect of E_2 on the UEBP content in the liver: first, this effect can be released without endogenous GH at the early period after hyphophysectomy; second, in hyphophysectomised animals the inhibitory effect of E_2 can be

Table 2. The effect of hGH on the liver ER and UEBP contents and on the efficiency of E_2 action on the UEBP content in the liver of hypophysectomized male rats (M ± m)

Experimental group	N_{ER} (fmol/mg protein)	N_{UEBP} (pmol/mg of protein)
I. Intact	109.2 ± 17.1 (14)	6.83 ± 0.49 (48)
II. Hypophysectomized[a]	35.7 ± 10.7 (4)	3.20 ± 0.33 (6)
	$P_{I-II} < 0.05$	$P_{I-II} < 0.01$
III. Hypophysectomized + hGH[b]	163.0 ± 33.5 (8)	9.04 ± 1.18 (7)
	$P_{II-III} < 0.001$	$P_{II-III} < 0.01$
IV. Hypophysectomized + hGH + E_2[c]	—	3.68 ± 0.19 (4)
		$P_{III-IV} < 0.05$

[a]The animals were used within 28 days of hypophysectomy. [b]hGH (60 μg, twice a day) was injected during days 21–26 after hypophysectomy. [c]E_2 (10 μg) was injected after hGH during day 27–32 after hypophysectomy. The UEBP and ER contents were determined 1 day after the last injection of the hormones.

restituted by a successive administration of GH and E_2. It should be emphasized that GH itself in used conditions (interrupted injections) does not possess feminizing effect typical of E_2 but rather induces an increase in the UEBP content in the liver of hypophysectomized males (Table 2). Presumably, GH performs a dual role in the regulation of hepatic sex-dependent proteins, such as liver UEBP. On the one hand, this hormone may have its own direct stimulating (intermittent administration—Table 2) or direct inhibiting (continuous infusion—[26]) effects on the liver UEBP content. On the other hand, GH may be a permissive factor for the realization of primary estrogen effect on hepatic sex-differentiated proteins, when up-regulating the liver ER level.

The direct effect of E_2 on cultured hepatocytes

To reveal the direct effect of E_2 on liver cells we examined the changes in the UEBP content in hepatocytes cultured in the presence of E_2. In previous experiments it was shown that cultured hepatocytes of sexually mature male rats have an essentially high ER content (Fig. 3) which can be compared with that in the liver of intact males. It should be mentioned that 0.15 sodium thiocyonate lowered the estimated values of K_a for [^{3}H]E_2 to ER interaction by 4–6-times, which is consistent with the data of others [24]. Thus, proceeding from the ER content the sensitivity of cultured hepatocytes to E_2 should be essentially high.

It can be seen from Fig. 4 that a 3-day culturing of mature male rat hepatocytes in the presence of E_2 resulted in dose-dependent decrease in the UEBP content. The addition of the synthetic estrogen-hexestrol (10^{-7} M) that does not bind to UEBP led to the inhibition of the UEBP level to the same extent (Fig. 4). High concentration of cholesterol (10^{-5} M)

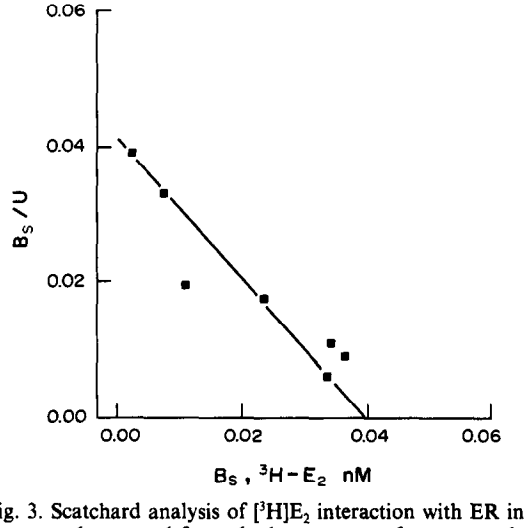

Fig. 3. Scatchard analysis of [^{3}H]E_2 interaction with ER in the cytosol prepared from the hepatocytes of mature male rats ($K_a = 0.39 \times 10^9\ M^{-1}$, $N_{ER} = 95.0$ fmol/mg protein; B_S, specifically bound; U, free [^{3}H]E_2). The hepatocytes were cultured for 4 days.

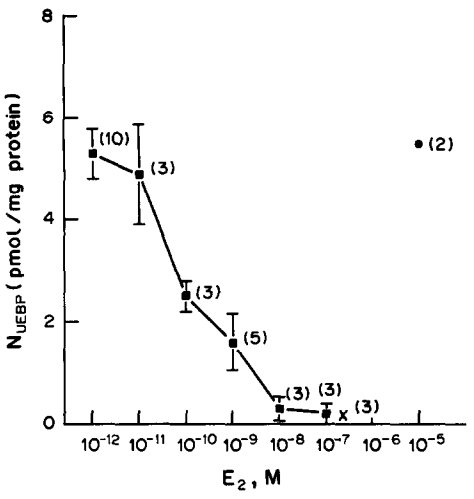

Fig. 4. The effect of various concentrations of E_2 on the UEBP level in the cytosol prepared from cultured mature male rat hepatocytes (M ± m). Various concentrations of E_2 were added to the culturing media on day 1–4 of culturing. (X) the effect of 10^{-7} M of hexestrol; (●) the effect of 10^{-5} M cholesterol. The number of determinations is given in brackets.

in the culturing medium produced no changes in the UEBP content in hepatocytes. It may be concluded that at the concentrations close to physiological levels estrogens may elicit specific effects on certain metabolic pathways in hepatocytes.

Comparison of effects of E_2 on the liver UEBP content *in vivo* [11] and in cultured hepatocytes revealed that the common characteristic in both cases is the sensitivity of the liver to physiological levels of E_2. The investigation of the dynamics of the inhibitory effect of E_2 on the UEBP content in the cultured hepatocytes showed that a 1-day incubation of hepatocytes with 10^{-9} M E_2 produced a statistically significant ($P < 0.01$) decrease in the UEBP content to 44% compared with the corresponding control (Fig. 5). However, this decrease is 1.5-fold less prominent than that observed on a 3-day incubation under the same conditions (see Fig. 4). These results are consistent with the fact that the maximal effect of E_2 *in vivo* is achieved by repeated injections of this hormone [11].

As known, at the organism level the regulatory effect of E_2 is reversible. To find out whether this effect is reversible in cultured hepatocytes, they were incubated with 10^{-9} M E_2 for 24 h, then the hormone was removed from the medium and the UEBP content was determined 1–3 days after. It can be seen from Fig. 5 that under these conditions the inhibitory effect of E_2 is reversible. On the 3rd day after the removal of E_2 a statistically significant ($P < 0.02$) increase in the UEBP content from 44.0 to 66.2% was observed. We failed to document the further restitution of the UEBP content, since after 6 days in culture the hepatocytes began to degrade (see Materials and Methods). Previously, it was shown that after the *in vivo* administration of E_2 the restitution of the UEBP

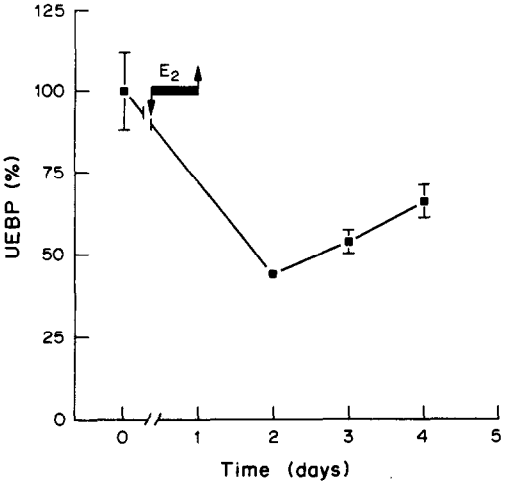

Fig. 5. The dynamics of the changes in the UEBP content in the cytosol prepared from cultured hepatocytes of mature male rats after different periods of a 1-day incubation with E_2 ($M \pm m$). The arrows indicate the addition and removal of 10^{-9} M E_2. Ordinate, N_{UEBP} at various periods after removal of E_2 as the percentage of its level under standard incubation conditions at the same periods of culturing.

content is slow and accomplished in 10 days [11]. Presumably, there are similar features in the regulatory effect of E_2 in cultured hepatocytes and *in vivo*.

DISCUSSION

The direct effect of E_2 on the liver was examined in the experiments on the regulation of the liver UEBP level. The evidence that the liver is the primary target for E_2 was obtained in 3 variants of experiments. Estrogens can efficiently reduce the UEBP content in liver cells without any direct involvement of hypophysial hormones when (1) injected at the early period after hypophysectomy; (2) administered at the later period after hypophysectomy if the animals preliminary received GH; (3) introduced in the medium in which mature male rate hepatocytes are cultured. Our previous results [11] and those presented here show that physiological concentrations of estrogens can alter the UEBP content in the liver. Thus, the primary estrogen action on the content of UEBP and probably of other sex-dependent proteins in the liver may be naturally occurring. Recent data show that the direct estrogen regulation of liver processes is relevant not only in the case of UEBP. It has been demonstrated that physiological concentrations of E_2 produce a direct effect on mRNA of certain apolipoproteins and their secretion by Hep-G2 cells [8], on the synthesis and secretion of vitellogenin by cultured frog hepatocytes [27], and, when used at higher concentrations, on the activity of 5α-reductase of cultured mature female rate hepatocytes [28]. The direct estrogen regulation of at least a number of sex-dependent liver metabolic pathways is confirmed by ER functional activity in liver cells under normal conditions (1, 2, 8). In some cases (for example, UEBP) this way may be the main. Presumably, the same situation may be observed in the case of SHBP in men. It has been shown that human liver contains ER and that E_2 can directly affect the SHBP secretion by human hepatocarcinoma cells [29, 30].

Our results suggest that upon sex differentiation of the liver functions, estrogens and hypophysial factors interact in more diverse ways than it was assumed earlier [4–7, 9]. The modulation of the direct effect of E_2 by GH (via the regulation of the ER content in the liver) may be of considerable importance in sex differentiation of the liver functions. This effect of GH could be compared with its direct feminizing [4–7, 9] or masculinizing [1, 2, 4, 11] hepatotropic effect due to a sex-specific pattern of its hypophysial secretion induced by sex steroids [5–7]. Presumably, the *in vivo* expression of the sex dependence of liver functions with regards to the content of UEBP and other sex differentiated proteins depends on the concentration ratios of sex steroids and GH and arises from their interrelated primary action on the liver. It can be suggested that the significant of GH participation in sex differentiation of different hepatic functions is coordination of adaptive and reproductive processes of the whole organism at the liver level.

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