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# Effects of oestrogen on adenylate cyclase system and glucose output in rat liver

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Effects of chronic oestrogen treatment on catecholamine- and glucagon-sensitive adenylate cyclase activity and glucose output in hepatocytes of castrated male rats were studied. In hepatocytes from male intact or castrated rats, the β-adrenergic agonist isoprenaline did not stimulate adenylate cyclase activity and glycogenolysis, but glucagon markedly stimulated all these activities. Treatment of castrated animals with 17β-oestradiol for 7 days led to the appearance of β-adrenergic-stimulated increases in both cyclic AMP generation and glucose output. The basal, glucagon- or fluoride-stimulated activities of adenylate cyclase of hepatic membranes prepared from oestrogen-treated rats were similar to those of control animals. Treatment with oestrogen did not influence the number or affinity of β-adrenergic receptors. In hepatic plasma membranes from control rats, GTP failed to decrease the affinity of β-adrenergic receptors for agonists, whereas the GTP-induced shift was apparently observed in those from oestrogen-treated animals. These results suggest that oestrogen is able to facilitate the coupling of hepatic β-adrenergic receptors to the enzyme by increasing the effectiveness of receptor–guanine nucleotide regulation.

## INTRODUCTION

Catecholamines appear to stimulate hepatic glycogenolysis by a β-adrenergic-receptor-mediated increase in cyclic AMP as well as by a $Ca^{2+}$-dependent α-adrenergic pathway (Guellaen *et al.*, 1978; Blair *et al.*, 1979; Studer & Borle, 1982; Morgan *et al.*, 1983). An age-related decrease in the β-adrenergic response and a reciprocal increase in the α-adrenergic-mediated effect in hepatocytes from male rats have been reported (Blair *et al.*, 1979; Studer & Borle, 1982; Morgan *et al.*, 1983; Nakamura *et al.*, 1983, 1984). Significant differences between male and female rats in the stimulation of hepatic adenylate cyclase by adrenaline were observed during development (Bitensky *et al.*, 1970). Studer & Borle (1982) have demonstrated that, in hepatocytes isolated from adult female rats, adrenaline utilizes both α- and β-adrenergic pathways which activate phosphorylase via $Ca^{2+}$ or cyclic AMP respectively, whereas in mature male rats adrenaline increases phosphorylase activity by an α-adrenergic-receptor-mediated, $Ca^{+}$-dependent, cyclic AMP-independent pathway. Bitensky *et al.* (1970) noted that the greater response of hepatic adenylate cyclase to adrenaline in weanling female rats as compared with males was suppressed by chronic administration of androgen. They also reported an elevated adrenaline-responsive component of the enzyme in weanling male rats treated with diethylstilboestrol, the synthetic oestrogen, compared with androgen treatment. The present studies were designed to investigate effects of chronic oestrogen treatment on catecholamine- and glucagon-sensitive adenylate cyclase activity and glycogenolysis in hepatocytes of castrated mature male rats, in which β-adrenergic responsiveness is almost absent.

## MATERIALS AND METHODS

### Chemicals

The following drugs were generously given: (−)-isoprenaline (Nicken Chemicals, Tokyo, Japan), (−)-propranolol (Japan ICI Pharma, Osaka, Japan), cyclic AMP and dibutyryl cyclic AMP (Daiichi Seiyaku, Tokyo, Japan), phentolamine (Japan Ciba Geigy, Tokyo, Japan), and phenylephrine (Kowa Co., Nagoya, Japan). Glucagon, 5′-guanylyl imidodiphosphate (p[NH]ppG) and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Calbiochem–Hoechst, Tokyo, Japan, and Japan Boehringer-Mannheim, Tokyo, Japan. Cyclic [8-$^3$H]AMP (32.0 Ci/mmol), [$^3$H]CGP-12177 (42.6 Ci/mmol) and [$^3$H]prazosin (30.0 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.

### Animals and membrane preparation

Male rats weighing 180–200 g, of Donryu strains (Nippon Rats Co., Tokyo, Japan), were castrated via the scrotal route under diethyl ether anaesthesia. Daily injections of 0.9% NaCl or oestradiol benzoate (50 μg/100 g body wt.) to castrated rats were carried out for 7 days from the first day of operation. Liver plasma membranes were prepared by the procedure of Neville (1968) up to step 11. Membrane protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

### Adenylate cyclase assay

Adenylate cyclase activity was assayed by incubation of the membrane in a total volume of 0.6 ml of 40 mM-

Abbreviations used: p[NH]ppG, 5′-guanylyl imidodiphosphate; IBMX, 3-isobutyl-1-methylxanthine; CGP-12177, 4-t-butylamino-2-hydroxypropoxybenzimidazole.

Tris/HCl buffer (pH 7.4) containing 4 mM-$MgCl_2$, 10 mM-theophylline, 2 mM-ATP, 100 $\mu$g of pyruvate kinase/ml and 5 mM-phosphoenolpyruvate for 10 min, as previously described (Shima *et al.*, 1980, 1983). Cyclic AMP formed during incubation was measured by a competitive binding assay, by using a protein purified from rabbit skeletal muscle to the DEAE-cellulose column step (Gilman, 1970).

### Receptor-binding assay

Samples of the membrane preparation were assayed for $\beta$-adrenergic-receptor binding with [$^3$H]CGP-12177. Membranes were incubated with [$^3$H]CGP-12177 (0.5–10 nM) in a buffer (50 mM-Tris/HCl, pH 7.4, 5 mM-$MgCl_2$) for 30 min at 30 °C. $\alpha_1$-Adrenergic-receptor binding was performed by using [$^3$H]prazosin as described by Geynet *et al.* (1981). Incubation of membranes with 0.2–10 nM-[$^3$H]prazosin in 50 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-$MgCl_2$, 1 mM-sodium (−)-ascorbate and 1 mM-pyrocatechol was carried out for 30 min at 25 °C.

### Hepatocyte preparation, glucose output and cyclic AMP contents

Hepatocytes were prepared by collagenase digestion of the liver by the procedure of Berry & Friend (1969), as modified by Tolbert *et al.* (1980). Glucose output was estimated by measuring glucose during incubation of hepatocytes (30–50 mg wet wt.) for 60 min at 37 °C in Krebs–Ringer bicarbonate buffer (119 mM-NaCl/4.8 mM-KCl/1.2 mM-$KH_2PO_4$/1.2 mM-$MgCl_2$/1.3 mM-$CaCl_2$/25 mM-$NaHCO_3$) in the absence of glucose and in the presence of 1% bovine serum albumin at pH 7.4 under $O_2/CO_2$ (1:19).

Glucose in the medium was determined in protein-free $HClO_4$ extracts by using glucose oxidase (Bergmeyer & Bernt, 1974). To measure cyclic AMP, isolated hepatocytes were preincubated at 37 °C for 30 min in Krebs–Ringer bicarbonate buffer, pH 7.4, with 40 mM-glucose, 1 mg of bovine serum albumin/ml (KRBGA buffer) and 0.1 mM-IBMX, followed by further incubation with the drug for 3 min. Incubation was terminated by adding 0.4 M-HCl, followed by quick immersion of tubes into boiling water. The cells and medium were homogenized by sonication (Ultrasonic Disrupter model UR-200 P/Set 7/10 sec.).

Student's *t* test was used for statistical analysis. For all Figures, data were obtained from two animals in each treatment group.

## RESULTS

### Cyclic AMP generation in oestrogen-treated rat hepatocytes

Increases in cyclic AMP accumulation and activation of adenylate cyclase in response to the $\beta$-adrenergic agonist isoprenaline (at concentrations up to 0.1 mM)

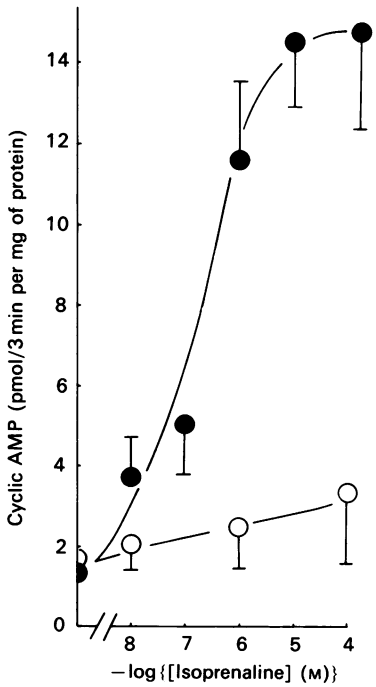


**Fig. 1. Effects of isoprenaline on cyclic AMP accumulation in hepatocytes isolated from castrated and oestrogen-treated male rats**

After preincubation with 0.1 mM-IBMX for 30 min, hepatocytes from castrated male rats treated with (●) or without (○) oestrogen were incubated for 3 min with increasing concentrations of isoprenaline. Data shown represent means ± S.E.M. for four incubations.

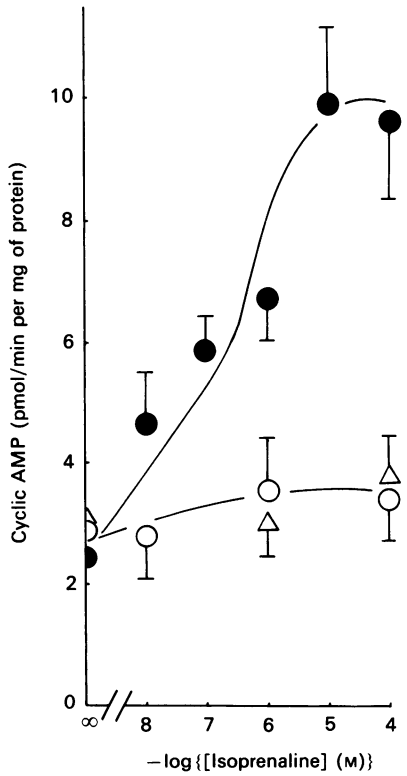


**Fig. 2. Effects of isoprenaline on adenylate cyclase activity in liver membranes from control and castrated rats with or without oestrogen treatment**

Hepatic membranes from control male rats (△) or from castrated male rats treated with (●) or without (○) oestrogen were incubated with various concentrations of isoprenaline for 10 min. Results shown are means ± S.E.M. for triplicate incubations.

**Table 1. Hormonal stimulations of cyclic AMP accumulation in hepatocytes isolated from castrated and oestrogen-treated rats**

After preincubation with 0.1 mM-IBMX for 30 min, hepatocytes were incubated for 3 min with or without 10 $\mu$M-isoprenaline, 50 $\mu$M-propranolol, 10 $\mu$M-phenylephrine and 100 nM-glucagon. Cyclic AMP accumulated in hepatocytes was determined as described in the Materials and methods section. Data shown represent the means ± S.E.M. for triplicate incubations: *significantly different from castrated ($P < 0.01$).

| Additions | Cyclic AMP formed (pmol/3 min per mg of protein) | |
|---|---|---|
| | Castrated | Castrated, oestrogen-treated |
| None | 1.18±0.51 | 1.50±0.25 |
| Isoprenaline | 2.55±1.11 | 11.88±3.15* |
| Isoprenaline+ propranolol | 2.75±0.88 | 4.73±0.88 |
| Phenylephrine | 1.43±0.98 | 1.82±0.12 |
| Glucagon | 18.79±4.22 | 22.01±5.01 |

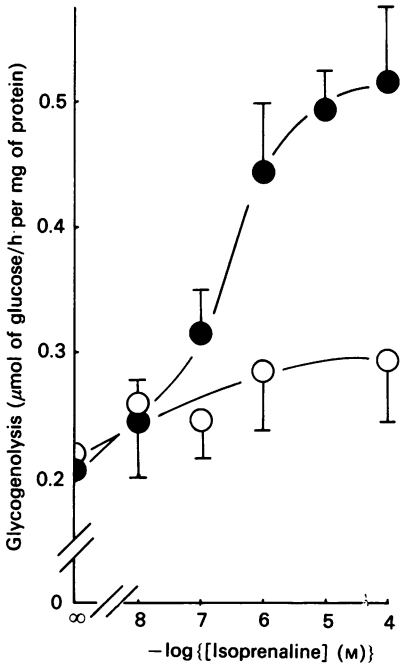


**Fig. 3. Effects of isoprenaline on glucose output in hepatocytes isolated from castrated and oestrogen-treated male rats**

Glucose in the medium was measured as glucose output from cells of castrated male rats with (●) or without (○) oestrogen treatment as described in the Materials and methods section. Values are means ± S.E.M. for four incubations.

were either very small or absent in hepatocytes from castrated adult male rats (Figs. 1 and 2). In oestrogen-treated animals, the effect of isoprenaline could be observed at 0.1 $\mu$M and was maximal at 10 $\mu$M (Figs. 1 and 2). These effects of isoprenaline on the cyclic AMP system were antagonized by the $\beta$-adrenergic antagonist propranolol, indicating activation of $\beta$-adrenergic receptors functionally coupled to adenylate cyclase systems after the oestrogen treatment (Table 1). Stimulation of adenylate cyclase by glucagon, NaF and p[NH]ppG in the oestrogen-treated rats was similar to that in castrated controls (results not shown).

**Table 2. Hormonal stimulations of glucose output in hepatocytes isolated from castrated and oestrogen-treated rats**

Hepatocytes were incubated for 60 min with or without 10 $\mu$M-isoprenaline, 50 $\mu$M-propranolol, 10 $\mu$M-adrenaline, 10 $\mu$M-phenylephrine, 50 $\mu$M-phentolamine or 100 $\mu$M-dibutyryl cyclic AMP. Glucose in the medium was determined as described in the Materials and methods section. Results are means ± S.E.M. for triplicate incubations: *significantly different from castrated ($P < 0.01$).

| Addition | Glucose output ($\mu$mol/h per mg of protein) | |
|---|---|---|
| | Castrated | Castrated, oestrogen-treated |
| None | 0.23±0.03 | 0.21±0.01 |
| Isoprenaline | 0.28±0.06 | 0.53±0.08* |
| Isoprenaline+ propranolol | 0.22±0.05 | 0.28±0.04 |
| Adrenaline | 0.49±0.01 | 0.55±0.03 |
| Phenylephrine | 0.67±0.12 | 0.57±0.11 |
| Phenylephrine+ phentolamine | 0.37±0.12 | 0.32±0.10 |
| Glucagon | 0.75±0.22 | 0.81±0.19 |
| Dibutyryl cyclic AMP | 0.68±0.11 | 0.58±0.13 |

### Glucose output in oestrogen-treated rat hepatocytes by adrenergic agonists or glucagon

A glycogenolytic response to isoprenaline was also revealed at 0.1 $\mu$M in hepatocytes from oestrogen-treated castrated rats, but was negligible in hepatocytes from castrated adult male rats (Fig. 3). Isoprenaline stimulation of glucose output by hepatocytes with oestrogen treatment was competed with propranolol (Table 2). No differences in glucose output induced by the $\alpha$-adrenergic agonist phenylephrine, glucagon or dibutyryl cyclic AMP were observed between hepatocytes from the castrated control and the oestrogen-treated rats (Table 2).

### Adrenergic receptors in hepatic membranes from oestrogen-treated rats

The $\beta$-adrenergic-receptor population of membranes derived from livers treated with or without oestrogen was examined (Fig. 4). Scatchard analysis of [$^3$H]CGP-12177 binding showed no differences in the binding affinities or capacities between membranes derived from the control and the oestrogen-treated animals. The affinities for isoprenaline determined with membranes from the control and the oestrogen-treated rats were similar. With addition of GTP, the competition curves were not altered in the castrated controls, but were shifted to the right 10-fold in the hormone-treated rats (Fig. 5). The quantity

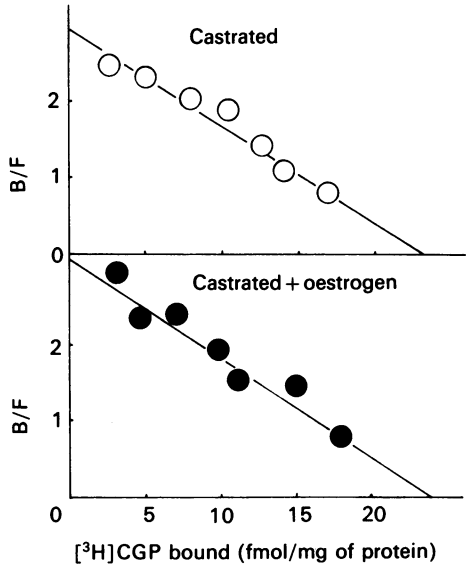


**Fig. 4. Scatchard analysis of [$^3$H]CGP-12177 binding to liver membranes from castrated and oestrogen-treated rats**

Membranes from castrated male rats treated with (●) or without (○) oestrogen were incubated with [$^3$H]CGP-12177. Conditions for the binding assay were as described in the Materials and methods section. The ratio B/F of bound CGP-12177 (fmol/mg of protein) to free CGP-12177 (nM) is plotted as a function of bound CGP-12177 (fmol/mg of protein). Points are means of triplicate incubations.

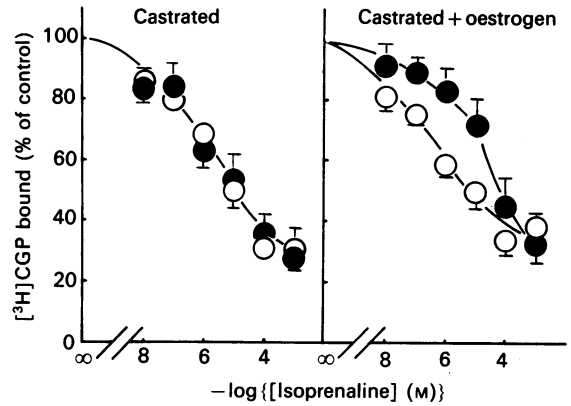


**Fig. 5. Isoprenaline competition curves for [$^3$H]CGP-12177 binding in membranes derived from livers of castrated rats with or without oestrogen treatment**

Membranes from castrated male rats treated with or without oestrogen were incubated in the presence (●) or the absence (○) of 100 $\mu$M-GTP in the binding assay as described in the Materials and Methods section. Maximum specific binding to membranes from castrated and oestrogen-treated rats in the absence of isoprenaline was taken as 100%. Values shown represent means of triplicate incubations.

of $\alpha_1$-adrenergic receptors, as revealed by the binding of saturating concentrations of [$^3$H]prazosin, was $986 \pm 121$ fmol/mg of protein ($n = 4$) in the castrated control membranes and $882 \pm 98$ fmol/mg of protein ($n = 4$) in those of hormone-treated rats.

## DISCUSSION

An age-associated decrease in g-adrenergic receptors and the $\beta$-adrenergic responsiveness of cyclic AMP generation and glycogenolysis is apparent in livers of male rats (Hutson *et al.*, 1976; Saitoh & Ui, 1976; Cherrington *et al.*, 1976; Birnbaum & Fain, 1977; Blair *et al.*, 1979; Morgan *et al.*, 1983; Nakamura *et al.*, 1983, 1984; Tsujimoto *et al.*, 1986). Moreover, the activity of the $\beta$-adrenergic-sensitive component of the hepatic cyclic AMP system was significantly higher in female than in male rats at all ages (Bitensky *et al.*, 1970; Malbon, 1981; Studer & Borle, 1982). Bitensky *et al.* (1970) have previously reported that the adrenaline-sensitive adenylate cyclase system in weanling male rats treated with diethylstilboestrol, a synthetic oestrogen, exhibited higher activity than in those treated with testosterone propionate. The present studies showed a significant appearance of $\beta$-adrenergic responses of adenylate cyclase activity and glycogenolysis in hepatocytes by chronic oestrogen treatment of adult rats. Stimulation of the cyclic AMP-generating system and glycogenolysis by glucagon was minimally affected in the oestrogen-treated rats, indicating that the $\beta$-adrenergic-receptor-mediated responses were exclusively enhanced by the hormone treatment. Moreover, a similar activation of hepatic adenylate cyclase by glucagon, NaF or p[NH]ppG in castrated controls and the oestrogen-treated rats suggests that guanine nucleotide regulatory and catalytic components of adenylate cyclase are functionally and quantitatively intact after the hormone treatment. The absence of a change in receptor affinity for the $\beta$-adrenergic agonist on addition of GTP suggests some defects in coupling efficiency of the stimulatory guanine nucleotide regulatory sites ($N_s$) between the receptor and catalytic moiety in membranes from the adult male rat. In oestrogen-treated liver membranes, GTP decreased the affinity of $\beta$-adrenergic-agonist binding, without any change in the number of receptor sites. These results suggest that the oestrogen treatment is involved in restoring a functional lesion in the $N_s$ mechanism in liver membranes from adult male animals, leading to the appearance of $\beta$-adrenergic-receptor-mediated responses. In liver membranes of male rats above 20–30 days of age, the number of $\beta$-adrenergic receptors appears not to be crucial to demonstrate $\beta$-adrenergic activation of adenylate cyclase. In contrast with the foetal- and suckling-rat liver, there was a large decrease in $\beta$-adrenergic-receptor sites in liver membranes of rats over 20–30 days of age (McMillian *et al.*, 1983). Responses of the cyclic AMP–adenylate cyclase system to $\beta$-adrenergic agonists are also prominent in 20–30-day-old male rats, but not in adult rats (60 days of age); little change in $\beta$-adrenergic receptor number occurs during this time span (Blair *et al.*, 1979; McMillian *et al.*, 1983; Shima *et al.*, 1987). The mechanisms whereby oestrogen treatment restores the interaction of $N_s$ with $\beta$-adrenergic receptors are unidentified at present. Oestrogens have been reported to induce cholestasis with bile-secretory failure and dysfunction (Plaa & Priestly, 1976; Klaassen & Watkins, 1984; Berr *et al.*, 1984). Extrahepatic cholestasis induced by common-bile-duct ligation resulted in a specific increase in the $\beta$-adrenergic-receptor density and the coupling efficiency of $N_s$ to the receptors, which was accompanied by an increase in catecholamine-stimulated adenylate cyclase in the rat

liver (Schmelck *et al.*, 1979; Aggerbeck *et al.*, 1983; Okajima & Ui, 1984). The present study supports an explanation that the cholestatic state with alterations in hepatic membrane lipid composition induced by oestrogen treatment (Berr *et al.*, 1984) is responsible for enhanced $\beta$-adrenergic stimulation of the cyclic AMP–adenylate cyclase system and subsequent glycogenolytic action. Kunos *et al.* (1984) have shown a development of increased $\beta$-adrenergic-receptor responsiveness in hepatocytes, with no change in the receptor density during incubation, and speculated an increased efficacy of $\beta$-adrenergic receptors contributing to changes in membrane phospholipase $A_2$ activity. Potentiation of adrenaline-stimulated lipase activity by oestrogen treatment has been reported in rat adipocytes (Benoit *et al.*, 1982). Meanwhile, no direct hormonal activation of the lipase has been reported in homogenized hepatocytes (Debeer *et al.*, 1982). A possibility of stimulatory effects by long-term treatment *in vivo* with oestrogens on the hepatic enzyme systems involving lipolysis, which also involve $\beta$-adrenergic-receptor function, could also not be excluded.

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