Regulation by oestrogen of carnitine palmitoyltransferase in hepatic mitochondria

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Treatment of female rats with ethynyloestradiol decreased both the activity of carnitine palmitoyltransferase and its K_i value for malonyl-CoA compared with pair-fed control rats. In starved rats, carnitine palmitoyltransferase activity and its K_i value for malonyl-CoA were both elevated as expected. Oestrogen treatment had no effect on carnitine palmitoyltransferase activity in the starved state, but the K_i value for malonyl-CoA was substantially decreased by oestrogen. Treatment with a more physiological concentration of ethynyloestradiol (15 $\mu\text{g/kg}$ body wt. per day) also produced a decrease in the K_i value for malonyl-CoA. These data suggest that the oestrogen may produce its anti-ketogenic effect by increasing the affinity of carnitine palmitoyltransferase for its physiological inhibitor, malonyl-CoA.

INTRODUCTION

Isolated perfused livers from female rats oxidize less fatty acid and esterify more fatty acid to triacylglycerol compared with livers from male rats (Soler-Argilaga & Heimberg, 1976), suggesting that oestrogen may play a role in the disposition of fatty acids by the liver. Indeed, livers from female rats that have been treated with ethynyloestradiol have an increased rate of esterification of fatty acids to triacylglycerol and a diminished capacity for fatty acid oxidation (Weinstein *et al.*, 1979). Comparable results were reported in pregnant rats on day 19 of gestation, when oestrogen concentrations were greatly elevated (Wasfi *et al.*, 1980). Understanding the mechanism of the anti-ketogenic effect of oestrogen may be important to our understanding of the physiological regulation of fatty acid oxidation and may have additional significance in therapy with oral contraceptives containing oestrogen.

Hepatic fatty acid oxidation appears to be regulated by modulation of the affinity of carnitine palmitoyltransferase for its physiological inhibitor, malonyl-CoA. Carnitine palmitoyltransferase from starved rats is much less sensitive, compared with the enzyme from fed rats, to inhibition by malonyl-CoA (Bremer, 1981; Saggerson & Carpenter, 1981). Starvation causes a modest (60%) increase in activity of carnitine palmitoyltransferase, but decreases the affinity for malonyl-CoA by 10-fold, a change in affinity so great that physiological concentrations of malonyl-CoA may no longer have any inhibitory effect (Cook, 1984). Experimental insulin deficiency similarly diminishes the affinity of carnitine palmitoyltransferase for malonyl-CoA, but treatment of diabetic rats with insulin restores the enzyme to normal (Gamble & Cook, 1985). Data presented here indicate that the anti-ketogenic effect of oestrogen is exerted through a mechanism that is very similar to the mechanism responsible for the regulation of fatty acid oxidation in the starvation–feeding cycle and in diabetes. The primary effect of oestrogen in these experiments was to increase the affinity of carnitine palmitoyltransferase for malonyl-CoA.

EXPERIMENTAL

Treatment of animals

Female rats (175–225 g) of the Sprague–Dawley strain were obtained from Harlan Industries (Indianapolis, IN, U.S.A.). One group was allowed free access to food (Purina Lab Chow for Rats; Ralston Purina Co., Richmond, IN, U.S.A.) and was not treated with ethynyloestradiol. Another control group, that also received no oestrogen, was given the same quantity of food consumed by the group that received oestrogen. This group (pair-fed controls) was included because rats treated with a high dose of oestrogen consumed less food than did untreated rats (Weinstein *et al.*, 1986). A third group of rats received 5 mg of ethynyloestradiol/kg body wt. per day for 5 days by subcutaneous injection. Ethynyloestradiol (100 mg) was dissolved in ethanol (0.5 ml) and diluted with propylene glycol (19.5 ml) for injection. All control animals received daily injections of the diluting solvents. In order to avoid problems arising from differences in food consumption in the high-dose oestrogen-treated group (see the Results section), additional experiments were carried out using rats that were treated and pair-fed as before but were starved for the 48 h immediately before enzyme assays were conducted. An additional group of rats received 15 μg of ethynyloestradiol/kg body wt. per day subcutaneously for 14 days and were also starved for 48 h before enzyme assays. Control rats for this group were also pair-fed along with the oestrogen-treated group before starvation. All animals were allowed free access to water.

Enzyme Assay

Rats were decapitated before livers were removed for the isolation of mitochondria (Johnson & Lardy, 1967). Mitochondrial pellets were suspended to a protein concentration of 20 mg/ml in 0.25 M-sucrose/1 mM-EDTA/3 mM-Tris, pH 7.2. Protein was measured by a biuret procedure summarized by Layne (1975). Respiratory control ratios (Estabrook, 1967) were 5 or greater with 10 mM-glutamate and 0.5 mM-malate as substrates.

The outer carnitine palmitoyltransferase was measured by a modification (Stephens *et al.*, 1983) of the method of Bremer (1981). Each assay contained, in a total volume of 1 ml, 82 mM-sucrose, 70 mM-KCl, 35 mM-imidazole, 35 mM-Hepes, 5 mM-GSH, 4 mg of bovine serum albumin, 0.5 mM-L-carnitine (0.4 μ Ci of L-[methyl- 3 H]carnitine/ μ mol) and 1 μ g of antimycin A; oleoyl-CoA was present at 200 μ M for measuring activity or at 75, 100 or 150 μ M for measuring K_i values; malonyl-CoA was present at 0, 1, 2, 5, 15, 25 or 40 μ M. All assays were conducted at 30 °C and pH 7.0. K_i values were determined by the graphical method of Dixon (1953). Four experiments were averaged for the determination of each K_i value or estimate of activity.

Fatty acid oxidation rates

At the end of 5 days of treatment with the higher dose of ethynyloestradiol, one group of rats was anaesthetized lightly with diethyl ether and livers were removed for perfusion in a recycling perfusion system (Heimberg *et al.*, 1964, 1965; Weinstein *et al.*, 1979). A complex of oleic acid and albumin was infused to give a steady-state concentration of approx. 0.4 mM-oleate in the erythrocyte-free perfusate. Samples of perfusate were taken for the determination of ketone bodies (Michaels *et al.*, 1951). Statistical evaluation of differences between means were determined by a two-tailed Student's *t* test.

Materials

Ethynyloestradiol, oleoyl-CoA, malonyl-CoA, L-carnitine hydrochloride, GSH, EDTA, imidazole, Hepes, and essentially fatty acid-free bovine serum albumin (used untreated for enzyme assays and treated to remove fatty acids for liver perfusions) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Oleic acid for liver perfusions was obtained from Nu Chek Prep (Elysian, MN, U.S.A.). L-[methyl- 3 H]Carnitine hydrochloride was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.).

RESULTS

The rate of fatty acid oxidation in perfused livers from control rats that were fed *ad libitum* was 2.5 ± 0.3 μ mol of ketone bodies produced/min per g wet wt. of liver, but livers from rats that were pair-fed along with the oestrogen-treated group oxidized oleate at a rate of 5.7 ± 0.5 μ mol of ketone bodies/min per g. The increase in ketogenic rate probably resulted from restricted food consumption by the pair-fed group. Treatment of rats with 5 mg of ethynyloestradiol/day for 5 days decreased (compared with the pair-fed control group) the rate of oleate oxidation to 3.0 ± 1.3 μ mol of ketone bodies/min per g. These results with a high dose of oestrogen agree with previous data with a much lower (15 μ g/kg per day for 14 days) dose of oestrogen (Weinstein *et al.*, 1979) and indicate that oestrogen has an anti-ketogenic effect over a wide range of concentrations.

Inhibition of carnitine palmitoyltransferase by malonyl-CoA was somewhat greater in hepatic mitochondria from rats fed *ad libitum* compared with rats pair-fed along with the oestrogen-treated group (Fig. 1). Treatment with oestrogen caused greater sensitivity to inhibition by malonyl-CoA at all concentrations of inhibitor compared with the pair-fed control. The concentration of malonyl-CoA causing 50% inhibition in

Fig. 1. Malonyl-CoA inhibition of carnitine palmitoyltransferase in intact liver mitochondria

Hepatic mitochondria were isolated from rats that were fed *ad libitum* (●), pair-fed along with the oestrogen-treated group (■) or treated with ethynyloestradiol (▲). Mitochondria were incubated with 40 μ M-oleoyl-CoA and the concentrations of malonyl-CoA indicated in the Figure. Each point represents the mean $\pm$ S.E.M. for four rats. Statistical differences ($P < 0.05$) were found at each malonyl-CoA concentration between the pair-fed group and the other two groups. Percentage inhibition refers to inhibition of total measured activity. Maximum inhibition was estimated by extrapolating the line obtained by plotting $(\% \text{ inhibition by malonyl-CoA})^{-1}$ versus $[\text{malonyl-CoA}]^{-1}$ to infinite inhibitor concentration (see Stephens *et al.*, 1985). Maximum inhibition was 101% for rats that were fed *ad libitum*, 98% for the oestrogen-treated rats and 96% for rats pair-fed along with the oestrogen-treated group.

oestrogen-treated rats was only 4 μ M, compared with 13 μ M in the pair-fed group.

The activity of carnitine palmitoyltransferase in hepatic mitochondria from rats that were pair-fed along with the oestrogen-treated group was greater than the activity in hepatic mitochondria from rats that were fed *ad libitum* (Table 1). Oestrogen treatment resulted in activity that was identical with that in rats fed *ad libitum*, but less than in the appropriate pair-fed controls, suggesting that oestrogen treatment decreased the activity of carnitine palmitoyltransferase. The differences in enzyme paralleled differences in rates of ketogenesis in the perfused liver, but the differences in those rates of fatty acid oxidation were much greater than differences in activity. The K_i of carnitine palmitoyltransferase for malonyl-CoA (Table 1) changed in concert with differences in activity and with rates of fatty acid

Table 1. Activity of carnitine palmitoyltransferase and K_i values for malonyl-CoA in intact mitochondria

Hepatic mitochondria were isolated from the five groups of rats indicated and assayed for carnitine palmitoyltransferase activity at the optimum concentrations of oleoyl-CoA (100 μ M) and carnitine (0.5 mM). K_i values were determined as described in the Experimental section. Results are presented as means $\pm$ S.E.M. for each group of rats ($n = 4$). There were statistical differences ($P < 0.05$) in activities between the pair-fed control group and the other two fed groups. There were statistical differences ($P < 0.001$) in K_i values between the pair-fed control group and the other two fed groups and between the two starved groups.

Treatment of rats	Carnitine palmitoyltransferase activity (nmol/min per mg of protein)	K_i for malonyl-CoA (μ M)
Fed (<i>ad libitum</i>)	7.2 $\pm$ 2.1	1.5 $\pm$ 0.3
Pair-fed	10.4 $\pm$ 0.4	3.5 $\pm$ 0.5
Fed + oestrogen	8.5 $\pm$ 0.3	1.3 $\pm$ 0.3
Starved	10.4 $\pm$ 0.7	6.7 $\pm$ 0.7
Starved + oestrogen	11.2 $\pm$ 0.7	3.0 $\pm$ 0.2

oxidation, exactly as seen previously during starvation (Cook, 1984) and experimental diabetes (Gamble & Cook, 1985), i.e. modest increases in activity were associated with quantitatively more significant changes in the K_i for malonyl-CoA.

The effects of oestrogen treatment seem quite clear when comparisons are made with the pair-fed control group, but the fact that the oestrogen-treated group was almost identical with the group fed *ad libitum* in all aspects stimulates questions regarding how rigorously one can control the nutritional status of animals in the various groups that were used in our experiments. To substantiate the effects of oestrogen on carnitine palmitoyltransferase, additional experiments were conducted using rats that were treated with ethynloestradiol or the dissolving vehicle exactly as before, except that the animals were starved for 48 h before they were used for enzyme measurements. Starvation caused a 30% increase in carnitine palmitoyltransferase activity, but the K_i for malonyl-CoA was increased by 350% (Table 1) over the values observed in the group fed *ad libitum*. Oestrogen did not significantly lower enzyme activity in the starved rats, but the K_i value for malonyl-CoA was decreased substantially by the oestrogen treatment.

We also confirmed the effect of oestrogen on K_i values for malonyl-CoA, using another group of rats that were treated with a much lower dose of ethynloestradiol (15 μ g/kg body wt. per day) and starved for 48 h before use. Results of that experiment showed that the K_i for malonyl-CoA was again substantially decreased by oestrogen (7.7 $\pm$ 1.1 μ M in the starved control rats and 5.0 $\pm$ 0.7 μ M with low-dose oestrogen treatment; $P < 0.05$).

DISCUSSION

This study indicates that oestrogen is anti-ketogenic at both high and low doses and that it acts through changes in carnitine palmitoyltransferase, decreasing both activity

of the enzyme and the K_i value for malonyl-CoA. It is difficult to assess the relative importance of these two changes, since both effects would be expected to inhibit fatty acid oxidation. Oestrogen produced both changes in carnitine palmitoyltransferase of the fed rat, but in the starved rat the high dose of oestrogen substantially increased the affinity of carnitine palmitoyltransferase for malonyl-CoA without affecting activity, indicating that the two changes in enzyme properties may not always be inseparable.

The effects of oestrogen on carnitine palmitoyltransferase may explain the stimulatory actions of oestrogen on triacylglycerol formation by the liver (Watkins *et al.*, 1972; Weinstein *et al.*, 1979) and the hypertriglyceridaemia that is associated with oral contraceptives containing oestrogens (Aurell *et al.*, 1966; Wynn *et al.*, 1966). Inhibition of carnitine palmitoyltransferase has been shown by Ide & Ontko (1981) to stimulate hepatic triacylglycerol synthesis and lipoprotein secretion. Those authors stressed the importance of carnitine palmitoyltransferase in the reciprocal relationship that exists between fatty acid oxidation and very-low-density lipoprotein synthesis.

Oestrogen apparently had no effect on the positive co-operative inhibition characteristic of malonyl-CoA inhibition of hepatic carnitine palmitoyltransferase. Dixon plots confirmed those effects, i.e. upwardly curved lines were observed in plots of data from rats fed *ad libitum* but not in rats on food-restricted (pair-fed) diets or in rats that were starved for 48 h. The difference in curvature of lines of Dixon plots from pair-fed rats compared with rats fed *ad libitum* indicated that the two control groups were quite different. Those differences, and the fact that the pair-fed rats are deprived of energy, make them suspect controls for the oestrogen-treated fed rat, even though high-dose oestrogen treatment also induces decreased food consumption. It was because of those considerations that we repeated oestrogen treatment with a group of rats that were starved for 48 h before use. The data from that experiment confirmed the anti-ketogenic effect of oestrogen and suggested that oestrogen does have a regulatory effect on carnitine palmitoyltransferase that is not related to feeding behaviour. Furthermore, treatment of female rats with the low dose of estrogen substantially decreased the K_i for malonyl-CoA, without having any significant effect on food consumption. Because we used normal female rats in these experiments, the effect of oestrogen may be underestimated, owing to the presence of endogenous oestrogen. Consideration of that fact supports the conclusion that the effect of oestrogen on carnitine palmitoyltransferase is indeed part of a physiological mechanism for regulating rates of hepatic fatty acid oxidation in the female rat.

The data presented here suggest that oestrogens should be considered with other hormones such as glucagon and insulin as being important in the regulation of fatty acid oxidation in the liver. Oestrogen apparently has its primary effect on the K_i for malonyl-CoA, but the mechanism causing this change is still to be elucidated. It will be interesting to determine how these hormones interact to produce a co-ordinated control of hepatic fatty acid oxidation.

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