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Effect of ovarian hormones upon liver mitochondrial function in diabetic rats

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Summary

In the present study it is shown that streptozotocin (SZ)-induced chronic diabetes of female albino rats produced significant alterations in liver mitochondrial function after 30–35 days of diabetes. The disturbances were as follows: (1) a significant fall of the mean values of the respiratory control ratio and of state 3 of respiration using three substrates, 3-hydroxybutyrate, malate-glutamate and succinate, and (2) a significant increase of the mean damping factor of the oscillatory osmotic variations (with valinomycin as K^+ ionophore and succinate as substrate). The same mitochondrial function parameters were analyzed for comparison in control non-diabetic rats (group N) and in the following groups of female rats with chronic diabetes: intact (group I), oophorectomized (6 days after the injection of SZ) (group O), and oophorectomized with restitution therapy of 17β-estradiol (from the operation until the day before killing) (group O + Eol). The O group showed significantly higher values of the respiratory control ratio and of state 3 of respiration and significantly lower damping factors than group I. The restitution treatment in the O + Eol group restored the mitochondrial functions assayed to values similar to those of group I. These data provide strong evidence that estrogens exert a negative effect at the molecular level upon impaired liver mitochondrial functions in SZ-induced diabetes.

Introduction

Houssay et al. [1] showed that the administration of estrogens during the first month after large subtotal pancreatectomy of rats increased the incidence and intensity of diabetes mellitus. The intake of oral

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contraceptives (artificial estrogens and progesterones) by millions of women over long periods has evoked disturbances of glucidic metabolism [2]. Pregnant diabetic women, during the advanced stages when serum ovarian hormone levels are high, have been shown to need increased dosages of insulin for therapy and may display abnormal glucose tolerance with associated higher perinatal mortality rates [3–5]. In chronic diabetes mellitus we find hyperglycemia, hyperlipemia and ketonemic acidosis besides alterations in endocrine and hepatic tissues at the molecular level. Vomachka et al. [6] demonstrated variations of endocrine functions and an increase of 17β-estradiol in the blood of immature female streptozotocin (SZ)-induced diabetic rats. Leaming et al. [7], also using female rats with SZ-induced diabetes, found a significant increase of blood testosterone and androsterone compared with normal controls. This androgen increase is reduced to normal levels by ovariectomy and adrenalectomy of the diabetic rats. Skett [8] has recently shown that experimentally induced diabetes modifies to a significant degree the activities of certain microsomal sex-dependent enzymes of steroid metabolism. Using male rats rendered diabetic either by injection of SZ or by subtotal pancreatectomy Brignone et al. [9] showed significant alterations of hepatic mitochondrial functions including respiration with 3-hydroxybutyrate substrate, oscillatory behavior and the activities of certain mitochondrial enzymes. These alterations were reversed by treatment with insulin. Thus diabetes mellitus is a metabolic disorder resulting in degenerative and functional changes of endocrine glands and other metabolic tissues at the molecular level. The aim of the present study was to investigate the effects of endogenous and exogenous ovarian hormones upon certain mitochondrial functions in the liver of SZ-induced diabetic rats.

Materials and methods

Animals and treatment

Female albino rats (210–220 g body weight) from the Department of Biochemistry were fed a balanced diet of Nutrimentos S.A. (No. 3). Food and tap water were administered ad libitum. The animals were kept in a temperature-controlled room maintained at 22–24°C. All animals were subject to a daily light cycle of 12 h light and 12 h darkness. They were rendered diabetic by intraperitoneal injection of SZ (55 mg/kg body weight), dissolved in 0.1 M citrate buffer at pH 4.5, 2–3 min before use. The volume administered was 0.3 ml per rat. During the 24 h before SZ injection all the animals drank a solution of glucose 5% in place of the tap water. Control rats received the corresponding volume of citrate solution. Diabetic rats were killed 30–35 days after the injection of SZ. Intact female and bilaterally ovariectomized albino rats were used. The latter group was operated on 6 days after the injection of SZ under ether anesthesia. Sham-operated rats were used as controls. The female diabetic animals were categorized into the following groups: (1) intact rats, (2) ovariectomized, (3) ovariectomized and treated with 17β-estradiol (40 μg/kg day^{-1}). The hormone was given dissolved in edible oil (0.1 ml) by subcutaneous injection. Restitution therapy to oophorectomized rats was applied daily from ovariectomy to the day before death (30–35 days after the injection of SZ). Non-diabetic intact and oophorectomized controls of similar age and weight were used. Those rats not treated with 17β-estradiol were injected with 0.1 ml of the vehicle of the hormone (edible oil) for the same period as the other animals.

Assay methods

Glucose and ketone bodies in the urine were determined by Ketodiastix (Ames) twice a week. Blood sugar was determined on the day of death by the method of Hyvarinen and Nikkila [10]. Mitochondrial protein was measured by the method of Gornall et al. [11], using crystalline bovine serum albumin as a standard.

Mitochondrial preparation

Liver mitochondria were prepared according to Gooch and Packer [12] using 0.33 M sucrose plus 0.25 mM Tris–EDTA (pH 7.4) during homogenization and for the first high-speed centrifugation

and only 0.33 M sucrose plus 0.25 mM Tris (pH 7.4) for the second and third high-speed centrifugations.

Mitochondrial oscillation

This was registered as described by Brignone et al. [9] by continuous measurement of periodic changes in mitochondrial volume by light absorption at 520 nm, 30°C, in a Beckman DU-2 or Shimadzu UV-Vis (180–190) double-beam spectrophotometer attached to a recorder. The mitochondria (0.66 mg protein/ml) were suspended in a medium containing 0.1 M sucrose, 28.6 mM acetate, 10 mM Tris–HCl (pH 7.9), 0.4 μg rotenone per mg of mitochondrial protein, 14 ng/ml valinomycin and to start the oscillatory mechanism the substrate Tris–succinate 3.3 mM (pH 7.9) was added. The final volume of the reaction mixture was 3.0 ml.

Mitochondrial respiration assay

Oxygen consumption and the respiratory control ratio were measured polarographically with a Gilson Medical Electronics oxygraph at 30°C using a vibrating platinum electrode. The reaction mixture (1.9 ml) contained 0.24 M sucrose, 34 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 9 mM Tris–HCl, 6 mM KH_2PO_4–K_2HPO_4 and 1.4 mg mitochondrial protein, pH 7.4. The following substrates and additional chemicals were used: DL-3-hydroxybutyrate 5 mM, pH 7.4, L-malate and L-glutamate 6 mM each and malonate 3 mM, pH 7.4, succinate 5 mM (plus rotenone 0.2 μM), pH 7.4.

Respiratory velocities of mitochondria at rest (state 4) and during active metabolism (state 3) were determined as nanogram atoms oxygen per minute per milligram of mitochondrial protein. Active state respiration was induced by adding adenosine diphosphate (ADP), final concentration 500 μM. The respiratory control ratio, which is an indicator of mitochondrial membrane integrity and of the capacity for phosphorylation, is defined as the ratio of the ADP stimulated velocity (state 3) to the velocity after exhaustion of ADP (state 4).

Chemicals

The commercial source of the following was Sigma Chemical Co., St. Louis, MO: NAD^+ (grade 3), DL-3-hydroxybutyric acid, malic acid, L-glutamic acid, L-malonic acid, Trizma base, EDTA, succinic acid, crystalline bovine serum albumin, sucrose, ADP (grade 1), valinomycin, rotenone, streptozotocin, 17β-estradiol. All other chemicals were of reagent grade.

Statistical analysis

The mean ± SD for the various parameters of mitochondrial functions employed in the different groups of rats were calculated and the differences were analyzed by Student's two-tailed *t*-test.

Results

Effect of ovariectomy and 17β-estradiol restitution upon oscillatory variations of liver mitochondria in diabetic rats

Fig. 1 shows a typical tracing of damped harmonic oscillations obtained with normal rat liver mito-

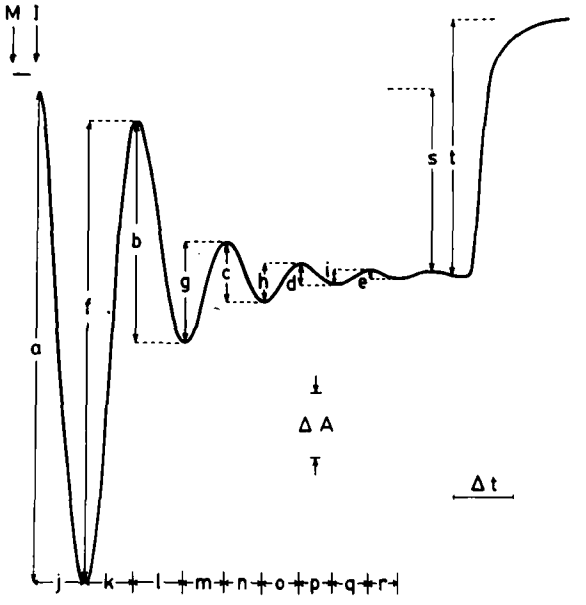

Fig. 1. Curve of normal tracing of liver mitochondrial oscillations showing different possible measurement parameters. a, b, c, d, etc. represent peaks; f, g, h, i, etc. represent troughs. Damping factors of peaks are a/b, b/c, c/d, etc. and of troughs f/g, g/h, h/i, etc. ΔA = absorption scale at 520 nm, Δt = time scale j, k, l, etc., represent half-periods of the waves. For other data see [9].

chondria. Gooch and Packer [9] recommended certain parameters for measuring these oscillatory variations. Among them is the damping factor which was used to compare the different experimental conditions assayed. The damping factor is defined as the ratio of successive oscillation amplitudes of peaks (a/b, b/c, c/d) and troughs (e/f, f/g, g/h). Peaks correspond to mitochondrial contraction and troughs to mitochondrial expansion. The amplitude was measured as ΔA_{520}/mg protein. Higher values of these ratios correspond to a smaller damped oscillatory response of mitochondria, i.e., to lesser elasticity in the membranes of these particles when their volume changes.

Fig. 2 shows the mean values of damping factors of the four groups of rats assayed, in a bar graph. Firstly, comparing the mean damping factor derived from the peaks and troughs of intact diabetic rats with that of normal non-diabetic rats, it is evident that it is higher in diabetic animals. Secondly, among the diabetic rats, those with ovaries removed showed significantly lower ratios than those with ovaries intact, and those with ovaries removed further showed values similar to those of non-diabetic animals. Finally, diabetic animals treated with 17β-estradiol showed the same mitochondrial damping factors as did intact diabetic rats.

The simultaneous restitution of progesterone and 17β-estradiol in physiological doses to ovariectomized diabetic rats produced the same increase of the damping factor as the administration of 17β-estradiol alone (results not shown).

Fig. 3 demonstrates typical tracings of oscillatory variation of hepatic mitochondria obtained with: (1) intact diabetic rats, (2) oophorectomized diabetic rats, (3) oophorectomized diabetic rats with restitution of 17β-estradiol, and (4) normal non-diabetic rats. The curves show a clearly visible difference between intact diabetic and oophorectomized diabetic rats. The curve corresponding to the diabetic rat with restitution of 17β-estradiol is very similar to that of the intact diabetic rat. It may be

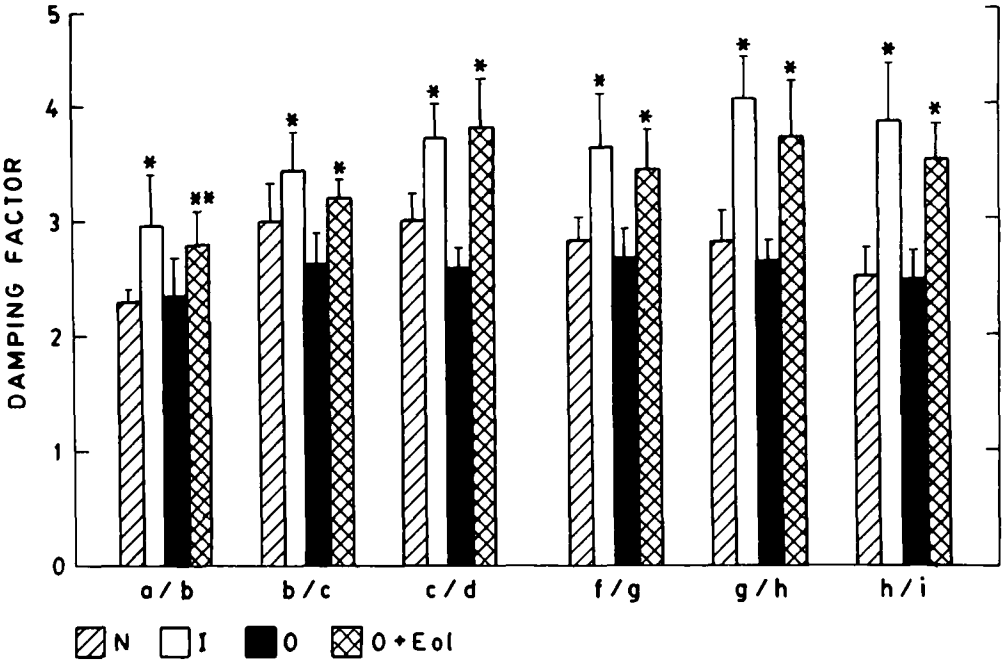

Fig. 2. Representation in bars of damping factors of the oscillatory variation of liver mitochondria in female diabetic rats: intact, oophorectomized, and oophorectomized treated with 17β-estradiol. N = normal, I = intact diabetic, O = oophorectomized diabetic, O + Eol = oophorectomized diabetic treated with 17β-estradiol. For other conditions see Materials and methods. The bars are the means ± SD, which are represented as lines above the bars. The number of animals was 11 per group. Analysis of variance: * $P < 0.001$, ** $P < 0.01$ compared with the oophorectomized diabetic rats.

noted that the tracing corresponding to the ovariectomized diabetic rat mimics that of the normal non-diabetic rat. Another way to demonstrate the significant differences between the groups of rats in the oscillatory behavior of liver mitochondria is the graphic method displayed in Fig. 4. Observing the pairs of curves in A and B it can be seen that the curve for normal rats and the similar one for diabetic oophorectomized rats have both quite different curvatures from the respective curves for intact diabetic rats and oophorectomized diabetic rats treated with 17β-estradiol. Taking as a landmark a 50% drop in damping, we see that diabetic rats and those with oophorectomies plus hormonal restitution reached the halfway mark more quickly than did normal or oophorectomized diabetic rats with no hormonal restitution. Also, the oscillatory variations in liver mitochondria of the intact group were more strongly damped than in the oophorectomized group.

Effect of ovariectomy upon liver mitochondrial respiration in diabetic rats

In this study we used a strain of albino rats rendered diabetic with the injection of streptozotocin and observed a decrease in liver mitochondrial respiration (state 3 and respiratory control ratio) just as observed in the Sprague–Dawley strain [13]. Table 1 shows that there is no significant difference in control respiratory ratio or active state 3 with the substrates 3-hydroxybutyrate, malate–glutamate or succinate, between intact non-diabetic and oophorectomized non-diabetic rats. These values may be considered normal standard values of these parameters. In Table 2 three groups of female diabetic rats were considered: intact (I), oophorectomized (O) and oophorectomized with restitution of 17β-estradiol (O + Eol). The O group demonstrated significantly higher values of the respiratory control ratio and state 3 of respiration of liver mitochondria with the three substrates mentioned

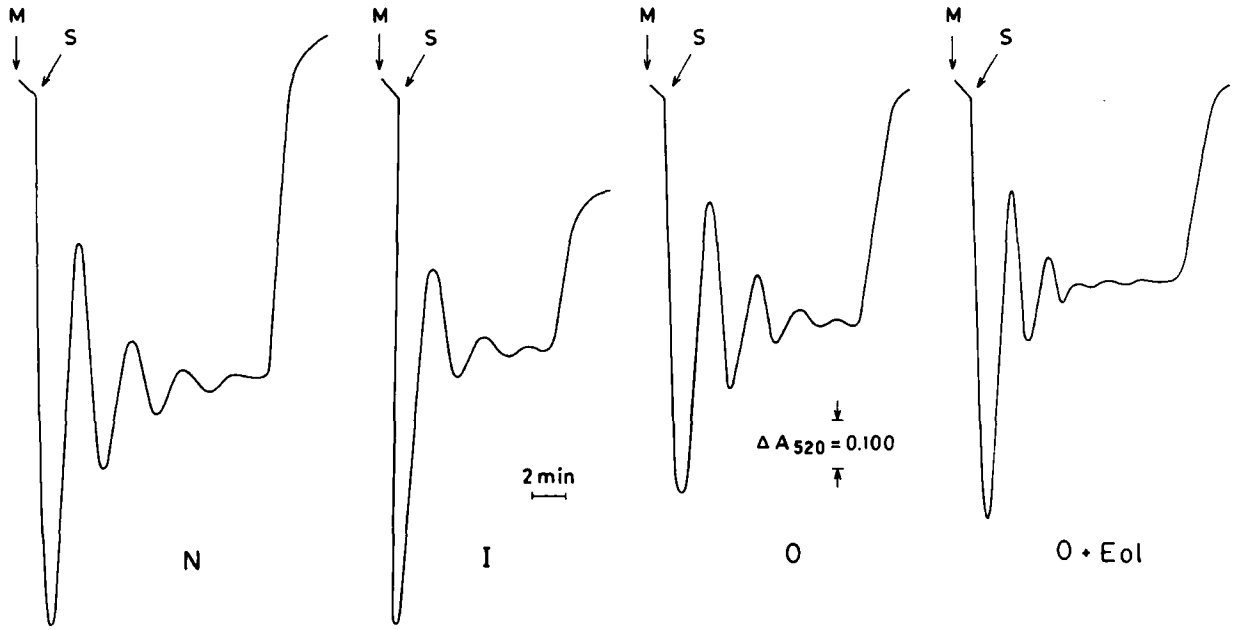

Fig. 3. Tracings of the oscillatory behavior of liver mitochondria in normal rat (N), oophorectomized diabetic rat (O), intact diabetic rat (I) and oophorectomized diabetic rat with restitution of 17β-estradiol (O + Eol). In all four experiments the same protein concentration (2 mg) was used. Values for initial absorption were similar in all the experiments. For other experimental conditions see Materials and methods.

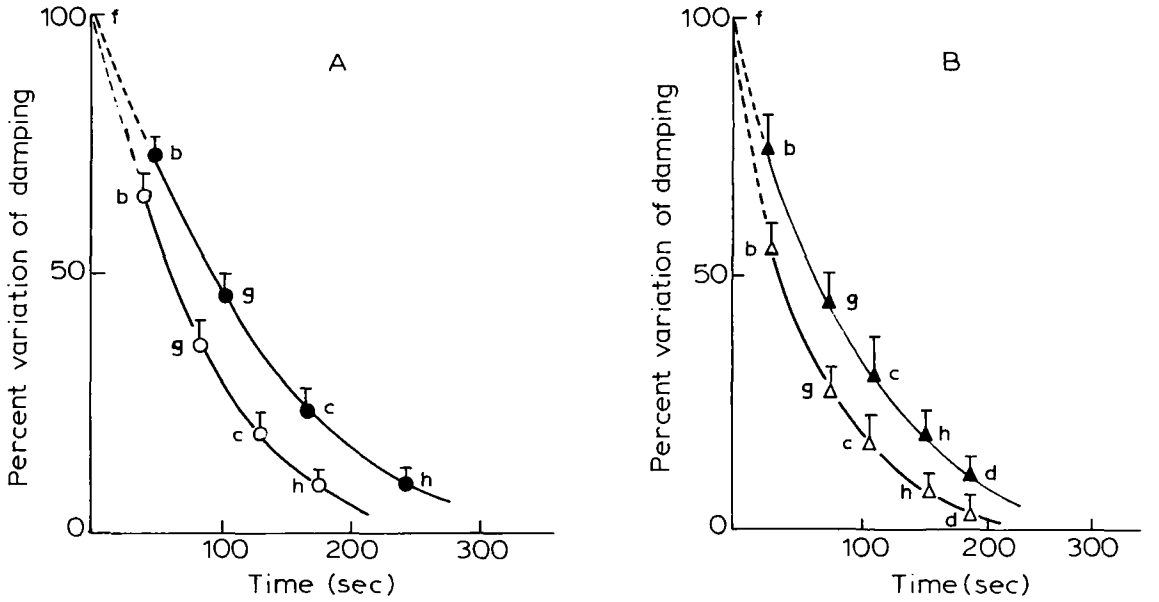

Fig. 4. Curves showing the percentage variations of damping in the oscillatory tracings of liver mitochondria of normal rats (●), intact diabetic (○), oophorectomized diabetic (▲) and oophorectomized diabetic rats with restitution of 17β-estradiol (△). The x axis gives time in seconds, after the first complete wave f (see Fig. 1) (time 0). The y axis represents the mean (three experiments) of the percentage variation of amplitude taking the first complete wave (f) as 100%. Each point in the curve represents the mean percentage amplitude decrease of subsequent waves (g,h,i, etc.).

above than the other two groups (I and O + Eol). As can be seen, the values of the O group were found to be similar to the normal standard values among mitochondrial parameters during respiration (see Table 1). The values of these parameters in the group O + Eol fell significantly and they were similar to those of the I group.

TABLE 1

EFFECT OF OVARIECTOMY ON MITOCHONDRIAL RESPIRATION PARAMETERS OF NORMAL NON-DIABETIC RATS

N = normal rats, O = ovariectomized rats, RCR = respiratory control ratio. State 3, state 4 and other experimental conditions are described in Material and methods. Values represent averages ± SD. Number of animals was six per group.

Substrate		N	O
3-Hydroxybutyrate	State 3	65 ± 12.00	66 ± 11.80
	State 4	15 ± 1.80	16 ± 2.00
	RCR	4.33 ± 0.55	4.20 ± 0.50
Malate–glutamate	State 3	127 ± 18.70	121 ± 10.26
	State 4	23 ± 2.35	22.83 ± 1.95
	RCR	5.60 ± 0.56	5.30 ± 0.66
Succinate	State 3	142 ± 16.70	142 ± 16.00
	State 4	32 ± 3.35	34 ± 3.10
	RCR	4.43 ± 0.12	4.22 ± 0.15

TABLE 2

EFFECT OF OVARIECTOMY AND 17β-ESTRADIOL RESTITUTION UPON LIVER MITOCHONDRIAL RESPIRATION OF DIABETIC RATS

I = intact diabetic rats, O = oophorectomized diabetic rats, O + EoI = oophorectomized diabetic rats treated with 17β-estradiol. The number of rats was ten per group. Conditions are as in Table 1.

Substrate		I	O	O + EoI
3-Hydroxybutyrate	State 3	29 ± 5.10[a]	55 ± 7.20[b]	30 ± 4.46[c]
	State 4	11 ± 1.45	14 ± 1.65	12 ± 2.10
	RCR	2.63 ± 0.45[d]	3.92 ± 0.61[e]	2.53 ± 0.46[f]
Malate–glutamate	State 3	80 ± 9.90[a]	121 ± 12.80[b]	65 ± 10.00[c]
	State 4	29 ± 2.00	23 ± 2.55	18 ± 1.82
	RCR	4.20 ± 0.39[d]	5.36 ± 0.81[e]	3.74 ± 0.95[f]
Succinate	State 3	95 ± 17.50[a]	135 ± 18.20[b]	95 ± 14.00[c]
	State 4	26 ± 2.41	30 ± 2.62	27 ± 3.00
	RCR	3.65 ± 0.52[g]	4.50 ± 0.51[e]	3.5 ± 0.45[f]

Analysis of variance: in brackets are the pairs of values compared [b,a], [b,c], [e,d], [e,f], $P < 0.001$; [e,g], $P < 0.01$.

Effect of ovariectomy upon blood glucose and the ratio of liver weight to body weight

Table 3 lists the mean values of blood glucose concentration and the ratio of liver weight to body weight in the two experimental groups of rats, intact and oophorectomized diabetic. It may be observed that there is no difference among the values of the above-mentioned parameters when comparing the two groups of animals.

Discussion

The results of this study demonstrated that female rats rendered diabetic by the injection of SZ had impaired liver mitochondrial functions.

The alterations registered were the following: (1) a significant decrease of the mean respiratory control ratio and the active state 3 of respiration with the substrates 3-hydroxybutyrate, malate–glutamate, and succinate; (2) significant alterations of the tracings of the osmotic harmonic damping oscillations of the organelle.

The impairment of the mitochondrial functions produced by SZ-induced diabetes in intact female rats was improved by ovariectomy. Furthermore, 17β-estradiol restitution restored the assayed parameters of mitochondrial functions to values similar to those of intact female diabetic rats.

The results shown in Table 3 show no significant variations in the blood sugar of intact diabetic rats

TABLE 3

COMPARISON OF BLOOD GLUCOSE CONCENTRATION AND RATIO OF LIVER WEIGHT TO BODY WEIGHT IN INTACT DIABETIC AND OOPHORECTOMIZED DIABETIC RATS

The values are the mean ± SD. The body and the liver of rats were weighed just before and just after death. The number of rats was 11 per group. For other experimental conditions see Material and methods.

Treatment	Blood glucose (mM)	$\frac{\text{Liver weight (g)}}{\text{Body weight (g)}} \times 10^3$
Intact diabetic	21 ± 3.05	44 ± 3.5
Oophorectomized diabetic	20 ± 2.22	45 ± 3.8

when compared with ovariectomized diabetics. This fact demonstrates that oophorectomy in diabetic rats does not modify the utilization of blood glucose by insulin-dependent tissues. In the same Table no variation is seen in the ratio of liver weight to body weight. Thus, a different food intake would not play any role in the favorable effect of ovariectomy upon liver mitochondrial functions of diabetic rats.

It is to be emphasized that ovariectomy in normal non-diabetic rats neither produced any variation in values of liver mitochondrial respiration (Table 1) nor influenced the damping factor of the oscillatory variation of the particles (results not shown).

These data demonstrate that in the diabetic state estrogens negatively affect very important mitochondrial functions. Mitochondrial functions depend on the composition of phospholipids and fatty acids of the membranes [14–18] and this composition determines the fluidity of these membranes. In experimental diabetes, impairment of the mechanism of liver microsomal enzymatic desaturation of fatty acids has been described [19–22]. The lesser supply of polyunsaturated fatty acids to mitochondrial membranes in the diabetic state must alter the fluidity and functions of the organelle. This diabetic alteration was reversed by treatment with insulin [23,24]. According to Brignone et al. [25] and González et al. [26] endogenous and exogenous estrogen modified the fatty acid composition and phospholipid turnover of mitochondrial and microsomal membranes. In addition, these steroids affect other endocrine glands which likewise regulate the fluidity of liver mitochondria [27–29]. Directly or indirectly, ovariectomy in diabetic rats may modify the phospholipid and fatty acid composition of liver mitochondrial membranes. Membrane fluidity may increase and mitochondrial functions improve as was found by the current authors. It has even been shown that estrogen reduces in vivo the activity of hepatic mitochondrial carnitine palmitoyltransferase [30] with less transference of acyl CoA to the matrix of the organelle. This effect consequently decreases the liver's oxidation of fatty acids and ketone body formation. Here diabetic oophorectomized rats with no circulating estrogen would appear to experience an increase in this metabolic pathway which would produce more available $NADH + H^+$ and $FADH_2$ in the liver mitochondrial matrix. These nucleotides will provide more reduction equivalents to the respiratory chain affected by the diabetic state. The data are consistent with the significant increase in activity of the enzyme 3-hydroxybutyrate dehydrogenase (NAD^+-dependent) in oophorectomized diabetic rats (diabetes induced by injection of SZ) as compared with intact diabetic rats, as was demonstrated recently in our laboratory (results not shown). Moreover, ovarian hormones potentiate the hyperactivity of the adrenal glands in experimentally induced diabetes [31–33]. Furthermore Albrecht et al. [34] showed that adrenalectomy of diabetic animals significantly decreases the concentration of free fatty acids in the blood (FFA) and as has been demonstrated these FFA induce alterations of mitochondrial functions at the membrane level [35,36]. Consequently ovariectomy of diabetic rats will produce a diminution of adrenal hyperactivity and this will contribute towards the improvement of the lipid environment of the mitochondria that will ameliorate the functions of these particles. This fits in well with our preliminary results [37] which showed that adrenalectomy of female rats (under the same conditions as in the present study), rendered diabetic by the injection of SZ, had the same favorable effect on the mitochondrial functions described in this paper.

The endocrine relationship of estrogens and diabetes investigated in the present work only constitutes a small part of a more complex mechanism involved in diabetic hormonal homeostasis. Additional research is needed to clarify the role of estrogen and other steroid hormones in this metabolic interrelationship.

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