

Effect of Alloxan-Diabetes and Subsequent Treatment with Insulin on Kinetic Properties of Succinate Oxidase Activity from Rat Liver Mitochondria

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We evaluated early and late effects of alloxan-diabetes and subsequent insulin treatment on the kinetic properties of succinate oxidase (SO) in rat liver mitochondria. Diabetic state lowered the SO activity; insulin treatment was effective in restoring the activity only in one-week diabetic rats. The energies of activation in low and high temperature ranges (E_H and E_L) decreased significantly in diabetic animals; once again insulin treatment was partially effective only in the one-week diabetic group. The total phospholipids (TPL) and cholesterol (CHL) contents did not change in one-week groups. In one-month diabetic animals TPL decreased while CHL increased; insulin treatment induced further changes without restoring normality. The lysophospholipid (Lyso), sphingomyelin (SPM), phosphatidylinositol (PI) and phosphatidylserine (PS) content increased in the diabetic state while phosphatidylcholine (PC) and phosphatidylethanolamine (PE) decreased. Insulin treatment had a partial restorative effect. The changes in E_H correlated negatively with SPM. The phase transition temperature, T_i , decreased in diabetic and insulin-treated groups. These changes correlated positively with the ratios of TPL/PI and TPL/PS. The membrane fluidity decreased in the diabetic state; insulin had a restorative effect only in the one-week group.

Key words: Alloxan-Diabetes, Succinate Oxidase, Arrhenius Kinetics

Introduction

The role of membrane structure, phospholipids composition and dynamics in regulation of membrane-bound enzymes is well recognized (Vidal *et al.*, 1983; Daum, 1985; Taryshkin *et al.*, 1986; Yeagle, 1989; Kuwahara *et al.*, 1997). Requirement of specific phospholipid classes for several mitochondrial enzyme and Na^+, K^+ -ATPase activity in the microsomes is well documented (Schwartz, 1976; Zelenka, 1984; Daum, 1985; Cornelius *et al.*, 2001). The phospholipid composition and content of mitochondria and microsomes is significantly influenced by the hormonal status of the organism. Thyroid hormones and insulin have been shown to significantly influence the phospholipids composition and make-up in sub-cellular membranes (Pasquini *et al.*, 1980; Ruggiero *et al.*, 1984; Bangur *et al.*, 1995; Parmar *et al.*, 1995; Kuwahara *et al.*, 1997; Brenner *et al.*, 2000; Cabello *et al.*, 2001; Ferreira *et al.*, 2003). Thus, it may be anticipated that the hormonal status can indirectly modulate the ki-

netic properties of membrane-bound enzymes, which is attributable to changes in membrane lipid/phospholipid make-up.

Earlier studies from our laboratory (Patel and Katyare, 2006) have shown that the insulin status plays a significant role in altering the kinetic behavior of FoF_1 ATPase in rat liver mitochondria. The FoF_1 ATPase on its part plays a significant role in energy coupling and has a restricted micro-domain where it is localized but is not a component of the electron transport chain (ETC) (Mathews and van Holde, 1996). The enzyme system succinate oxidase, on the other hand, spans a major portion of the ETC from succinate dehydrogenase (SDH) to cytochrome oxidase. Evaluation of its kinetic properties as influenced by the insulin status can give broad based, deeper insights into the regulatory role of membrane lipids. The present communication summarizes these aspects.

Materials and Methods

Chemicals

Bovine serum albumin (BSA) fraction V, 1,6-diphenyl-1,3,5-hexatriene (DPH) and sodium salt of ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Sodium salt of succinic acid was purchased from SRL, Mumbai, India. Silica gel G was from E. Merck, Darmstadt, Germany. Neutral protamine Hagedorn (NPH) insulin (40 U/ml) was obtained from Lilli, Fegersheim, France S.A.S. All other chemicals were of analytical-reagent grade and purchased locally.

Animals

Adult male albino rats of Charles-Foster strain (weighing 200–250 g) were used. The animals were fasted overnight and the diabetic state was induced by injecting subcutaneously (s.c.) 12 mg alloxan/100 g body weight (Kumthekar and Katyare, 1992; Satav *et al.*, 2000; Dave and Katyare, 2002). Alloxan solutions were prepared freshly prior to use in saline solution. The control rats received an equivalent volume of saline solution.

The diabetic state was confirmed by hyperglycemia, polyurea and glucoseurea and the results were consistent with the previously described observations (Kumthekar and Katyare, 1992; Khandkar *et al.*, 1995; Dave and Katyare, 2002). Hence, for sake of brevity, these confirmatory data are not given here.

Experiments were carried out at the end of one-week and one-month of induction of diabetic state to ascertain the early onset and long-term effects (Satav and Katyare, 2004; Katyare and Satav, 2005). Animals falling in the one-week diabetic group received insulin from the fifth day of induction of diabetes for 3 d and the rats in the one-month diabetic group received insulin from starting of the fourth week of induction of diabetes for 7 d at a dose of 0.8 units of NPH insulin/100 g body weight twice daily (around 6 A.M. and 6 P.M.) by the s.c. route (Satav *et al.*, 2000; Dave and Katyare, 2002).

Isolation of mitochondria

Isolation of liver mitochondria was carried out by the procedures described earlier using an isolation medium comprising 0.25 M sucrose, 10 mM Tris [tris(hydroxymethyl)aminomethane]-HCl buffer, pH 7.4, and 1 mM EDTA; 250 μ g BSA/ml of isolation

medium were included (Katewa and Katyare, 2004).

Succinate oxidase assay

Measurement of succinate oxidase activity was carried out polarographically using a Clarke-type oxygen electrode. The assay medium (final volume 1.6 ml) consisted of 50 mM potassium phosphate buffer, pH 7.4, containing 0.4 mM each of CaCl_2 and AlCl_3 (Katyare *et al.*, 1971) and saturating amount of sodium succinate (10 mM). The measurements were carried out over the temperature range from 5 to 53 °C with an increment of 4 °C at each step. The activity (v) is expressed as $\text{nmol O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$.

The data on temperature kinetics were analyzed for determination of energies of activation in the high and low temperature ranges (E_H and E_L , respectively) and phase transition temperature (T_t) according to the methods described previously (Dave *et al.*, 1999; Patel *et al.*, 2000; Dave and Katyare, 2002).

The extraction of mitochondrial lipids/phospholipids, estimation of cholesterol, determination of phospholipid profile and membrane fluidity were as described previously (Pandya *et al.*, 2004).

The regression analysis was carried out across the groups using Jandel Sigmastat Statistical Software, version 2.0 (Jandel Corporation, San Rafael, California, USA).

Estimation of protein was done according to Lowry *et al.* (1951) using BSA as the standard.

Statistical evaluation of data was by Students' t-test.

Results and Discussion

Data in Table I show that succinate oxidase activity of rat liver mitochondria decreased by 67 to 70% in the one-week diabetic group. Treatment with insulin restored the activity to near control values. At the end of one-month of diabetic state, the decrease in activity amounted to 42–50% which is indicative of partial recovery. However, in long-term diabetic animals, insulin treatment failed to restore the activity. Also, measurements at 25 °C and 37 °C indicated that the increment in the activity with temperature was different and of lesser magnitude in the diabetic groups.

These observations prompted us to investigate in detail the temperature-dependence of the enzyme activity under different experimental condi-

Table I. Effects of alloxan-diabetes and subsequent treatment with insulin on succinate oxidase activity in rat liver mitochondria.

Group	Treatment	Activity [nmol O ₂ min ⁻¹ mg protein ⁻¹]	
		25 °C	37 °C
One-week	Control	34.24 ± 1.12	59.55 ± 3.94
	Diabetic	11.15 ± 0.43 ^a	17.66 ± 0.92 ^a
	Diabetic + insulin	28.79 ± 0.48 ^{a,c}	54.26 ± 1.16 ^{a,c}
One-month	Control	31.35 ± 1.48	53.21 ± 2.18
	Diabetic	18.21 ± 1.26 ^a	26.39 ± 1.21 ^a
	Diabetic + insulin	14.21 ± 0.18 ^{a,b}	23.24 ± 0.29 ^{a,b}

The results are given as mean ± SEM of 6–8 independent experiments.

^a $p < 0.001$ compared to the corresponding control.

^b $p < 0.05$ and ^c $p < 0.001$ compared to the corresponding diabetic.

tions. The typical activity versus temperature curves and corresponding Arrhenius plots for the one-week and the one-month groups, respectively, are shown in Figs. 1 and 2. As can be noted the

activity versus temperature curves are consistent with the data in Table I. Thus, the activities at any given temperature were low in the diabetic groups (Figs. 1 and 2, panels A, B and C). Insulin treatment restored the activities in the one-week diabetic but not in the one-month diabetic group. However, the most interesting feature was a shift in optimum temperature in the diabetic or insulin-treated animals. Thus, the optimum temperature for the control group was 37 °C which increased to 41–45 °C in diabetic groups and remained elevated at 45 °C in insulin-treated diabetic animals. The differences in the profiles of activity versus temperature were clearly evident in the corresponding Arrhenius plots (Figs. 1 and 2, panels D, E and F).

The values of energies of activation derived from Arrhenius plots are given in Table II. The values of E_H and E_L , respectively, were around 42 and 100 kJ/mol for the controls with phase transition occurring at around 20 °C. In diabetic animals the energies of activation E_H and E_L decreased significantly in the one-week diabetic group. In one-month diabetic animals a similar trend was noted although the extent of decrease was not as

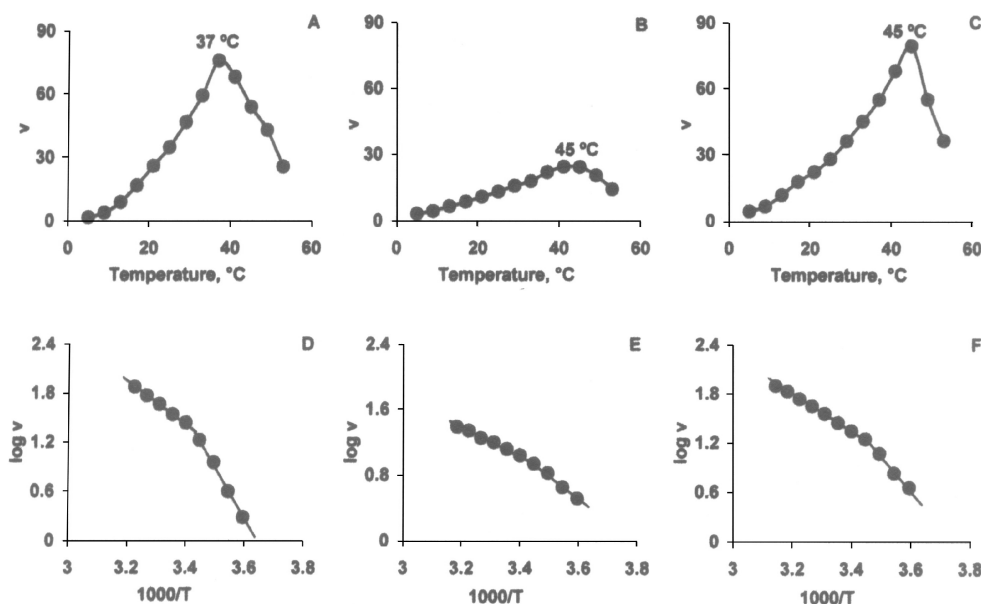


Fig. 1. Typical plots depicting dependence of enzyme activity on temperature and the corresponding Arrhenius plots for one-week groups. In the temperature curves, the enzyme activity v on abscissa is plotted versus temperature T (°C) on ordinate. A, B and C represent the control, diabetic and insulin-treated diabetic groups. In Arrhenius plots, $\log v$ on abscissa is plotted against $1000/T$ on ordinate where v represents the activity at corresponding absolute temperature T . D, E and F represent the control, diabetic and insulin-treated diabetic groups. The plots are typical of 6–8 independent experiments in each group.

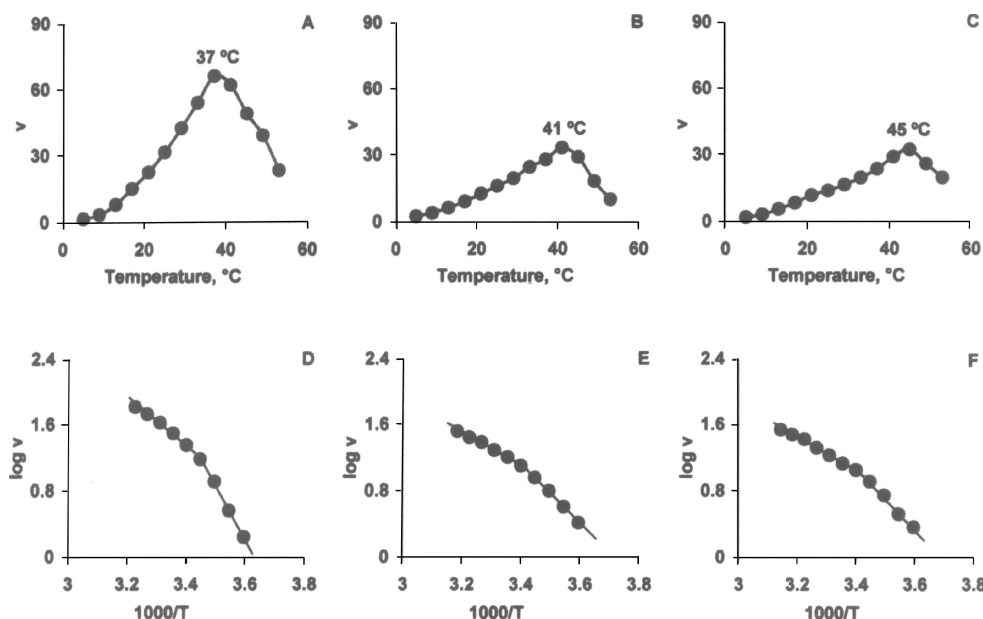


Fig. 2. Typical plots depicting dependence of enzyme activity on temperature and the corresponding Arrhenius plots for one-month groups. In the temperature curves, the enzyme activity v , on abscissa is plotted versus temperature T ($^{\circ}\text{C}$) on ordinate. A, B and C represent the control, diabetic and insulin-treated diabetic groups. In Arrhenius plots, $\log v$ on abscissa is plotted against $1000/T$ on ordinate where v represents the activity at corresponding absolute temperature T . D, E and F represent the control, diabetic and insulin-treated diabetic groups. The plots are typical of 6–8 independent experiments in each group.

appreciable. In both the groups, phase transition temperature T_t decreased significantly. Treatment with insulin of the one-week diabetic group completely restored E_H with partial restoration in E_L ; in one-month diabetic animals insulin treatment was ineffective in this respect. The T_t values were not restored to control levels and remained lower than the control values. The low value of T_t under

both the experimental conditions, *i.e.* diabetic and insulin-treated diabetic groups, seems to be paradoxical especially in view of the fact that the fatty acid desaturase activity and unsaturation index decrease in diabetes (Kuwahara *et al.*, 1997; Coste *et al.*, 1999). It may hence be suggested that the observed changes in lowered values of E_H and E_L as well as T_t may represent compensatory mecha-

Group	Treatment	Energy of activation [kJ/mol]		Phase transition temperature [$^{\circ}\text{C}$]
		E_H	E_L	T_t
One-week	Control	42.98 ± 2.31	105.7 ± 3.76	19.48 ± 0.642
	Diabetic	29.30 ± 1.14^d	48.85 ± 1.37^d	17.13 ± 0.544^a
	Diabetic + insulin	43.12 ± 2.00^f	$71.24 \pm 1.84^{d,f}$	15.72 ± 0.396^d
One-month	Control	40.37 ± 2.64	98.45 ± 4.83	21.05 ± 0.696
	Diabetic	32.83 ± 1.43^a	79.38 ± 2.73^b	15.55 ± 0.675^d
	Diabetic + insulin	34.09 ± 0.75^a	81.29 ± 2.34^b	$60 \pm 0.481^{c,e}$

The results are given as mean $\pm$ SEM of 6–8 independent experiments.

^a $p < 0.05$; ^b $p < 0.01$; ^c $p < 0.002$ and ^d $p < 0.001$ compared to the corresponding control.

^e $p < 0.05$ and ^f $p < 0.001$ compared to the corresponding diabetic.

Table II. Effects of alloxan-diabetes and subsequent treatment with insulin on Arrhenius kinetics properties of succinate oxidase in rat liver mitochondria.

Table III. Effects of alloxan-diabetes and subsequent treatment with insulin on total phospholipids (TPL), cholesterol (CHL), and content of rat liver mitochondria.

Group	Treatment	TPL [$\mu\text{g}/\text{mg}$ protein]	CHL [$\mu\text{g}/\text{mg}$ protein]	TPL/CHL [mol/mol]	Fluorescence polarization <i>p</i>
One-week	Control	178.42 $\pm$ 4.55	55.11 $\pm$ 2.41	1.62 $\pm$ 0.07	0.157 $\pm$ 0.007
	Diabetic	180.55 $\pm$ 4.97	57.22 $\pm$ 2.63	1.57 $\pm$ 0.08	0.201 $\pm$ 0.004 ^c
	Diabetic + insulin	185.38 $\pm$ 9.77	52.99 $\pm$ 1.55	1.74 $\pm$ 0.07	0.150 $\pm$ 0.004 ^c
One-month	Control	173.65 $\pm$ 5.66	53.44 $\pm$ 2.00	1.56 $\pm$ 0.09	0.159 $\pm$ 0.008
	Diabetic	122.49 $\pm$ 7.11 ^c	80.81 $\pm$ 2.54 ^c	0.76 $\pm$ 0.05 ^c	0.250 $\pm$ 0.006 ^c
	Diabetic + insulin	140.55 $\pm$ 6.27 ^{c,d}	46.81 $\pm$ 1.56 ^{b,c}	1.50 $\pm$ 0.08 ^{a,c}	0.207 $\pm$ 0.005 ^{c,e}

The results are given as mean $\pm$ SEM of 6–8 independent experiments in each group.

^a $p < 0.05$; ^b $p < 0.01$ and ^c $p < 0.001$ compared to the corresponding control.

^d $p < 0.05$ and ^e $p < 0.001$ compared to the corresponding diabetic.

Table IV. Effects of alloxan-diabetes and subsequent treatment with insulin on phospholipid composition of rat liver mitochondria.

Phospholipid class	Composition (% of total)					
	One-week			One-month		
	Control	Diabetic	Diabetic + insulin	Control	Diabetic	Diabetic + insulin
Lyso	1.50 $\pm$ 0.04	4.59 $\pm$ 0.34 ^c	1.88 $\pm$ 0.11 ^{e,h}	1.84 $\pm$ 0.11	5.11 $\pm$ 0.35 ^c	1.21 $\pm$ 0.08 ^{e,h}
SPM	2.87 $\pm$ 0.19	5.37 $\pm$ 0.24 ^c	2.86 $\pm$ 0.14 ^h	2.77 $\pm$ 0.08	4.74 $\pm$ 0.18 ^c	5.33 $\pm$ 0.31 ^e
PC	45.40 $\pm$ 0.52	44.13 $\pm$ 0.54 ^a	45.14 $\pm$ 0.54	44.66 $\pm$ 0.38	41.22 $\pm$ 0.85 ^c	43.06 $\pm$ 0.52 ^a
PI	1.61 $\pm$ 0.12	2.24 $\pm$ 0.13 ^d	3.29 $\pm$ 0.20 ^{e,h}	1.89 $\pm$ 0.12	4.59 $\pm$ 0.20 ^c	3.70 $\pm$ 0.24 ^{e,h}
PS	1.65 $\pm$ 0.08	2.34 $\pm$ 0.12 ^c	2.98 $\pm$ 0.20 ^{b,f}	1.67 $\pm$ 0.08	5.46 $\pm$ 0.22 ^c	2.72 $\pm$ 0.20 ^{e,f}
PE	36.09 $\pm$ 0.49	30.55 $\pm$ 0.73 ^c	30.99 $\pm$ 0.56 ^c	35.84 $\pm$ 0.46	24.51 $\pm$ 0.46 ^c	31.89 $\pm$ 0.39 ^{e,h}
DPG	10.89 $\pm$ 0.18	10.79 $\pm$ 0.40	12.86 $\pm$ 0.21 ^{e,h}	11.47 $\pm$ 0.18	14.36 $\pm$ 0.75 ^b	12.10 $\pm$ 0.24 ^g

The results are given as mean $\pm$ SEM of 6–8 independent experiments in each group.

Lyso, lysophospholipid; SPM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol.

^a $p < 0.05$; ^b $p < 0.02$; ^c $p < 0.01$; ^d $p < 0.002$ and ^e $p < 0.001$ compared to the corresponding control.

^f $p < 0.01$; ^g $p < 0.002$ and ^h $p < 0.001$ compared to the corresponding diabetic.

nisms to improve the catalytic efficiency of the enzyme system under experimental conditions.

The observed changes in the temperature kinetic properties prompted us to examine the effects of insulin status on lipid/phospholipid profiles of the mitochondria. These results are given in Tables III–V. In one-week diabetic animals the content of total phospholipids (TPL) and cholesterol (CHL) did not change appreciably and insulin treatment had no effect. Nevertheless, the membrane fluidity decreased significantly in the diabetic group which was corrected by insulin treatment. By contrast, in one-month diabetic animals the TPL content decreased (30% decrease) while the CHL content increased (51% increase). This was reflected in terms of a decreased molar

ratio of the two entities and lowering of membrane fluidity. Insulin treatment resulted in partial restoration of the TPL content whereas the CHL content decreased below the control value. As a consequence there was only a partial restoration of the membrane fluidity to normality (Table III).

Analysis of the phospholipid profile (Table IV) revealed that the diabetic state had a generalized effect of increasing lysophospholipid (Lyso), sphingomyelin (SPM), phosphatidylinositol (PI) and phosphatidylserine (PS) and a tendency to decrease phosphatidylcholine (PC) and phosphatidylethanolamine (PE) components. Insulin treatment could correct the Lyso component and partially correct the PC and PE composition (Table IV). The computed contents of the individual

Table V. Effects of alloxan-diabetes and subsequent treatment with insulin on content of individual phospholipids in rat liver mitochondria.

Phospholipid class	Content [$\mu\text{g}/\text{mg}$ protein]					
	One-week			One-month		
	Control	Diabetic	Diabetic + insulin	Control	Diabetic	Diabetic + insulin
Lyso	2.62 ± 0.10	$8.32 \pm 0.72^{\text{d}}$	$3.48 \pm 0.25^{\text{c,g}}$	3.21 ± 0.19	$5.49 \pm 0.62^{\text{d}}$	$1.53 \pm 0.10^{\text{d,g}}$
SPM	5.02 ± 0.35	$9.72 \pm 0.58^{\text{d}}$	$5.26 \pm 0.30^{\text{g}}$	4.85 ± 0.20	5.11 ± 0.52	$6.93 \pm 0.44^{\text{d,e}}$
PC	79.55 ± 2.17	79.75 ± 2.51	83.55 ± 4.28	77.94 ± 1.47	$45.32 \pm 5.12^{\text{d}}$	$59.45 \pm 2.00^{\text{d,e}}$
PI	2.83 ± 0.21	$4.03 \pm 0.24^{\text{c}}$	$6.05 \pm 0.48^{\text{d,f}}$	3.31 ± 0.23	$5.07 \pm 0.65^{\text{c}}$	$5.33 \pm 0.32^{\text{d}}$
PS	2.88 ± 0.16	$4.25 \pm 0.28^{\text{d}}$	$5.54 \pm 0.51^{\text{d,e}}$	2.91 ± 0.15	$5.99 \pm 0.69^{\text{d}}$	$3.99 \pm 0.26^{\text{b,g}}$
PE	63.11 ± 1.43	$55.29 \pm 2.29^{\text{d}}$	$57.25 \pm 2.75^{\text{a}}$	62.54 ± 1.31	$26.72 \pm 2.84^{\text{d}}$	$44.62 \pm 2.00^{\text{d,g}}$
DPG	19.06 ± 0.53	19.57 ± 1.05	$23.84 \pm 1.32^{\text{b,e}}$	20.02 ± 0.48	$15.13 \pm 1.20^{\text{d}}$	$16.40 \pm 0.51^{\text{d}}$

The results are given as mean $\pm$ SEM of 6–8 independent experiments in each group.

Lyso, lysophospholipid; SPM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol.

^a $p < 0.02$; ^b $p < 0.01$; ^c $p < 0.002$ and ^d $p < 0.001$ compared to the corresponding control.

^e $p < 0.05$; ^f $p < 0.002$ and ^g $p < 0.001$ compared to the corresponding diabetic.

phospholipid classes were generally consistent with the above data (Table V).

Since energies of activation and phase transition temperature showed significant insulin-status-dependent changes, it was of interest to find out if a correlation with lipid/phospholipids make-up existed. Regression analysis across the groups indicated that SPM showed a strong negative correlation with E_{H} ($r = -0.715$). On the other hand T_{t} showed a positive correlation with TPL/PI and TPL/PS ($r = +0.620$ and $+0.631$, respectively). Our earlier studies have shown that the SPM, PI and PS components increased in diabetic animals and remained elevated even after insulin treatment (S. P. Patel and S. S. Katyare, unpublished work). However, the succinate oxidase activity by itself did not seem to be correlated with any of the lipid/phospholipids classes. This is consistent with earlier reported observations that the enzyme succinate oxidase has a non-specific requirement for phospholipids in general (Daum, 1985). Therefore, the bulk membrane lipids seem to meet the general requirements (Daum, 1985) and SPM, PI and PS emerged as modulatory factors.

The enzyme SDH which is responsible for initiating the process of electron transfer is a rate-limiting step in succinate oxidase activity (Singer *et al.*, 1973). The enzyme is activated by several physiologic activators which include ATP, NADH, Co

Q and Pi (Singer *et al.*, 1973). Cytochromes of the electron transport chain are other rate limiting step. Insulin-status-dependent changes in the contents of Co Q and cytochromes in mitochondria have been demonstrated (Ferreira *et al.*, 2003; Satav and Katyare, 2004; Katyare and Satav, 2005).

The enzyme SDH is made up of two subunits, both of which are coded by nuclear DNA (Singer *et al.*, 1973; Poyton and McEwen, 1996). As is evident from the data presented, the succinate oxidase activity was low in diabetic animals and could not be restored by insulin treatment in the one-month diabetic group. Based on these observations, it may be suggested that in the diabetic state, besides insulin, other hormones may be involved in the expression of subunits of SDH. A parallel relationship between insulin and thyroid hormones has been demonstrated. Thus, the thyroid hormones could be an additional regulatory factor. We have earlier noted thyroid-status-dependent alterations in mitochondrial lipid/phospholipid profiles, membrane fluidity and enzyme kinetics parameters (Bangur *et al.*, 1995; Parmar *et al.*, 1995).

In conclusion, results of our present studies besides demonstrating the regulatory role of specific phospholipids also emphasize that regulation of succinate oxidase in diabetes is a complex process which may involve hormonal interplay.

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