Chapter 12

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Ageing-Induced Alterations in Lipid/Phospholipid Profiles of Rat Brain and Liver Mitochondria: Implications for Mitochondrial Energy-Linked Functions

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Abstract Effects of ageing on the lipid/phospholipid profile of brain and liver mitochondria from rats were examined. In the brain mitochondria the contents of total phospholipid (TPL) and cholesterol (CHL) increased with simultaneous increase in the TPL/CHL (mole:mole) ratio. The proportion and contents of lysophospholipid (Lyso), sphingomyelin (SPM), phosphatidylinositol (PI), phosphatidylserine (PS) and diphosphatidylglycerol (DPG) components increased, with maximal increases seen for PS and PI; phosphatidylcholine (PC) and phosphatidylethanolamine (PE) components registered decrease. In the liver mitochondria contents of TPL and CHL increased. However, the TPL/CHL (mole:mole) ratio was not altered. Lyso, PI and PS increased. However, the magnitude of increase was competitively lower; PE and DPG decreased. SPM and PC did not change as a consequence of ageing. These changes altered the contents of individual phospholipids in the two membrane systems. Respiration with glutamate, pyruvate + malate, succinate and ascorbate + *N,N,N',N'*-tetramethyl-*p*-phenylenediamine was significantly impaired in brain mitochondria from old animals. For liver mitochondria the respiratory activity declined with glutamate and succinate. Correlation studies by regression analysis revealed that the lipid/phospholipid classes regulate respiratory function differently in the mitochondria from the two tissues. The respiration-related parameters in the brain mitochondria were dependent on multiple lipid/phospholipid components, and the process of regulation was complex compared to the liver mitochondrial functions.

Keywords Age · Brain mitochondrial lipid · Liver mitochondrial lipid · Brain mitochondrial phospholipid profile · Liver mitochondrial phospholipid profile · Mitochondrial respiration

Introduction

Ageing, a unique feature of all organisms, is accompanied by impaired functional capacity of many systems. With ageing there is a gradual decline in the capacity of various cell types including neurons (Toescu, Myronova & Verkhratsky, 2000). Also, the levels of several hormones, including the thyroid hormones insulin, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), testosterone, estradiol and leptin, as well as others, decline with ageing (Elmlinger et al., 2003; Moreira et al., 2005; Baranowska et al., 2007). It has been shown that the thyroid hormones insulin and DHEA significantly influence the lipid/phospholipid makeup of subcellular organelles including mitochondria (Pasquini et al., 1980; Ruggiero et al., 1984; Hostetler, 1991; Bangur, Howland & Katyare, 1995; Parmar et al., 1995; Dugan & Porter, 1997; Patel & Katyare, 2006a, b). It may hence be anticipated that age-related changes could occur in the lipid/phospholipid profiles of mitochondria.

It has also been reported that the oxidative energy metabolism of mitochondria from various tissues including brain and liver is significantly diminished in aged animals (Hansford, 1983; Kim et al., 1988a; Kim, Shrago & Elson, 1988b; Patel & Katyare, 2006b; Patel, Modi & Katyare, 2007). Since several components of the electron transport chain require specific lipids/phospholipids for their function (Daum, 1985), the ageing-related changes in lipid/phospholipid profiles can in turn lead to alteration(s) in the function(s) of the components of the electron transport chain.

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Age-related changes in the contents of lipids in brain regions in experimental animals and in humans have been documented (Giusto, Roque & Ilincheta de Boschero, 1992; Soderberg et al., 1990; Delion et al., 1997). Changes in the enzymes involved in lipid metabolism in rat liver have also been reported (Bourre, 2004). In particular, the emphasis has been on alterations in the fatty acid composition (Ilincheta de Boschero et al., 2000; Carver et al., 2001). A few reports describe the effect of ageing on the lipid/phospholipid composition of mitochondria. However, the results are equivocal (Kim et al., 1988a, 1988b; Ruggiero et al., 1992).

Therefore, in the present study, we examined the effect of ageing on lipid/phospholipid profiles of rat brain mitochondria. Parallel studies were also carried out on liver mitochondria since liver is the major site of metabolism and lipid biosynthesis. It was felt that these studies will help to address the question as to whether the ageing-related changes are tissue-specific. We also attempted to correlate the observed changes with respiratory functions of the mitochondria. Results of our studies indicate that ageing differentially affected the mitochondrial lipid/phospholipid makeup and respiratory functions of mitochondria from brain and liver. Our studies also point out that the effects were more prominent and pronounced for brain mitochondria. Our present results also suggest that altered lipid/phospholipid profiles of brain mitochondria have a major influence on the respiratory function of the brain mitochondria.

Materials and Methods

Chemicals

ADP, rotenone, NAD$^+$, NADH, 4-morpholinopropanesulfonic acid (MOPS), dichlorophenolindophenol (DCIP), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and bovine serum albumin (BSA) fraction V were purchased from Sigma (St. Louis, MO). Sources of the substrates used for measurement of oxidative phosphorylation were as detailed previously (Patel & Katyare, 2006b; Patel et al., 2007). Silica gel G was from E. Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade and were purchased locally.

Animals

Male young adult (8–10 weeks, body weight 220–230 g) and old (18–24 months, body weight 350–370 g) albino rats of Charles-Foster strain were used. The experimental protocol was approved by the Departmental Animal Ethics Committee.

Isolation of Mitochondria

Isolation of brain and liver mitochondria was essentially according to the procedures described previously with some modifications (Katyare, Balasubramanian & Parmar, 2003; Katewa & Katyare, 2004; Pandya, Dave & Katyare, 2004; Katyare & Rajan, 2005; Patel & Katyare, 2006a, b; Patel et al., 2007).

Lipid Analysis

The extraction of mitochondrial lipids/phospholipids with freshly prepared chloroform and methanol (2:1vol/vol) was according to the procedures described in detail previously (Folch, Lees & Sloane-Stanley, 1957; Pandya et al., 2004).

Separation of phospholipid classes by thin layer chromatography (TLC) and estimations of cholesterol (CHL) and phospholipid phosphorus were according to the procedures described earlier (Zlatkis, Zak & Boyle, 1953; Bartlett, 1954; Skipski et al., 1967; Pandya et al., 2004).

The content of individual phospholipid classes were calculated by multiplying the values of total phospholipid (TPL) with the percent composition of the individual phospholipid classes (Pandya et al., 2004).

Oxidative Phosphorylation

Measurements of oxidative phosphorylation were carried out at 25°C using a Clark-type oxygen electrode as described previously (Katewa & Katyare, 2004; Patel & Katyare, 2006b; Patel et al., 2007). Briefly, the respiration medium (total volume 1.6 ml) consisted of 225 mM sucrose, 20 mM KCl, 10 mM MOPS (pH 7.4), 5 mM potassium phosphate buffer (pH 7.4), 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 160 mg BSA (i.e., 0.1 mg BSA/ml).

Determination of state 3 and state 4 respiration rates and calculation of ADP/O ratio and ADP phosphorylation rates were as described previously (Ferreira & Gil, 1984; Katyare & Satav, 1989).

Enzyme Assays

Glutamate dehydrogenase (GDH), malate dehydrogenase (MDH) and succinate DCIP reductase (SDR) activities were determined as described previously (Patel & Katyare, 2006a). The ATPase activity in the brain mitochondria was determined using the assay medium (total volume 0.1 ml) containing 350 mM sucrose, 10 mM MOPS (pH 7.4), 10 mM KCl, 0.2 mM EDTA, 2 mM $MgCl_2$ and 50 μM dinitrophenol

(DNP). For liver mitochondria the assay medium (total volume 0.1 ml) consisted of 50 mM MOPS (pH 7.4), 75 mM KCl, 0.4 mM EDTA, 6 mM $MgCl_2$ and 100 μM DNP (Katewa & Katyare, 2004; Patel & Katyare, 2006b; Patel et al., 2007). Estimation of inorganic phosphate was according to the procedure described (Katewa & Katyare, 2004). Protein estimation was by the method of Lowry et al. (1951) using BSA as the standard. Regression analysis was performed using Jandel Sigmastat Statistical Software, version 2.0 (Jandel, San Rafael, CA). Results are given as mean ± standard error of the mean (SEM). Statistical evaluation of the data was by Students' *t*-test.

Results

Data in Table 1 show that with increase in age the body and the brain weights increased, respectively, by 61% and 17%. This disproportionate increase was also reflected in the lowering of the relative brain weight (27% decrease). Compared to this, both liver weight as well as relative liver weight decreased significantly with ageing (21% and 51%, respectively).

Data in Table 2 show that with ageing the TPL and CHL content of the brain mitochondria increased by 33% and 22%, respectively. This disproportionate increase was also reflected in a small but reproducible increase in the TPL/CHL (mole:mole) ratio.

Table 1 Effect of ageing on body weight, brain weight and liver weight

Parameters	Young adult (12)	Old (10)
Body weight (g)	233.9 ± 7.43	376.5 ± 8.62*
Brain weight (g)	1.52 ± 0.03	1.78 ± 0.01*
Brain weight (% of body weight)	0.66 ± 0.02	0.48 ± 0.01*
Liver weight (g)	8.62 ± 0.30	6.79 ± 0.25*
Liver weight (% of body weight)	3.69 ± 0.06	1.80 ± 0.03*

Experimental details are given in the text. Results are mean ± SEM of the number of observations indicated in parentheses

* $P < 0.001$ compared with the corresponding young adult group

Examination of the phospholipid profile revealed that in old animals the proportion of lysophospholipid (Lyso), sphingomyelin (SPM) and diphosphatidylglycerol (DPG) components increased by 30–54%. However, the most dramatic increase was in the proportion of the acidic phospholipids (APL) phosphatidylinositol (PI) and phosphatidylserine (PS) components (2.7-fold increase in both). The phosphatidylcholine (PC) and phosphotidylethanolamine (PE) components were reduced by 19% and 27% in old animals, respectively (Table 3).

Data in Table 4 detail the content of individual phospholipids in the brain mitochondria. It is clear that in the old animals the content of Lyso and SPM increased by 99% and 74%, respectively. As is to be expected, a pronounced 3.9-fold increase in the content of PI and PS was evident; under these conditions the DPG content doubled. Interestingly, the contents of PC and PE were unchanged (Table 4).

For liver mitochondria, compared to brain mitochondria, an opposite picture was noted with respect to TPL and CHL contents. Thus, in old animals the TPL content increased by 23% and the CHL content increased by 37%. The TPL/CHL (mole:mole) ratio was somewhat lowered, but the decrease was not statistically significant (Table 2).

The phospholipid composition of liver mitochondria was also affected by age. Thus, in old animals the Lyso component increased by 82%. Of the acidic phospholipids, the increase in PI was marginal (46%), whereas PS components increased by 1.31-fold. Paradoxically, the DPG component showed a 20% decrease. SPM and PC were unchanged, while PE decreased by 9% (Table 3).

The data in Table 4 show the contents of individual phospholipids in the liver mitochondria. Thus, in old animals the contents of Lyso, SPM and PC increased, respectively, by 1.25-, 0.36- and 0.24-fold. The increase in the contents of acidic phospholipids PI and PS was 82% and 81%, respectively; the content of DPG was not changed. Also, PE content was unchanged (Table 4).

The foregoing results prompted us to evaluate if the observed changes in the lipid/phospholipid profiles correlated with and had bearing on the energy-linked functions in the mitochondria from the two tissues. With a view to

Table 2 Effect of ageing on TPL and CHL contents of rat brain and liver mitochondria

Mitochondria	Animals	TPL (μg/mg protein)	CHL (μg/mg protein)	TPL/CHL (mole:mole)
Brain	Young adult	423.4 ± 12.93	385.0 ± 6.74	0.55 ± 0.01
	Old	563.1 ± 7.76*	470.8 ± 7.49**	0.60 ± 0.02*
Liver	Young adult	180.7 ± 7.71	48.6 ± 1.63	1.86 ± 0.08
	Old	222.9 ± 6.28**	66.7 ± 3.41**	1.69 ± 0.08

Experimental details are given in the text. Results are mean ± SEM of eight independent observations in each group

* $P < 0.05$, ** $P < 0.001$ compared with the corresponding young adult group

Table 3 Effect of ageing on phospholipid composition of rat brain and liver mitochondria

Phospholipid class	Composition (% of total)			
	Brain		Liver	
	Young adult	Old	Young adult	Old
Lyso	3.59 ± 0.24	5.29 ± 0.37**	1.55 ± 0.04	2.82 ± 0.27**
SPM	6.47 ± 0.10	8.43 ± 0.57**	3.19 ± 0.09	3.50 ± 0.22
PC	41.73 ± 0.16	33.65 ±1.06**	46.21 ±1.04	46.42 ± 1.09
PI	2.41 ± 0.08	8.87 ± 0.63**	2.40 ± 0.17	3.51 ± 0.25*
PS	2.66 ± 0.08	9.77 ± 0.55**	1.83 ± 0.11	4.23 ± 0.28**
PE	39.86 ± 0.17	28.98 ± 1.60**	32.42 ± 0.59	29.62 ± 0.30**
DPG	3.27 ± 0.14	5.02 ± 0.24**	12.40 ± 0.69	9.91± 0.43*

Experimental details are given in the text. Results are mean ± SEM of eight independent observations in each group

* $P < 0.01$, **$P < 0.001$ compared with the corresponding young adult group

Table 4 Effect of ageing on phospholipid content of rat brain and liver mitochondria

Phospholipid class	Content (μg/mg protein)			
	Brain		Liver	
	Young adult	Old	Young adult	Old
Lyso	15.06 ± 0.75	29.93 ± 2.31**	2.81 ± 0.17	6.31 ± 0.68**
SPM	27.45 ± 1.11	47.72 ± 3.76**	5.76 ± 0.32	7.83 ± 0.58*
PC	176.74 ± 5.67	189.56 ± 6.93	83.47 ± 3.85	103.47 ± 3.78*
PI	10.24 ± 0.53	49.97 ± 3.61**	4.30 ± 0.30	7.81 ± 0.58**
PS	11.25 ± 0.50	55.06 ± 3.31**	3.32 ± 0.26	9.34 ± 0.44**
PE	168.82 ± 5.39	162.73 ± 8.06	58.63 ± 2.91	6.06 ± 2.30
DPG	13.82 ± 0.67	28.17 ± 1.19**	22.38 ± 1.61	22.05 ± 1.00

Experimental details are given in the text. Results are mean ± SEM of eight independent observations in each group

* $P < 0.01$, **$P < 0.001$ compared with the corresponding young adult group

further explore this point, we examined the effect of ageing on oxidative energy metabolism and related enzyme activities. These data are given in Tables 5–7

From the data in Table 5 it becomes evident that in the brain mitochondria state 3 respiration (in the presence of added ADP) with all the substrates tested decreased significantly (18–36%). The effect on state 4 respiration was variable; increase with pyruvate + malate (54% increase) and decrease with succinate and ascorbate + TMPD (17% and 50%, respectively). The respiratory control ratio (RCR) decreased for all the substrates, indicating that in old animals the mitochondria were less tightly coupled. Although the ADP/O ratios were unchanged, the potential for ATP synthesis, i.e., ADP phosphorylation rates, decreased significantly (24–38%). The levels of dehydrogenases also decreased significantly in old rats (21–73%), with maximal decrease seen for SDR activity. ATPase activity was somewhat low, but the change was not statistically significant (Table 7).

In liver mitochondria, state 3 respiration decreased only with glutamate and succinate (25% and 33%, respectively) and state 4 respiration decreased with glutamate by 36%. The values of RCR were unchanged. ADP phosphorylation rates with glutamate and succinate as the substrates decreased by 24% and 30%, respectively (Table 6). The trend for the dehydrogenases and ATPase activities was similar to that for the brain mitochondria. However, ATPase activity decreased drastically by 41% (Table 7).

The foregoing results thus emphasize that ageing differentially affected the oxidative energy metabolism of the mitochondria from brain and liver and that the effects were more pronounced on the cerebral mitochondria. These results are in general agreement with our previously reported observations (Patel & Katyare, 2006b; Patel et al., 2007).

We tried to correlate the changes in lipid/phospholipid profile and the respiratory functions of the mitochondria from the two tissues by regression analysis. These data are given in Tables 8 and 9. As can be noted, in brain mitochondria state 3 respiration rates in general correlated positively with PC and PE, whereas TPL, CHL, PS, PI, DPG and acidic phospholipid/basic phospholipid (APL/BPL) ratio seemed to be the negative modulators. Additionally, SPM/PC and SPM/PE ratios seemed to be negative modulators except for glutamate. State 4 respiration with glutamate was independent of lipid/phospholipid modulation, as was the case even for succinate. In the case of pyruvate + malate, TPL, CHL, PS, PI, DPG, SPM/PC and APL/BPL were positive modulators, while PE was a negative modulator. For ascorbate + TMPD system, the role was reversed and TPL, CHL, PI, PS, PC/PE, SPM/PE and APL/BPL became negative modulators; PS was a positive modulator.

Table 5 Effect of ageing on oxidative phosphorylation in rat brain mitochondria using glutamate, pyruvate+malate, succinate and acsorbate+TMPD as substrates

Substrate	Animals	ADP/O ratio	Respiration rate (nmole O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (nmole/min/mg protein)
			+ ADP	-ADP		
Glutamate	Young adult (12)	3.17 ± 0.10	19.9 ± 1.0	5.2 ± 0.3	3.86 ± 0.14	125.9 ± 7.5
	Old (12)	3.07 ± 0.12	12.8 ± 0.5***	5.2 ± 0.5	2.64 ± 0.20***	77.6 ± 2.2***
Pyruvate+malate	Young adult (12)	3.17 ± 0.19	21.7 ± 1.3	5.0 ± 0.5	4.41 ± 0.54	135.8 ± 7.6
	Old (10)	2.92 ± 0.15	17.9 ± 0.8*	7.7 ± 0.7***	2.44 ± 0.17**	102.6 ± 3.2***
Succinate	Young adult (12)	2.07 ± 0.06	24.2 ± 0.8	14.0 ± 0.8	1.83 ± 0.17	99.9 ± 3.7
	Old (10)	2.07 ± 0.16	16.9 ± 0.8***	11.6 ± 1.1***	1.53 ± 0.10	69.6 ± 6.0***
Ascorbate+TMPD	Young adult (16)	0.70 ± 0.03	23.7 ± 1.7	14.3 ± 1.0	1.67 ± 0.04	32.6 ± 2.1
	Old (12)	0.75 ± 0.06	16.1 ± 0.8***	11.9 ± 0.6*	1.36 ± 0.04***	24.1 ± 2.1***

The respiration medium (total volume 1.6 ml) consisted of 225 mM sucrose, 20 mM KCl, 10 mM MOPS (pH 7.4), 5 mM potassium phosphate buffer (pH 7.4), 0.2 mM EDTA and 160 mg BSA (i.e., 0.1 mg BSA/ml). Concentration of glutamate was 10 mM. State 3 respiration rates initiated by addition of 80–200 nmoles of ADP and state 4 rates ensuing after its depletion were recorded. Other experimental details are given in the text. Results are mean ± SEM of the number of observations indicated in parentheses

* $P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the young adult group

Table 6 Effect of ageing on oxidative phosphorylation in rat liver mitochondria using glutamate, pyruvate+malate, succinate and ascorbate+TMPD as substrates

Substrate	Animals	ADP/O ratio	Respiration rate (nmole O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (nmole/min/mg protein)
			+ ADP	-ADP		
Glutamate	Young adult (12)	3.11 ± 0.07	27.3 ± 1.1	10.2 ± 0.4	2.70 ± 0.08	168.4 ± 5.5
	Old (10)	3.11 ± 0.11	20.6 ± 0.9*	6.5 ± 0.4*	3.26 ± 0.15	128.1 ± 7.5*
Pyruvate+malate	Young adult (12)	3.25 ± 0.09	16.8 ± 0.6	7.7 ± 0.3	2.21 ± 0.09	109.1 ± 4.5
	Old (12)	3.11 ± 0.15	15.4 ± 0.7	8.1 ± 0.4	1.97 ± 0.14	97.0 ± 8.1
Succinate	Young adult (12)	2.17 ± 0.07	57.0 ± 2.6	22.1 ± 1.2	2.65 ± 0.15	246.3 ± 11.1
	Old (12)	2.30 ± 0.14	38.0 ± 2.3*	20.5 ± 1.3	1.88 ± 0.08	172.0 ± 11.9*
Ascorbate+TMPD	Young adult (14)	0.44 ± 0.03	26.7 ± 1.3	20.7 ± 1.2	1.30 ± 0.04	23.3 ± 1.5
	Old (21)	0.44 ± 0.03	27.8 ± 1.2	21.1 ± 1.1	1.33 ± 0.03	24.1 ± 1.2

The respiration medium (total volume 1.6 ml) consisted of 225 mM sucrose, 20 mM KCl, 10 mM MOPS (pH 7.4), 5 mM potassium phosphate buffer (pH 7.4), 0.2 mM EDTA and 160 mg BSA (i.e., 0.1 mg BSA/ml) Concentration of glutamate was 10 mM. State 3 respiration rates initiated by addition of 80–200 nmoles of ADP and state 4 rates ensuing after its depletion were recorded. Other experimental details are given in the text. Results are mean ± SEM of the number of observations indicated in parentheses

* $P < 0.001$ compared with the young adult group.

Table 7 Effect of ageing on mitochondrial enzymes

Enzyme	Brain		Liver	
	Young adult	Old	Young adult	Old
GDH	60.2 ± 1.33	47.8 ± 2.07*	202.9 ± 5.60	145.8 ± 8.68*
MDH	4,680 ± 141	2,954 ± 146*	4,214 ± 146	2,901 ± 154*
SDR	14.6 ± 0.40	4.0 ± 0.11*	20.4 ± 0.68	5.6 ± 0.31*
ATPase	5.4 ± 0.31	4.6 ± 0.27	20.1 ± 0.86	11.8 ± 0.49*

Experimental details are given in the text. Enzyme activities are given as nanomoles of substrate transformed per minute per milligram of protein. Results are given as mean ± SEM of 12–15 observations

* $P < 0.001$ compared with the corresponding young adult group

For liver mitochondria, PE positively modulated state 3 and state 4 respiration rates with glutamate. For succinate PE and DPG were the positive modulator, whereas for ascorbate + TMPD system CHL seemed to be the positive modulator. The state 4 respiration rates with succinate correlated positively with DPG and APL/BPL ratio. On the negative modulator list of state 3 and state 4 respiration rate of glutamate were CHL, PI and APL/BPL; SPM/PE was an additional negative modulator for state 4 respiration. The state 3 and state 4 respiration rates with succinate were negatively correlated with PC, PI, PC/PE and PC. For ascorbate + TMPD system, both respiration rates correlated negatively with TPL/CHL ratio. Interestingly, state 3 and state 4 respiration rates were independent of any lipid modulation.

Regression analysis for enzyme activities revealed that the positive and negative modulators for GDH in brain mitochondria were PE and PS. For MDH, SDR and ATPase, PC and PE were common positive modulators, whereas TPL, CHL, SPM, PS, PI, DPG, TPL/CHL, SPM/PC, SPM/PE and APL/BPL were common negative modulators. For liver mitochondria, PE emerged as the common positive modulator for MDH, SDR and ATPase. Additionally, DPG correlated positively with the latter two enzymes. SPM/PC and PI were common negative modulators for SDR and ATPase. PS and SPM/PE correlated negatively with SDR activity.

The foregoing results thus suggest that the lipid/phospholipid components of the membranes differentially modulate the respiratory function–related parameters in a tissue-specific manner. The result also points out that the regulation of brain enzymes is a more complex process than that in the liver mitochondria.

Discussion

Reports in the literature indicate that in the brain from old rats the pathways of phospholipid synthesis as well as the composition of phospholipid-specific fatty acids are altered significantly (Ilincheta de Boschero et al., 2000). The changes include decrease in docosahexaenoic acid and arachidonic acid and increase in the content of 18:1 monounsaturated fatty acid (MUFA) (Lopez et al., 1995). In the rat hippocampus, loss of docosahexaenoic acid in PE, plasmenylethanolamine (PmE) and PS with increasing age has been reported (Favreliere et al., 2003). Also, ageing significantly reduced PE levels in the frontal cortex of rat brain (Favreliere et al., 2000). The aforementioned changes can thus alter membrane fluidity. The alternative pathways for phospholipid synthesis referred to above have been suggested to represent the compensatory mechanism to provide a pool of phospholipid classes for the maintenance of cellular membrane lipid composition during ageing (Ilincheta de Boschero et al., 2000). However, no significant changes occurred in the composition or content of phospholipid classes in the whole brain (Ilincheta de Boschero et al., 2000). Interestingly, Soderberg et al. (1990) reported that in human brain the TPL content decreased marginally (5–10%) with age and the phospholipid composition changed differently in the various brain regions, whereas CHL content showed a 0–40% decrease.

The changes in liver lipid synthesis in aged rats have been suggested to relate to modifications in lipid homeostasis induced by altered hormonal balance (Ilincheta de Boschero et al., 2000; Favreliere et al., 2000; Toescu et al., 2000). Toescu et al. (2000) suggested that gradual, age-dependent impairment of mitochondrial function is an important factor in the decrease of this "homeostatic reserve." The differential changes which we observe here (Tables 2–4) may also relate to differential responses of the two tissues to hormonal changes in old rats.

The results of our present studies have shown that the TPL and CHL contents of the brain and liver mitochondria increased in old rats in a tissue-specific manner. Consequently, the TPL/CHL (mole:mole) ratio increased in the brain mitochondria but was not affected in the liver mitochondria. Our results differ from the observation of Grinna (1977a), who reported that the TPL/CHL (mole:mole) ratio decreased in liver and kidney mitochondria. The observed difference may be attributed to the differences in the strain of rats; Grinna used Sprague-Dawley rats for these studies. Strain-dependent variations in the age-related changes in lipid profiles of plasma and liver lipids of Yoshida and Wistar rats have been reported (Masella et al., 1995).

The phospholipid profiles of the mitochondria from the two tissues also changed in a tissue-specific manner (Tables 3 and 4). Although there were similarities and differences in the observed changes, it became evident that ageing influenced maximally the lipid/phospholipid profiles of the brain mitochondria (Tables 2–4). The common feature of ageing was a significant increase in the Lyso component in the mitochondria from both tissues, which may be attributed to increased phospholipase activity associated with ageing. Altered phosphatidate phosphohydrolase and phospholipase D activities in the aged brain have been reported (Pasquare, Ilincheta de Boschero & Giusto, 2001). Although SPM, PI and PS contents increased in mitochondria from both tissues, the magnitude of increase was always higher for brain mitochondria. Significantly increased levels of SPM in the whole brain as well as in specific brain regions of old rats have been reported (Delion et al., 1997; Giusto et al., 1992; Aureli et al., 2000). Also, the contents of PI and PS increased significantly in the hippocampus of old rats (Delion et al., 1997). The increased synthesis of PS in the cerebral cortex and cerebellum of aged rats has been attributed to increased serine base-exchange activity (Giusto et al., 2002). The content of DPG in brain mitochondria doubled in aged rats, whereas in liver mitochondria the proportion of DPG decreased. A similar 20% decrease in the content of DPG in liver and kidney mitochondria from old rats has been reported, although the changes were not statistically significant (Grinna, 1977a). The contents of PC and PE were unchanged in brain mitochondria, while in liver mitochondria PC content increased.

The increases in TPL and CHL contents would result in an increased ratio of lipid/protein and add to the lipid bulk in the membrane. Additionally, alterations in the

Table 8 Correlation between respiratory activity of rat brain and liver mitochondria with membrane lipid/phospholipid composition

Substrate	Respiration	Brain mitochondria		Liver mitochondria	
		Positive	Negative	Positive	Negative
Glutamate	State 3	PC ($r = +0.707$) PE ($r = +0.696$)	TPL, CHL, PS, PI, DPG, APL/BPL ($r \leq -0.650$) (range −0.797 to −0.650)	PE ($r = +0.596$)	CHL, PI, APL/BPL ($r \leq -0.553$) (range −0.766 to −0.553)
	State 4	–	–	PE ($r = +0.580$)	CHL, Lyso, PI, SPM/PE, APL/BPL ($r \leq -0.536$) (range − 0.734 to − 0.536)
Pyruvate+malate	State 3	PC ($r = +0.759$) PE ($r = +0.696$)	TPL, CHL, PS, PI, DPG, SPM/PC, APL/BPL, ($r \leq -0.621$) (range −0.688 to −0.621)	–	–
	State 4	TPL, CHL, PS, PI, DPG, SPM/PC, APL/BPL ($r \geq +0.591$) (range +0.591 to +0.701)	PE ($r = -0.736$)	–	–
Succinate	State 3	PC ($r = +0.711$) PE ($r = +0.761$)	TPL, CHL, PS, PI, DPG, SPM/PC, SPM/PE, APL/BPL ($r \leq -0.529$) (range −0.816 to −0.529)	PE ($r = +0.596$) DPG (+0.677)	PS, PI, PC/PE ($r \leq -0.598$) (range − 0.677 to −0.598)
	State 4	–	–	DPG (+0.592) APL/ BPL(+0.601)	PC (− 0.614)
Ascorbate+TMPD	State 3	PC ($r = +0.784$) PE ($r = +0.778$)	TPL, CHL, SPM, PS, PI, DPG, SPM/PC, SPM/PE, APL/BPL ($r \leq -0.571$) (range −0.881 to −0.571)	CHL ($r = +0.648$)	TPL/CHL ($r = -0.682$)
	State 4	PS ($r = +0.636$) DPG (+ 0.663)	TPL, CHL, PI, PE, PC/PE, SPM/PE, APL/BPL ($r \leq -0.586$) (range −0.773 to −0.586)	–	TPL/CHL ($r = -0.589$)

Values in parentheses represent regression coefficient r, which is based on eight independent experiments in each group

proportion and contents of individual phospholipid classes would lead to altered charge distribution across the membrane. This in turn could affect the permeability properties of the membrane, besides affecting the catalytic activity of the specific enzymes (e.g., Table 9). Requirement of phospholipid components for the proper functioning of the component electron transport chain has been well documented (Daum, 1985). In particular, it has been shown that cytochrome oxidase and FoF$_1$ ATPase have a specific requirement for DPG. Similarly, succinate oxidase has requirement for bulk phospholipids for its activity (Daum, 1985). In earlier studies we reported that the cytochrome aa_3 content of brain mitochondria decreased significantly in old rats, whereas that in the liver was not affected (Patel & Katyare, 2006b; Patel et al., 2007). Similarly, with ageing the FoF$_1$ ATPase activity decreased significantly in liver mitochondria (Patel et al., 2007). Viewed in this context, the increased content of DPG in brain mitochondria which we observed here may represent a compensatory mechanism to aid the enzyme activity, especially cytochrome oxidase. In the liver, where only FoF$_1$ ATPase activity decreased, the attempt is to retain the DPG content near the young adult level.

Another interesting feature of our studies was the increased content of PS. It has been shown that in experimental animals and in human trials nutritional supplementation with PS improved memory and cognitive functions, while PC was ineffective in this respect (McDaniel, Maier & Einstein, 2003). One therefore wonders whether increased PS and PI contents represent a compensatory mechanism to improve cognitive functions. Increased content of PS, however, raises some concern since externalization of PS is a signal for apoptosis (Mourdjeva et al., 2006).

Ruggiero et al. (1992) reported that in synaptic and nonsynaptic mitochondria the cholesterol and phospholipid contents decreased by 27% and 12%, respectively. Among the phospholipids, only the cardiolipin fraction showed a

Table 9 Correlation between enzymatic parameters of brain and liver with membrane lipid/phospholipid composition

Enzymes	Brain mitochondria		Liver mitochondria	
	Positive	Negative	Positive	Negative
GDH	PE (+ 0.564)	PS (−0.565)	–	SPM/PC (−0.535)
MDH	PC (+ 0.709) PE (+ 0.851)	TPL, CHL, SPM, PS, PI, DPG, TPL/CHL, SPM/PC, SPM/PE, APL/BPL, ($r \leq -0.592$) (range −0.888 to −0.592)	PE (+ 0.585)	PI (−0.605)
SDR	PC (+ 0.809) PE (+ 0.875)	TPL, CHL, SPM, PS, PI, DPG, TPL/CHL, SPM/PC, SPM/PE, APL/BPL, ($r \leq -0.554$) (range −0.938 to −0.554)	PE (+ 0.696) DPG (+ 0.599)	TPL, CHL, PS, PI, SPM/PE ($r \leq -0.559$) (range −0.877 to −0.559)
ATPase	PC (+ 0.778) PE (+ 0.785)	TPL, CHL, SPM, PS, PI, DPG, TPL/CHL SPM/PC, SPM/PE, APL/BPL, ($r \leq -0.554$) (range −0.857 to −0.554)	PE (+ 0.524) DPG (+ 0.599)	TPL, CHL, Lyso, PI, ($r \leq -0.679$) (range −0.874 to −0.679)

Values given in parentheses indicate regression coefficient r, which is based on eight independent experiments in each group

significant decrease (26%) in the nonsynaptic mitochondria from the brains of aged rats. Our results are at variance with these observations. In this context it may be pointed out that Ruggiero et al. (1992) achieved the separation of phospholipids by high-performance liquid chromatography (HPLC), which resolves in five peaks. In our studies separation by TLC enabled us to the resolve phospholipids into seven distinct classes. It is possible that resolution of phospholipid classes by HPLC in their studies might not have given a fine resolution, probably due to intermixing of the components (Ruggiero et al., 1992).

Our present studies also emphasize that ageing caused a significant decrease in the respiratory activity of brain mitochondria with all the substrates tested, thereby significantly impairing the energy potential (Table 5). Against this, in the liver mitochondria, respiration only with glutamate and succinate decreased (Table 6). It may hence be suggested that impairment in oxidation of glutamate and succinate may be a primary event in ageing. Nevertheless, the total energy potential of the liver tissue would also decrease significantly due to drastic reduction in the tissue weight. It may hence be suggested that hormonal imbalance in ageing may affect the energy metabolism of tissues in a differential manner (Tables 1, 5, 6).

The interesting observation that emerged from our attempts to correlate the changes in lipid/phospholipid profiles with respiratory functions was that the regulation of these functions is also tissue-specific. It also became apparent that, compared to liver mitochondria, the regulation of respiratory functions in brain mitochondria is a complex process involving several lipid/phospholipid components and molar ratios (Tables 8 and 9). As referred to above, requirement of specific lipid/phospholipid classes for function of several mitochondrial enzymes has been reported (Daum, 1985). These studies were carried out mainly with liver or heart mitochondria as the model system (Daum, 1985). Our present studies, however, suggest that, indeed, no common rule applies and the requirements seem to be tissue-specific. For example, while PE was important for SDR activity in mitochondria from both tissues, brain mitochondria had an additional requirement for PC. By contrast, for liver mitochondria, DPG was an additional requirement (Table 9). Also, the negative modulators for these two enzyme systems for mitochondria from the two tissues varied widely. A similar picture of involvement of different phospholipid classes for GDH and MDH also became evident (Table 9). The results thus suggest that these requirements, more likely than not, are tissue-specific and there may not be a general rule of thumb.

Morphological and biochemical alterations are associated with a progressive age-related cognitive deficit (Pasquare et al., 2001). Also, the turnover rates of the individual phospholipids are affected differentially with ageing in the synaptic membrane and liver mitochondria (Grinna, 1977b; Ando et al., 2002). When such changes, including the compositional changes, occur in the brain, the susceptibility to neurodegenerative diseases amplifies considerably. Although some of the neurodegenerative diseases are also associated with some degree of mitochondrial dysfunction, it is not yet clear if these changes are due to the underlying process of normal physiological ageing or to the specific pathophysiological agents responsible for the neurodegenerative processes. Furthermore, it was proposed that important differences exist between normal ageing and neurodegeneration (Toescu et al., 2000). Abnormalities in lipid/phospholipid profiles and activation of phospholipase A_2 are known to affect cerebral and mitochondrial functions in pathological conditions such as Parkinson disease, neural trauma, neurodegenerative disease and Alzheimer disease (Farooqui, Ong & Horrocks, 2004). Decrease in the levels of PI 18% and a 22% increase in SPM have been reported in Down syndrome patients (Murphy et al., 2000). It has been suggested that free radical also contribute to brain ageing by promoting lipid peroxidation, thus introducing modifications in membrane fluidity (Viani et al., 1991; Poon et al., 2004).

In conclusion, our present results show that as a consequence of ageing very major changes occur in the lipid/phospholipid components and respiratory functions of brain mitochondria. Compared to these changes the alterations in liver mitochondrial membranes are of lesser magnitude. The results thus suggest that brain mitochondria may be the primary target in the normal ageing process.

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Stimulation of oxidative energy metabolism in liver mitochondria from old and young rats by treatment with dehydroepiandrosterone (DHEA). A comparative study

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Abstract Effects of treatment with DHEA (0.2 or 1.0 mg/kg body weight for 7 days) on oxidative energy metabolism of rat liver mitochondria from old (18–24 month old) and young (8–10 weeks old) male albino rats belonging to Charles-Foster strain were examined. Treatment with 1.0 mg DHEA resulted in increased body weights of the young rats without change in the liver weight. In the old animals the liver weight increased progressively with increasing dose of DHEA without affecting body weight. The state 3 respiration rates in liver mitochondria from old animals were, in general, lower than those in the young rats. The state 3 and state 4 respiration rates increased following DHEA treatment in dose-dependent manner bringing them close to values for young animals or beyond that with the effect being more pronounced at 1.0 mg dose. Treatment with DHEA also stimulated state 3 and state 4 respiration rates in young rats in dose-dependent manner. Contents of cytochrome aa_3, b and $c+c_1$ increased significantly in old animals in dose-dependent manner. In the young rats the lower dose (0.2 mg) of DHEA was more effective in bringing about a maximum increase in the contents of the cytochromes; the effect declined at the higher dose (1.0 mg). DHEA treatment also stimulated the mitochondrial ATPase activity in the old as well as in the young rats. The dehydrogenases activities were considerably low in the old rats compared to the values for the young animals. Treatment with DHEA stimulated dehydrogenases activities in old rats in dose-dependent manner bringing them close to values for the young animals or beyond. Treatment with lower dose (0.2 mg) of DHEA maximally stimulated dehydrogenases activities in young animals.

Key words ATPase activity · cytochromes · dehydroepiandrosterone (DHEA) · dehydrogenases · liver mitochondria · oxidative energy metabolism

Introduction

Dehydroepiadrosterone (DHEA) and its sulfated ester DHEA-S show an age related pattern of synthesis and secretion in humans. The levels are low at young age, peak at adulthood and decline thereafter (Hinson and Raven 1999; Parker 1999). Based on this characteristic pattern DHEA is considered to be a youth hormone (Hinson and Raven 1999; Celec and Starka 2003). It is well recognized that the respiratory activity of mitochondria from different tissues declines with aging (Marcus et al. 1982; Sastre et al. 1996; Nakahara et al. 1998; Navarro and Boveris 2004). Earlier studies from our laboratory have shown that DHEA treatment stimulated respiratory functions in liver and brain mitochondria from young rats (Patel and Katyare 2007).

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Additionally we also found that DHEA treatment had a grater stimulatory effect on the respiratory functions in brain mitochondria from old rats than in the young animals (Patel and Katyare 2006a). The foregoing studies prompted us to examine as to whether treatment with DHEA would also influence the energy metabolism of liver mitochondria in old rats in a similar manner. These studies assume importance in view of the fact that the levels of DHEA decrease significantly in the older population and beneficial effects of exogenous supplementation with DHEA in elderly population have been claimed (Hinson and Raven 1999; Milgrom 1990; Buvat 2003). It is possible that observed beneficial effects may result due to enhancement in the energy potential of the liver which is the major site of metabolism. Thus, if DHEA is indeed a youth hormone (Hinson and Raven 1999; Celec and Starka 2003) it may be anticipated that the effects should also be manifested on the energy metabolism of liver mitochondria from the old rats.

We therefore examined the effects of DHEA treatment on energy metabolism of liver mitochondria from the old rats in comparison with the young animals. The results are summarized in the present communication.

Materials and methods

Chemical

Dehydroepiandrosterone (DHEA), (3*β*-Hydroxy-5-androsten-17-one (+)–dehydroisoandrosterone) was obtained from Sigma-Aldrich Corporation, St. Louis, MO, USA. Sodium salts of succinic acid, pyruvic acid, L-malic acid and ADP, rotenone, bovine serum albumin fraction V (BSA), 4-morpholinopropanesulfonic acid (MOPS), dicholrophenolindophenol (DCIP), NAD^+, NADH, and oxaloacetic acid were purchased from Sigma Chemical Co. St. Louis, MO, USA. Sodium salt of L-glutamic acid was purchased from E Merck, Darmstadt, Germany. N,N,N′,N′-Tetramethyl-*p*-phenylenediamine (TMPD) was obtained from British Drug Houses, Dorset, Poole, England. All other chemicals were of analytical-reagent grade and were purchased locally.

Animals and treatment with DHEA

Male young (8–10 week old) and old (18–24 month old) albino rats of Charles-Foster strain were used. At the start of the experiments the body weights of young rats was in the range of 220–230 g while that of the old rats was in the range of 350–370 g (Table 1). The animals received 0.2 or 1.0 mg DHEA/Kg body weight subcutaneously (s.c.) for 7 consecutive days. This dose regimen was decided on the basis of our earlier observation (Patel and Katyare 2007). Suspension of DHEA was prepared fresh in saline prior to injection. The controls received equivalent volume of saline vehicle. The animals were killed on the 8th day of treatment. Daily record of body weight was maintained. The experimental protocol was approved by the Departmental Animal Ethics Committee.

Isolation of mitochondria

Isolation of liver mitochondria was essentially according to the procedures described previously with some

Table 1 Effect of DHEA treatment on body weight and liver weight

Age group	Treatment	Body weight, g	Liver weight	
			g	% of body wt.
Young	Untreated (12)	243.1±6.33	8.66±0.56	3.50±0.08
	0.2 mg DHEA (12)	251.6±7.01	8.70±0.31	3.51±0.04
	1.0 mg DHEA (12)	268.3±5.98[a]	9.01±0.63	3.62±0.06
Old	Untreated (12)	378.2±7.26	6.73±0.21	1.72±0.03
	0.2 mg DHEA (15)	372.1±6.95	9.92±0.41[b]	2.66±0.09[b]
	1.0 mg DHEA (15)	381.7±10.0	12.35±0.59[b]	3.24±0.13[b]

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.

[a] $p<0.01$ and [b] $p<0.001$ compared with the corresponding control.

modifications (Katewa and Katyare 2004; Patel and Katyare 2007) The mitochondria were sedimented by centrifugation of the nuclei-free supernatant at 7000×g for 10 min and were washed once by suspending in the isolation medium (250 mM sucrose containing 5 mM MOPS and 1 mM EDTA all at pH 7.4 and 0.25 mg BSA/ml of isolation medium) and resedimenting. Finally the pellet was suspended in the isolation medium to give a protein concentration in the range of 25–30 mg/ml. Isolation of cytosolic fraction was as described previously (Kaushal et al. 1999).

Oxidative phosphorylation

Measurements of oxidative phosphorylation were carried out at 25°C using a Clark-type oxygen electrode as described previously (Katewa and Katyare 2004; Patel and Katyare 2007). State 3 respiration rates initiated by the addition of 80–200 n moles of ADP and state 4 rates ensuing after its depletion were recorded. Calculations of ADP/O ratio and ADP phosphorylation rates were as described previously (Ferreira and Gil 1984; Katyare and Satav 1989).

Cytochrome content

The contents of cytochromes were quantified from the difference spectra of dithionite reduced versus ferricyanide oxidized samples using the wavelength pairs and millimolar extinction coefficients as detailed previously. (Katewa and Katyare 2004; Patel and Katyare 2007; Subramaniam and Katyare 1990).

Assay of dehydrogenases

Glutamate dehydrogenase (GDH), malate dehydrogenase (MDH) and succinate DCIP reductase (SDR) activities were determined by the procedures described earlier (Katewa and Katyare 2004; Patel and Katyare 2007).

Assay of ATPase

The ATPase activity was determined using the assay medium (total volume 0.1 ml) consisting of 50 mM MOPS pH 7.4, 75 mM KCl and 0.4 mM EDTA. Activity determinations were carried out in the absence and presence of $MgCl_2$ (6 mM) and/or 100 μM DNP as detailed earlier (Katewa and Katyare 2004; Patel and Katyare 2007). Estimation of inorganic phosphate was according to the procedure described by Katewa and Katyare (2003).

Protein estimation was by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Results are given as mean ± SEM.

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

Table 2 Effect of DHEA treatment on oxidative phosphorylation in rat liver mitochondria using glutamate as the substrate

Age group	Treatment	ADP/O ratio	Respiration rate (n mole O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (n mole/min/mg protein)
			+ADP	−ADP		
Young	Untreated (12)	3.24±0.07	27.88±0.95	10.80±0.34	2.72±0.07	180.8±7.82
	0.2 mg DHEA (12)	3.06±0.11	38.34±1.53^{b}	14.49±0.68^{b}	2.67±0.05	231.8±9.07^{b}
	1.0 mg DHEA (12)	3.10±0.09	32.90±0.73^{b}	18.38±0.87^{b}	1.86±0.09	204.0±6.92^{b}
Old	Untreated (19)	3.23±0.19	23.27±1.03**	7.78±0.46**	3.07±0.11	149.4±10.08*
	0.2 mg DHEA (15)	3.11±0.13	25.73±1.46	11.59±0.46^{b}	2.23±0.11	161.3±8.51
	1.0 mg DHEA (9)	3.15±0.12	30.71±2.17^{a}	12.09±0.82^{b}	2.59±0.21	193.1±11.72^{a}

The respiration medium (total volume 1.6 ml) consisted of 225 mM sucrose, 20 mM KCl, 10 mM MOPS pH 7.4, 5 mM potassium phosphate buffer pH 7.4, 0.2 mM EDTA and 160 mg of BSA (i.e. 0.1 mg BSA/ml). Concentration of glutamate was 10 mM. State 3 respiration rates initiated by the addition of 80–200 n moles of ADP and state 4 rates ensuing after its depletion were recorded. Other experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.

[a] $p<0.01$ and [b] $p<0.001$ compared with the corresponding untreated group.

* $p<0.01$ and ** $p<0.001$ compared with the untreated young group

Table 3 Effect of DHEA treatment on oxidative phosphorylation in rat liver mitochondria using pyruvate + malate as the substrate

Age group	Treatment	ADP/O ratio	Respiration rate (n mole O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (n mole/min/mg protein)
			+ADP	−ADP		
Young	Untreated (12)	3.19±0.07	16.49±0.80	7.59±0.26	2.18±0.10	105.2±5.89
	0.2 mg DHEA (12)	3.00±0.08	17.36±0.82	9.43±0.32^a	1.85±0.04	104.1±6.09
	1.0 mg DHEA (12)	3.09±0.07	33.95±2.18^a	17.33±1.53^a	2.04±0.13	211.0±15.00^a
Old	Untreated (17)	3.14±0.15	15.25±0.97	7.76±0.45	2.03±0.12	94.2±5.69
	0.2 mg DHEA (12)	3.24±0.21	14.61±0.82	8.33±0.58	1.78±0.08	95.1±7.10
	1.0 mg DHEA (10)	3.23±0.25	22.81±1.47^a	13.75±0.57^a	1.66±0.08	147.1±9.31^a

Experimental details are as given in the text and in Table 2. Concentrations of pyruvate and malate were 10 and 1 mM respectively. Results are given as mean ± SEM of the number of observations indicated in the parentheses.

[a] $p<0.001$ compared with the corresponding untreated group.

Results

The data in Table 1 show that in the young rats treatment only with higher dose (1.0 mg) of DHEA resulted in 18% increase in the body weight without any change in the liver weight (Table 1). By contrast, in the old rats treatment with DHEA had no effect on the body weights but the liver weight increased progressively with increasing dose of DHEA (47% and 84% increase respectively by the two dose regimens).

The results on effect of DHEA treatment on oxidative energy metabolism are summarized in Tables 2, 3, 4 and 5. As can be noted, the state 3 respiration rates with glutamate, pyruvate+malate, succinate and ascorbate+TMPD were generally low (8–32% lower) in the old rats (Tables 2, 3, 4 and 5) which is consistent with the earlier reports by other researchers (Marcus et al. 1982; Sastre et al. 1996; Nakahara et al. 1998; Navarro and Boveris 2004). Treatment with 0.2 mg DHEA stimulated state 3 respiration rates in mitochondria from young rats with glutamate by 38%. However, the effect declined at higher dose (Table 2). In the old animals maximum stimulatory effect (32% increase) was obtained with 1.0 mg dose and the value became comparable to untreated young rats (Table 2). When pyruvate+malate was used as the substrate pair, in young rats state 3 and state 4 respiration rates almost doubled following treatment with 1.0 mg DHEA. Even in the old animals treatment with 1.0 mg DHEA resulted in 50% and 77% increase in state 3 and state 4 respiration rates (Table 3). With succinate as the substrate, treatment with 0.2 mg DHEA was able to

Table 4 Effect of DHEA treatment on oxidative phosphorylation in rat liver mitochondria using succinate as the substrate

Age group	Treatment	ADP/O ratio	Respiration rate (n mole O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (n mole/min/mg protein)
			+ADP	−ADP		
Young	Untreated (12)	2.34±0.10	56.88±2.92	22.55±1.29	2.56±0.09	264.1±15.34
	0.2 mg DHEA (12)	2.55±0.09	69.81±2.82^b	33.72±2.72^c	2.22±0.19	358.3±21.74^c
	1.0 mg DHEA (12)	2.33±0.12	53.91±2.96	33.55±2.62^c	1.67±0.10	252.7±21.35
Old	Untreated (18)	2.24±0.13	38.82±3.28*	23.04±0.68	1.69±0.09	174.2±11.39*
	0.2 mg DHEA (12)	2.17±0.12	45.37±3.21	24.67±1.85	1.87±0.06	189.5±12.25
	1.0 mg DHEA (10)	2.27±0.11	49.95±2.15^b	26.15±1.04^a	1.92±0.07	226.6±15.01^a

Experimental details are as given in the text and in Table 2. Concentration of succinate was 10 mM. Rotenone (1 μM) was included in the respiration medium. Results are given as mean ± SEM of the number of observations indicated in the parentheses.

[a] $p<0.02$; [b] $p<0.01$ and [c] $p<0.002$ compared with the corresponding untreated group.

* $p<0.001$ compared with the untreated young group.

Table 5 Effect of DHEA treatment on oxidative phosphorylation in rat liver mitochondria using ascorbate + TMPD as the substrate

Age group	Treatment	ADP/O ratio	Respiration rate (n mole O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (n mole/min/mg protein)
			+ADP	−ADP		
Young	Untreated (12)	0.44±0.03	28.12±1.90	21.46±1.65	1.33±0.03	24.52±1.57
	0.2 mg DHEA (12)	0.41±0.03	36.02 ± 1.84^{b}	26.36±2.19	1.43±0.10	29.99 ± 1.89^{a}
	1.0 mg DHEA (12)	0.42±0.02	45.24 ± 2.08^{d}	31.76 ± 1.52^{d}	1.44±0.07	38.61 ± 2.06^{d}
Old	Untreated (21)	0.40±0.02	24.71±1.12	19.27±0.80	1.28±0.02	19.92±1.18*
	0.2 mg DHEA (15)	0.42±0.03	27.92 ± 0.86^{a}	21.67 ± 0.72^{a}	1.30±0.05	23.64±1.59
	1.0 mg DHEA (12)	0.43±0.02	32.01 ± 1.38^{d}	24.40 ± 1.18^{c}	1.32±0.02	26.50 ± 1.63^{b}

Experimental details are as given in the text and in Table 2. Concentrations of ascorbate and TMPD were 10 and 0.1 mM respectively. Rotenone (1 μM) was included in the respiration medium. Results are given as mean ± SEM of the number of observations indicated in the parentheses.

[a] $p<0.05$; [b] $p<0.01$; [c] $p<0.002$ and [d] $p<0.001$ compared with the corresponding untreated group.

* $p<0.05$ compared with the untreated young group.

bring about 23% and 50% increase respectively in state 3 and state 4 respiration rates in the young rats; effect on state 4 respiration rate persisted at the higher dose (1.0 mg) of DHEA. Under these conditions, in the old animals there was a dose-dependent 17% and 29% increase in state 3 respiration rate. Corresponding increases in the state 4 respiration rates, respectively, were 7% and 14% (Table 4).. With ascorbate+TMPD used as electron donor system, treatment with increasing doses of DHEA brought about progressive increase in the state 3 and state 4 respiration rates (23 to 61% increase) in the young animals. In the old rats also a similar trend with 13–30% increase was evident (Table 5).

The contents of cytochrome aa_3, b and $c+c_1$ were comparable for the young and the old rats. Treatment with 0.2 mg DHEA resulted in 15–31% increase in the contents of the three cytochrome classes in young rats; the effect declined at higher dose (1.0 mg) of DHEA. On the other hand, in the old rats treatment with 1.0 mg DHEA brought about significant increase in the contents of all the cytochromes which ranged from 26–47%. The most important point was that at the highest dose employed (1.0 mg) the observed increase in the contents of the cytochromes in the old rats was of greater magnitude than that seen in the young rats (Table 6). The basal, Mg^{2+}-stimulated, DNP-stimulated and Mg^{2+} + DNP-stimulated, ATPase activities were significantly low in the old rats. DHEA treatment was able to stimulate the ATPase activities by 6–51%. As against this, in the young animals treatment with 0.2 mg DHEA resulted in substantial

Table 6 Effect of DHEA treatment on the cytochrome content of rat liver mitochondria

Age group	Treatment	Cytochrome content (pmoles/mg protein)		
		aa_3	b	$c+c_1$
Young	Untreated (6)	135.1±3.11	277.8±10.93	326.2±15.94
	0.2 mg DHEA (6)	167.7 ± 3.97^{b}	364.8 ± 12.90^{b}	375.3 ± 7.10^{b}
	1.0 mg DHEA (6)	151.7±7.44	310.5±21.00	313.0±13.10
Old	Untreated (17)	136.6±6.09	285.9±9.81	331.4±12.71
	0.2 mg DHEA (19)	137.5±10.37	309.3±9.93	364.5±15.90
	1.0 mg DHEA (12)	169.3 ± 9.57^{a}	421.1 ± 18.89^{b}	436.4 ± 19.90^{b}

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.

[a] $p<0.01$; and [b] $p<0.001$ compared with the corresponding untreated group.

Table 7 Effect of DHEA treatment on ATPase activity in rat liver mitochondria

Age group	Treatment	Activity (µmole Pi liberated/hr/mg protein)			
		Basal	+Mg^{2+}	+DNP	Mg^{2+} + DNP
Young	Untreated (12)	2.01 ± 0.13	6.33 ± 0.14	19.11 ± 0.76	22.08 ± 1.03
	0.2 mg DHEA (12)	5.11 ± 0.10^{d}	8.80 ± 0.46^{d}	32.51 ± 1.09	33.24 ± 1.19^{d}
	1.0 mg DHEA (12)	4.96 ± 0.07^{d}	8.12 ± 0.63^{a}	25.36 ± 0.98	22.98 ± 0.86
Old	Untreated (12)	$1.49\pm0.76^{*}$	$2.16\pm0.18^{**}$	$11.37\pm0.83^{**}$	$12.09\pm0.46^{**}$
	0.2 mg DHEA (12)	2.20 ± 0.18^{c}	2.29 ± 0.12	16.76 ± 0.84^{d}	15.89 ± 0.95^{c}
	1.0 mg DHEA (12)	2.21 ± 0.16^{d}	3.26 ± 0.19^{d}	14.94 ± 0.62^{b}	17.34 ± 1.06^{d}

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.

[a] $p<0.02$; [b] $p<0.01$; [c] $p<0.002$ and [d] $p<0.001$ compared with the corresponding untreated group.

* $p<0.01$ and ** $p<0.001$ compared with the untreated young group

39% to 2.5 fold increases in the ATPase activities. However, the effect declined at the higher dose of 1.0 mg (Table 7). The dehydrogenases activities were generally low in the old rats and DHEA treatments were able to restore the GDH and mitochondrial MDH activities near the level of untreated young animals. While the SDR activity was stimulated by 78% in young animals, interestingly, in the old rats treatment with 1 mg DHEA brought about a substantial 7.8 fold increase in the SDR activity. The cytosolic MDH activity increased marginally in young rats after treatment with 0.2 mg DHEA; higher dose of 1.0 mg had an adverse effect. By contrast, both the doses of DHEA resulted in 26–28% increase in cytosolic MDH activity in old rats (Fig. 1).

Discussion

The present studies were undertaken to examine if exogenous supplementation with DHEA has beneficial effect on oxidative energy metabolism of liver mitochondria from old rats. Studies were carried out with normal animals rather than using a rat model of aging such as Fischer 344 since we were interested in normal aging process. Complications and limitations associated with Fischer 344 strain have been documented Shimokawa et al. 1993). As is evident, the data of our present studies on the various respiratory parameters in the young animals (Tables 2, 3, 4, 5, 6 and 7, Fig. 1) are consistent with our previously published observations (Patel and Katyare 2006b, 2007). The results of our present studies also show that the respiratory functions of liver mitochondria, in general, declined in the old rats, although the respiration rates with pyruvate+malate and ascorbate+TMPD were not affected. Variable and equivocal effects on respiratory activity of mitochondria, depending on the strain of the animals have been documented (Hansford 1983).

From the data presented it is clear that the respiratory activities, contents of cytochromes, dehydrogenases activities and the ATPase activities were stimulated in the liver mitochondria from both young as well as old rats in a dose-dependent manner after treatment with DHEA. In general, the higher dose of DHEA seemed to have a greater stimulatory effect in the old rats (Tables 2, 3, 4, 5, 6 and 7). This may perhaps relate to the declining levels of DHEA in the old animals (Kazihnitkova et al. 2004; Ren and Hou 2005; Vallee et al. 2000; Weill-Engerer et al. 2003). From the data presented one may also tend to think that with respect to respiratory activities the effect was more pronounced in the young animals than in the old animals. A similar conclusion may be drawn even for the ATPase activity.

The differential increase in the contents of cytochrome aa$_3$, b and c+c$_1$ and ATPase and dehydrogenases activities in old versus young animals is of interest and deserves some comment. It is well recognized that while the dehydrogenases and cytochrome c+c$_1$ are coded by the nuclear genes, crucial polypeptides of cytochrome aa$_3$, cytochrome b and mitochondrial ATPase are mitochondrial gene products (Poyton and Mc Ewen 1996). It may hence be suggested that DHEA may have differential effects on activation of the nuclear and mitochondrial genes in the young and old rats. It has been reported that in the old animals initially there is up-regulation of the genes encoding

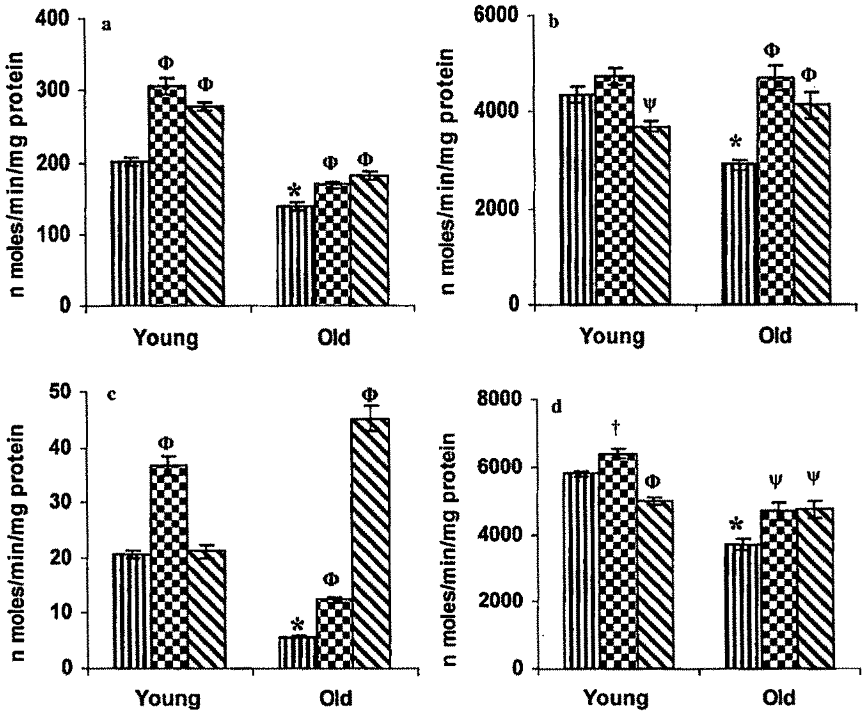

Fig. 1 Effect of DHEA treatment on mitochondrial and cytosolic dehydrogenases activities in rat liver. The results are given as mean ± SEM of 12 independent observations. **a** Glutamate dehydrogenase; **b** Malate dehydrogenase (Mitochondrial); **c** Succinate DCIP reductase and **d** Malate dehydrogenase (cytosolic); ▥, Untreated; ▦, 0.2 mg DHEA and ▧, 1.0 mg DHEA †, $p<0.01$; ψ, $p<0.002$ and Φ, $p<0.001$ compared with the corresponding untreated group. *, $p<0.001$ compared with the untreated young group

peptides in complex I, III, IV and V of the respiratory chain which is followed by down-regulation at later stage (Manczak et al. 2005).

The most significant observation of the present studies is the progressive increase in the liver weight in the old rats following treatment with DHEA (Table 1). Because of the significant increase in the liver weight the total potential of the tissue for respiratory activity and rates of ATP synthesis i.e. ADP-phosphorylation rates (Tables 2, 3, 4 and 5) would be significantly high in the old animals. Although these data are not given, an approximate estimation of respiratory potential and potential for ATP synthesis can be calculated by multiplying corresponding values with the respective liver weights. A similar picture would emerge even for the cytochromes contents and dehydrogenases activities. Thus the results of the present studies indicate that DHEA treatment specifically stimulates the proliferative potential of the liver cells in the old rats.

As cited above, in the humans the plasma levels of DHEA reach a peak in young adults and decline substantially in the older population (Hinson and Raven 1999; Parker 1999). Viewed in this context, data of our present studies would suggest that the plasma level of DHEA in the young rats receiving 0.2 mg dose of DHEA may represent the safe highest threshold value beyond which at higher dose of 1.0 mg the adverse effects become evident (e.g. see Tables 2 and 4). By contrast, in the old rats the maximum stimulatory effect was seen at the higher (1.0 mg) dose of DHEA (Tables 2, 3, 4, 5, 6 and 7). This is consistent with the reported low levels of DHEA (10% of adult value) in the old population (Hinson and Raven 1999; Parker 1999) and in rats (Kazihnitkova et al. 2004; Ren and Hou 2005; Vallee et al. 2000; Weill-Engerer et al. 2003). Of interest to note in this context is our earlier observation that high dose of 2.0 mg had adverse affects on respiratory activities of the liver as well as the brain mitochondria (Patel and Katyare 2007). This may relate to toxicity of DHEA given in higher doses. The age-dependent changes in the plasma and tissue levels of DHEA in the humans are well documented

(Hinson and Raven 1999; Parker 1999). However, no such data are available for plasma levels in the rats. What has been reported is that the plasma and tissue levels of DHEA in rats are comparatively very low and that the levels of DHEA in the brains of old rats decrease significantly (Kazihnitkova et al. 2004; Ren and Hou 2005; Vallee et al. 2000; Weill-Engerer et al. 2003). The low DHEA levels in the rat may possibly relate to high metabolic rate which could result in rapid turnover of the steroid. For example, it is well recognized that the life-span of erythocytes in humans is 120 days; in rats the life-span is 60 days (Alberts et al. 1994).

The steroids DHEA and DHEAS are synthesized in the highest concentrations by the adrenals. Additionally, these steroids are also synthesized in the brain (Racchi et al. 2003). However, there are no known receptors for either of the steroids (Natawa et al. 2002). DHEAS is metabolized to 7α hydroxy DHEA and δ 5 androstene 3 β, 17 β diol (Steckelbroeck et al. 2002; Weill-Engerer et al. 2003) and 7α hydroxy DHEA is considered as active metabolite (Steckelbroeck et al. 2002; Weill-Engerer et al. 2003). Thus based on our studies it may also be suggested that the metabolism of DHEAS may be differentially affected in aging.

In conclusion, the overall results of our present studies point out that DHEA treatment significantly stimulated the respiratory activity in the liver mitochondria from young rats as well as old rats. Although the effects were of lesser magnitude in old rats, the total potential of oxidative energy metabolism increased to a grater extent because of significant dose-dependent increase in the liver weight. The results thus suggest that treatment with DHEA can have beneficial effect on energy transduction potential in liver mitochondria even in the old rats.

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Effect of dehydroepiandrosterone (DHEA) treatment on oxidative energy metabolism in rat liver and brain mitochondria
A dose–response study

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Abstract

Objectives: Effects of treatment with dehydroepiandrosterone (DHEA) on oxidative energy metabolism in rat liver and brain mitochondria were examined.

Design and methods: Young adult rats were administered DHEA (0.1, 0.2, 1.0 or 2.0 mg/kg body weight) by subcutaneous route for 7 consecutive days.

Results: DHEA treatment resulted in general, in stimulation of state 3 respiration rates without having any uncoupling effect on ADP/O ratios. The stimulation of state 3 respiration rate for a given substrate was dose dependent in a tissue-specific manner. Parallel increases in the contents of cytochromes aa_3 and b were also noted. DHEA treatment stimulated the glutamate dehydrogenase (GDH) and succinate DCIP reductase (SDR) activities. Under the treatment conditions, mitochondrial ATPase activity was also stimulated.

Conclusions: Treatment with DHEA significantly stimulated oxidative energy metabolism in liver and brain mitochondria.

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Keywords: Dehydroepiandrosterone (DHEA); Oxidative energy metabolism; ATPase activity; Cytochromes; Dehydrogenases

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Introduction

Dehydroepiandrosterone (DHEA) and its sulfated conjugate DHEA-S are the two steroids synthesized in highest concentrations (DHEA, 6–8 mg/day; DHEA-S, 15–18 mg/day) by the adrenal cortex in the human adults [1,2]. The pattern of secretion is characteristically age related. Thus, the concentration of DHEA in the serum is low in early age, starts increasing two years before the onset of puberty, peaks at adult stage and declines after the age of 35–40 years [3,4]. Based on this characteristic age-related pattern DHEA is considered to be the youth hormone [3,5]. In the past few years a number of studies have been carried out to find usefulness of exogenous DHEA supplementation in health, disease, human well being and improvement of cognitive functions in the elderly population [5]. Although DHEA is freely available in the U.S. without prescription [3], claims for its beneficial effects in health, disease and improvement of memory and behaviour in elderly persons have not been clearly established and seem to be equivocal [3,6].

It has been reported that treatment with DHEA resulted in increased concentration of NAD^+ and $NADP^+$ in liver [7] and elevated the rate of malic enzyme gene transcription [8]. Hypertrophy of hepatocytes following treatment with DHEA has been attributed to proliferation of peroxisomes and mitochondria [9]. Antioxidant effects of DHEA have also been demonstrated [10].

DHEA and DHEA-S are present in the brains of male adult rats and DHEA-S has been shown to play a major role in brain development and aging by influencing the migration of neurons, arborization of dendrites and formation of new synapses [11]. In neural precursor culture nanomolar concentration of DHEA decreased apoptosis and activated serine–threonine protein kinase (Akt) [12]. DHEA treatment also stimulated the process of neurogenesis in a fraction of older rats [13].

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The foregoing results thus indicate that treatment with DHEA significantly influences metabolic activities and energy-dependent functions in the liver and the brain. However, in the studies cited above, various researchers have used different dose regimens and the routes of administration also differed [7,9, 10,13,14].

Therefore, it is important to carry out systematic studies to evaluate simultaneously the possible effects of DHEA treatment on oxidative energy metabolism potential of the liver and the brain mitochondria. Outcome of such studies can have implications for therapeutic application of exogenous DHEA supplementation since the metabolic activities, in general, are energy dependent [5,15,16].

In the present studies we decided to investigate the effects of treatment with various doses of DHEA on oxidative energy metabolism in liver and brain mitochondria from adult male rats. This aspect assumes importance since most of the cellular processes are energy-dependent and also because effects of DHEA on mitochondrial metabolism and proliferation have already been demonstrated [8,9,14].

Materials and methods

Chemicals

3β-Hydroxy-5-androsten-17-one (+)-dehydroisoandrosterone (DHEA) was purchased from Sigma-Aldrich, USA. Sodium salt of L-glutamic acid was obtained from E Merck, Germany. Sodium salts of succinic acid, pyruvic acid, L-malic acid and ADP, rotenone, bovine serum albumin fraction V (BSA), 4-morpholinopropanesulfonic acid (MOPS), dichlorophenolindophenol (DCIP), NAD$^+$, NADH, oxaloacetic acid and disodium salt of ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. USA. *N,N,N′,N′*-Tetra methyl-*p*-phenylenediamine (TMPD) was purchased from British Drug Houses, Dorset, Poole, England. Ascorbic acid was from Sarabhai Chemicals, Vadodara. All other chemicals were of analytical-reagent grade and were purchased locally.

Animals and treatment with DHEA

Adult male albino rats (8–10 weeks old) of Charles–Foster strain weighing between 200 and 250 g were used. The animals received daily injections of 0.1, 0.2, 1.0 or 2.0 mg DHEA/kg body weight subcutaneously (s.c.) for 7 consecutive days. Daily records of body weight were maintained. Suspension of DHEA was prepared fresh in saline prior to injection. The controls received equivalent volume of saline. The animals were killed on the 8th day.

Isolation of mitochondria

Isolation of liver and brain mitochondria was done according to the procedures described previously with some modifications [15,16,17,18]. Thus briefly, at the end of the experimental period, the animals were killed by decapitation and the liver and brain were quickly removed and placed in beakers containing chilled (0–4°C) isolation medium which consisted of 250 mM sucrose containing 5 mM MOPS and 1 mM EDTA all at pH 7.4; 0.25 mg BSA/mL of isolation medium was included. Liver tissue was minced with a pair of scissors. The tissues were washed repeatedly with the isolation medium to remove adhering blood. The tissues were then homogenized using a Potter–Elvehjem-type glass—Teflon homogenizer to obtain 10% and 20% (w/v) homogenates, respectively, for liver and brain. After removal of nuclei and cell debris at 650×*g* for 10 min, the mitochondria were sedimented by centrifugation at 7500×*g* for 10 min, washed once by gently suspending in the isolation medium and resedimenting and were finally suspended in the isolation medium to give a protein concentration in the range of 25–30 mg/mL or 10–15 mg/mL, respectively, for liver and brain mitochondria. All the steps in the isolation procedure were carried out at 0–4°C in a Sorvall RC 5B plus centrifuge. Cytosolic fraction was isolated from post-mitochondrial supernatant as described previously [19].

We have previously demonstrated that mitochondria isolated by these procedures are practically free from microsomal and/or cytosolic contaminations [19,20].

Oxidative phosphorylation

Measurements of oxidative phosphorylation were carried out at 25°C using a Clark-type oxygen electrode as described previously [15,16,17,18]. Briefly, the respiration medium (total volume 1.6 mL) consisted of 225 mM sucrose, 20 mM KCl, 10 mM MOPS pH 7.4, 5 mM potassium phosphate buffer pH 7.4, 0.2 mM EDTA and 160 μg of BSA (i.e., 0.1 mg BSA/mL). After introducing mitochondria (4–8 mg protein depending on the substrate used) in the electrode chamber, respiration was induced by addition of substrates. Final concentrations of the substrates used were glutamate (10 mM), pyruvate+malate (10 mM+1 mM), succinate (10 mM) and ascorbate+TMPD (10 mM+0.1 mM). Measurements with the latter two substrates were performed in the presence of 1 μM rotenone. State 3 respiration rates initiated by the addition of 80–200 nmol of ADP and state 4 respiration rates ensuing after the depletion of added ADP were recorded. Calculations of ADP/O ratio and ADP phosphorylation rates were as described previously [21,22].

Cytochrome content

The contents of cytochromes were calculated from the difference spectra as described previously [16,18]. Briefly, 6–8 mg mitochondrial protein was taken up in potassium phosphate-buffered sucrose and solubilized by adding 0.25 mL of 10% Triton X-100. The total volume was made up to 2.5 mL. The sample was then transferred to two 1-mL cuvettes. The sample in the reference cuvette was oxidized by adding small amount of potassium ferricyanide and the sample in the experimental cuvette was reduced by adding a few mg of sodium dithionite. The different spectra of reduced versus oxidized samples were recorded in a JASCO UV/VIS spectrophotometer model V-530. The contents of aa_3, b and $c+c_1$ cyto chromes were calculated using the wavelength pairs 604–624, 559–580 and 535–552 nm and millimolar extinction coefficients 24, 23.4 and 18.7, respectively [23].

Assay of dehydrogenases

Glutamate dehydrogenase (GDH), malate dehydrogenase (MDH) and succinate DCIP reductase (SDR) activities were determined as described previously [16,18].

Assay of ATPase

The ATPase activity in the liver mitochondria was measured in the assay medium (total volume 0.1 mL) containing 50 mM MOPS pH 7.4, 75 mM KCl and 0.4 mM EDTA. The assays were performed in the absence and presence of $MgCl_2$ (6 mM) and DNP 100 μM, or a combination of both. After pre-incubating the mitochondrial protein (40–60 μg) in the assay medium at 37°C for 1 min, the reaction was initiated by the addition of ATP at the final concentration of 5 mM. The reaction was carried out for 15 min and then terminated by the addition of 0.1 mL of 5% (w/v) SDS [16,18].

The ATPase activity in brain mitochondria was measured in the assay medium (total volume 0.1 mL) containing 350 mM sucrose, 10 mM MOPS pH 7.4, 10 mM KCl and 0.2 mM EDTA. The assays were performed in the absence and presence of $MgCl_2$ (2 mM) and DNP 50 μM, or a combination of both. After pre-incubating the mitochondrial protein (40–60 μg) in the assay medium at 37°C for 1 min, the reaction was initiated by the addition of ATP at the final concentration of 2 mM. The reaction was carried out for 15 min and then terminated by the addition of 1.1 mL of 5% w/v TCA [17]. The tubes were allowed to stand on ice and then centrifuged at 3000 rpm for 10 min and 0.8 mL aliquots of supernatant fluid were taken up for the estimation of liberated inorganic phosphate.

Estimation of inorganic phosphate was according to the procedure described by Katewa and Katyare [24].

Protein estimation was according to the method of Lowry et al. [25] using bovine serum albumin as the standard.

Results are given as mean±SEM.

Statistical evaluation of the data was carried out by Tukey test using Sigmastat version 2.0.

Results

Treatment with increasing doses of DHEA resulted in progressive increase in the body weight compared to the untreated controls, with maximum increase (23%) being noted for 1 mg dose. At higher dose (2.0 mg) of DHEA the increase in the body weight declined. Under these conditions, the liver and brain weights did not change (data not given).

Effect on oxidative phosphorylation

Liver mitochondria

Effects of DHEA treatment with different doses on oxidative phosphorylation in liver mitochondria are summarized in Tables 1–4. Thus, treatment only with 0.2 mg DHEA resulted in 26% increase in state 3 respiration rate with glutamate as the substrate. However, the increase was not statistically significant. By contrast, the state 4 respiration rate was stimulated by 73% at 1.0 mg dose. The ADP/O ratios were unchanged following DHEA treatment. The ADP phosphorylation rate increased by 25% in animals treated with 0.2 mg DHEA, which agrees well with the similar increase in state 3 respiration rate (Table 1). When pyruvate+malate was used as the substrate couple, maximum and greater stimulation of state 3 respiration rate (59% increase) was obtained in animals treated with 1.0 mg dose. Under these conditions the state 4 respiration rate was stimulated by 69%. The ADP/O ratios were not affected. An increase in ADP phosphorylation rate corresponding to a similar increase in state 3 respiration rate in animals treated with 1.0 mg dose was also noted (Table 2). When succinate was used as the respiratory

Table 1
Effect of DHEA treatment on oxidative phosphorylation in rat liver and brain mitochondria using glutamate as the substrate

Tissue	Treatment	ADP/O ratio	Respiration rate (nmol O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (nmol/min/mg protein)
			+ADP	−ADP		
Liver	Untreated [12]	3.13±0.15	28.45±1.35	10.30±0.77	2.90±0.17	178.1±7.74
	0.1 mg DHEA [10]	3.14±0.29	27.81±2.47	12.77±1.85	2.24±0.18	174.6±9.50
	0.2 mg DHEA [10]	3.11±0.16	35.88±1.89	12.81±1.59	2.88±0.17	223.2±12.74[a]
	1.0 mg DHEA [12]	2.97±0.27	31.10±2.70	17.77±2.08[a]	1.75±0.17[a,b]	184.7±11.69
	2.0 mg DHEA [10]	3.18±0.25	28.01±1.85	13.26±1.49	2.19±0.17[a]	179.0±18.04
p value between groups		–	–	0.022	<0.001	–
Brain	Untreated [14]	3.21±0.22	20.12±1.02	5.40±0.79	4.92±0.62	127.1±8.90
	0.1 mg DHEA [8]	3.26±0.24	28.46±2.28[a]	5.78±0.76	5.36±0.60	184.4±15.98[a]
	0.2 mg DHEA [12]	3.16±0.19	27.32±1.11[a]	5.74±1.01	4.61±0.80	171.4±10.16[a]
	1.0 mg DHEA [12]	3.26±0.23	28.98±1.88[a]	17.01±1.74[a,b,c]	1.85±0.11[a,b,c]	192.6±12.50[a]
	2.0 mg DHEA [10]	3.16±0.16	21.52±1.02[b,d]	6.02±0.62	3.85±0.29	133.4±4.75[b,d]
p value between groups	–	<0.001	<0.001	<0.001	<0.001	

Experimental details are as given in the text. Results are given as mean±SEM of the number of observations indicated in the parentheses.

[a] $p<0.05$ compared with control.

[b] $p<0.05$ compared with 0.1 mg DHEA.

[c] $p<0.05$ compared with 0.2 mg DHEA.

[d] $p<0.05$ compared with 1 mg DHEA groups.

Table 2
Effect of DHEA treatment on oxidative phosphorylation in rat liver and brain mitochondria using pyruvate+malate as the substrate

Tissue	Treatment	ADP/O ratio	Respiration rate (nmol O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (nmol/min/mg protein)
			+ADP	−ADP		
Liver	Untreated [12]	3.11±0.27	17.72±1.08	8.58±0.77	2.16±0.12	110.0±11.68
	0.1 mg DHEA [10]	3.02±0.10	16.85±0.64	7.95±0.45	2.18±0.14	102.3±6.58
	0.2 mg DHEA [10]	3.14±0.24	16.42±0.58	8.93±0.23	1.85±0.09	103.6±9.98
	1.0 mg DHEA [12]	3.14±0.18	28.16±2.25^{a,b,c}	14.50±1.22^{a,b,c}	1.97±0.16	177.3±18.43^{a,b,c}
	2.0 mg DHEA [10]	3.18±0.24	18.53±0.89^{d}	11.06±0.32^{d}	1.69±0.10	119.1±13.78^{d}
p value between groups		–	<0.001	<0.001	–	<0.001
Brain	Untreated [12]	3.13±0.15	22.27±1.24	5.67±0.49	3.93±0.25	138.9±9.19
	0.1 mg DHEA [12]	3.23±0.16	24.52±1.33	6.21±0.42	3.95±0.24	157.8±10.11
	0.2 mg DHEA [12]	3.19±0.17	22.19±1.21	6.14±0.58	3.92±0.28	141.5±6.80
	1.0 mg DHEA [12]	3.17±0.24	26.00±1.31	7.93±0.40^{a}	3.80±0.22	164.8±7.69
	2.0 mg DHEA [10]	3.17±0.23	21.45±0.81	7.20±0.35	3.59±0.20	136.0±8.62
p value between groups		–	–	0.007	–	–

Experimental details are as given in the text. Results are given as mean±SEM of the number of observations indicated in the parentheses.

[a] $p<0.05$ compared with control.

[b] $p<0.05$ compared with 0.1 mg DHEA.

[c] $p<0.05$ compared with 0.2 mg DHEA.

[d] $p<0.05$ compared with 1 mg DHEA groups.

substrate the maximum stimulation of state 3 respiration rate (21% increase) was seen in animals treated with 0.2 mg DHEA. The state 4 respiration rate was also maximally stimulated at this dose (54% increase); the stimulatory effect declined at higher doses. ADP/O ratios were unaffected by DHEA treatment. ADP phosphorylation rate increased by 21% in animals treated with 0.2 mg DHEA although the increase was nor statistically significant (Table 3). With ascorbate+TMPD as the electron donor system, the stimulation of state 3 respiration rate (53% increase) was noted at a higher dose of DHEA, i.e., 1 mg. A similar trend was also seen for state 4 respiration rate (40% increase). The ADP/O ratios were unchanged and ADP phosphorylation rates increased by 39% in animals treated with 1 mg DHEA although the increase was nor statistically significant (Table 4).

Treatment with 0.2 and 1.0 mg doses of DHEA resulted in significant increase in the content of cytochrome aa_3 (35–44% increase). Similarly, following the treatment with 0.2–2.0 mg doses of DHEA the content of cytochrome b increased from 15% to 23%. The content of cytochrome $c+c_1$ increased (11% increase) only in animals treated with 0.2 mg doses of DHEA. (Table 5).

Treatment with DHEA, in general, resulted in stimulation of basal ATPase activity while the Mg^{2+}-stimulated ATPase activity increased (37% increase) only in the animals treated with 0.1 mg dose. Maximum stimulation (58% increase) of the DNP-stimulated ATPase activity was observed in the animal treated with 0.2 mg DHEA. Also, the DNP+ Mg^{2+}-stimulated ATPase activity increased by 31% in 0.2 mg DHEA treated animals (Table 6).

Table 3
Effect of DHEA treatment on oxidative phosphorylation in rat liver and brain mitochondria using succinate as the substrate

Tissue	Treatment	ADP/O ratio	Respiration rate (nmol O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (nmol/min/mg protein)
			+ADP	−ADP		
Liver	Untreated [12]	2.15±0.11	59.52±2.83	24.62±1.45	2.47±0.10	256.2±9.90
	0.1 mg DHEA [9]	2.26±0.19	61.79±2.25	26.18±1.73	2.45±0.18	279.6±25.18
	0.2 mg DHEA [11]	2.15±0.18	71.83±4.92	37.97±3.04^{a,b}	1.98±0.12	309.3±17.20
	1.0 mg DHEA [9]	2.21±0.17	59.75±4.08	31.37±2.49	1.94±0.12^{a}	264.0±17.50
	2.0 mg DHEA [11]	2.25±0.11	56.56±5.00	29.90±1.34	1.99±0.13	251.2±22.66
p value between groups		–	–	<0.001	0.004	–
Brain	Untreated [13]	2.15±0.18	26.70±1.18	15.07±1.27	1.91±0.16	114.8±6.90
	0.1 mg DHEA [12]	2.02±0.18	28.24±1.42	17.39±0.89	1.63±0.05	114.1±8.19
	0.2 mg DHEA [10]	2.15±0.17	32.86±2.38	19.16±1.31	1.83±0.13	141.3±6.03
	1.0 mg DHEA [12]	2.11±0.15	29.22±1.88	18.32±1.67	1.67±0.08	125.5±10.84
	2.0 mg DHEA [9]	1.88±0.11	26.12±2.19	17.24±1.09	1.58±0.09	98.1±9.71^{c}
p value between groups		–	–	–	–	0.022

Experimental details are as given in the text. Results are given as mean±SEM of the number of observations indicated in the parentheses.

[a] $p<0.05$ compared with control.

[b] $p<0.05$ compared with 0.1 mg DHEA.

[c] $p<0.05$ compared with 0.2 mg DHEA groups.

Table 4
Effect of DHEA treatment on oxidative phosphorylation in rat liver and brain mitochondria using ascorbate+TMPD as the substrate

Tissue	Treatment	ADP/O ratio	Respiration rate (nmol O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (nmol/min/mg protein)
			+ADP	−ADP		
Liver	Untreated [12]	0.47±0.03	29.11±1.84	23.15±1.61	1.27±0.03	26.5±1.58
	0.1 mg DHEA [13]	0.44±0.07	30.10±1.28	22.10±1.24	1.38±0.03	26.0±3.58
	0.2 mg DHEA [14]	0.45±0.04	34.26±2.47	24.95±1.50	1.37±0.04	29.9±2.81
	1.0 mg DHEA [12]	0.42±0.02	44.42±2.63$^{a, b, c}$	32.49±1.82^{a,b,c}	1.37±0.04	36.6±2.69
	2.0 mg DHEA [12]	0.41±0.05	36.05±3.04	26.15±1.91	1.38±0.03	28.6±4.06
p value between groups		–	<0.001	<0.001	–	–
Brain	Untreated [12]	0.77±0.07	25.34±1.34	16.30±0.94	1.59±0.06	38.9±3.09
	0.1 mg DHEA [13]	0.78±0.05	29.77±1.18	19.81±1.27	1.49±0.03	45.9±2.20
	0.2 mg DHEA [12]	0.76±0.06	29.27±1.43	20.56±0.91^{a}	1.42±0.04	44.3±3.25
	1.0 mg DHEA [12]	0.75±0.04	28.94±1.30	20.82±0.76^{a}	1.39±0.03^{a}	44.1±2.23
	2.0 mg DHEA [13]	0.76±0.04	25.62±1.40	17.71±0.90	1.46±0.06	37.5±2.11
p value between groups		–	0.047	0.007	0.041	–

Experimental details are as given in the text. Results are given as mean±SEM of the number of observations indicated in the parentheses.

[a] $p<0.05$ compared with control.

[b] $p<0.05$ compared with 0.1 mg DHEA.

[c] $p<0.05$ compared with 0.2 mg DHEA groups.

Treatment with 0.1 mg and 0.2 mg DHEA stimulated GDH activity by 36% and 50%. The SDR activity was stimulated by 80% following treatment with 0.2 mg DHEA. The MDH activities both mitochondrial as well as cytosolic were generally unaffected (Fig. 1).

Brain mitochondria

Effects of treatment with DHEA on oxidative energy metabolism in brain mitochondria are summarized in Tables 1–4. Thus, when glutamate was used as the respiratory substrate treatment with DHEA (0.1 mg to 1.0 mg) brought about significant stimulation (36–44% increase) in state 3 respiration rate. Under these conditions, the state 4 respiration rate was stimulated by 3.2-fold in animals treated with 1.0 mg DHEA. The ADP/O ratios were not affected by DHEA treatment. Changes in state 3 respiration rates were reflected in corresponding increase in the ADP phosphorylation rates (35–52% increase) (Table 1). As opposed to the above, when pyruvate+malate was used as substrate couple the changes in state 3 respiration rates were marginal; only in animal treated with 1.0 mg DHEA the rate increased by 17%. However, the change was not statistically significant. Higher doses of DHEA (1.0 mg and 2.0 mg) stimulated state 4 respiration rates by 40% and 27%, respectively; the latter change was not statistically significant. The ADP phosphorylation rates did not change significantly by DHEA treatment (Table 2). When succinate was used as substrate the state 3 and state 4 respiration rates were generally unchanged by treatment with DHEA. Similarly, the ADP phosphorylation rates were also generally unaltered (Table 3). With ascorbate+TMPD as the electron donor system only the state 4 respiration rate increased (22–28% increase) in the animals treated with 0.1 mg to 1.0 mg DHEA. The ADP phosphorylation rates were somewhat high but the changes were not statistically significant (Table 4).

Table 5
Effect of DHEA treatment on the cytochrome content of rat liver and brain mitochondria

Tissue	Treatment	Cytochrome content (pmol/mg protein)		
		aa_3	b	$c+c_1$
Liver	Untreated [8]	117.6±8.17	257.5±8.58	327.0±4.07
	0.1 mg DHEA [6]	119.4±4.56	255.2±8.63	338.1±8.52
	0.2 mg DHEA [8]	169.7±6.58^{a}	317.1±12.54$^{a, b}$	366.0±10.24^{a}
	1.0 mg DHEA [10]	158.8±15.99^{a,b}	298.1±7.19^{a,b}	344.4±5.16
	2.0 mg DHEA [8]	177.8±13.02	295.9±5.86^{a,b}	352.4±6.71
p value between groups		0.002	<0.001	0.007
Brain	Untreated [6]	146.5±4.41	162.1±5.41	218.8±8.00
	0.1 mg DHEA [5]	177.3±4.38^{a}	194.2±8.85^{a}	251.8±11.33
	0.2 mg DHEA [8]	172.4±5.49^{a,b}	215.9±10.45^{a,b}	205.7±17.55
	1.0 mg DHEA [9]	211.6±3.45$^{a,b, c}$	291.3±8.13^{a,b,c}	203.8±15.19
	2.0 mg DHEA [5]	204.6±4.18$^{a,b,c, d}$	215.0±10.89^{a,b,c,d}	166.2±7.96^{b}
p value between groups		<0.001	<0.001	0.022

Experimental details are as given in the text. Results are given as mean±SEM of the number of observations indicated in the parentheses.

[a] $p<0.05$ compared with control.

[b] $p<0.05$ compared with 0.1 mg DHEA.

[c] $p<0.05$ compared with 0.2 mg DHEA.

[d] $p<0.05$ compared with 1 mg DHEA groups.

Table 6
Effect of DHEA treatment on ATPase activity in rat liver and brain mitochondria

Tissue	Treatment	Activity (μmol Pi liberated/h/mg protein)			
		Basal	+Mg^{2+}	+DNP	Mg^{2+}+DNP
Liver	Untreated [12]	1.67±0.13	5.14±0.20	18.98±1.75	21.42±1.46
	0.1 mg DHEA [8]	3.74±0.30^{a}	7.03±0.61^{a}	23.28±1.38	20.89±0.69
	0.2 mg DHEA [12]	4.43±0.40^{a}	7.39±0.68	30.00±2.64^{a}	28.07±2.01^{a,b}
	1.0 mg DHEA [14]	4.38±0.29^{a}	6.29±0.45	22.58±1.18^{b}	22.45±0.93
	2.0 mg DHEA [8]	4.03±0.37^{a}	6.12±0.56	17.81±1.68^{b}	21.60±2.44
p value between groups		<0.001	0.027	<0.001	0.012
Brain	Untreated [12]	0.47±0.02	6.06±0.40	0.39±0.02	5.76±0.49
	0.1 mg DHEA [8]	0.59±0.04	7.53±0.24	0.62±0.04^{a}	7.75±0.46^{a}
	0.2 mg DHEA [12]	0.88±0.05^{a,c}	6.70±0.17	0.94±0.05^{a,c}	7.32±0.29
	1.0 mg DHEA [14]	0.53±0.03^{b}	5.93±0.49	0.82±0.04^{a,c}	7.59±0.46^{a}
	2.0 mg DHEA [8]	0.54±0.02^{b}	7.15±0.57	0.35±0.02^{a,c,d}	6.35±0.33
p value between groups		<0.001	0.048	<0.001	0.007

Experimental details are as given in the text. Results are given as mean±SEM of the number of observations indicated in the parentheses.

[a] $p<0.05$ compared with control.

[b] $p<0.05$ compared with 0.2 mg DHEA.

[c] $p<0.05$ compared with 0.1 mg DHEA.

[d] $p<0.05$ compared with 1 mg DHEA groups.

DHEA treatment (0.2–2.0 mg) resulted in increase in the content of cytochrome aa_3 and b with the optimum increase (44% and 80% increase, respectively) seen at 1 mg dose. The effect declined at higher dose (2.0 mg) of DHEA. The content of cytochrome $c+c_1$ increased by 15% only in the animals treated with 0.1 mg DHEA. However, the change was not statistically significant. At the highest dose employed (2.0 mg), the content actually decreased by 24% (Table 5).

DHEA treatment had marginal effect on basal and Mg^{2+}-stimulated ATPase activities but significantly enhanced DNP-stimulated and DNP+ Mg^{2+}-stimulated ATPase activities. This effect was noted up to the dose of 1.0 mg DHEA (Table 6).

The GDH activity increased by 89% and 114%, respectively, following treatment with 0.1 and 0.2 mg dose of DHEA with the effect declining at higher doses. The SDR activity increased progressively with maximum effect (1.74-fold increase) being seen at the highest dose (2.0 mg) of DHEA. The mitochondrial MDH activity increased (28% increase) in the animals treated with 1.0 mg DHEA whereas the cytosolic MDH activity was unaltered (Fig. 1).

Discussion

The present studies were undertaken to evaluate the possible effects of treatment with DHEA on oxidative energy metabolism in rat liver and brain mitochondria. The dose regimen was essentially based on the report of Su and Lardy [14]. These authors reported that when the rats were fed a diet containing 0.01–0.2% DHEA for seven days the hepatic mitochondrial glycerophosphate dehydrogenase activity increased 3–5 times [14]. We decided to use s.c. route for administration of DHEA based on our earlier experiments with corticosterone and dexamethasone, where the steroids were injected s.c. to ensure a slow release in circulation [15,16]. This aspect is important especially in view of the short half-life of DHEA [26,27]. The additional advantage is that this eliminates the problem of variations in the absorption of DHEA ingested by oral route.

From the data presented it is clear that the treatment regimen that we followed significantly influenced the energy metabolism parameters in mitochondria from the liver as well as the brain. The effects were tissue specific and dose dependent. Broadly speaking, treatment with 0.1–2.0 mg DHEA affected respiration rates, cytochrome contents, ATPase activities and dehydrogenases levels in mitochondria from the two tissues in different manner. Most importantly with highest concentration of DHEA (2.0 mg), the effects, in general, declined.

Thus, in the liver mitochondria state 3 respiration with glutamate was maximally stimulated following treatment with 0.2 mg dose; in the brain mitochondria doses from 0.1 to 1.0 mg stimulated state 3 respiration rate with the extent of stimulation being greater than that observed for liver mitochondria. State 3 respiration rates with pyruvate+malate were stimulated maximally in liver mitochondria after treatment with 1 mg DHEA whereas only marginal changes were noted for the brain mitochondria which were not statistically significant. In the liver as well as brain mitochondria with succinate as the substrate, the state 3 respiration rates increased by about 20% with 0.2 mg dose of DHEA although the increase was not statistically significant State 3 respiration rate with ascorbate +TMPD was only marginally affected in the brain mitochondria whereas in the liver mitochondria about 50% stimulation was obtained with 1 mg dose. DHEA treatment had no uncoupling effect and ADP/O ratios were unchanged. Observed increases in state 3 respiration rate were also reflected in corresponding increases in ADP phosphorylation rates, which is indicative of increased energy potential (Tables 1–4).

DHEA treatment also resulted in significant increase in the contents of cytochrome aa_3 and b in mitochondria from both the tissues. The increase in the content of $c+c_1$ cytochromes was marginal. Once again the effects were tissue specific. In the case of liver mitochondria 0.2 mg dose was more effective whereas for brain mitochondria the effects were evident with the lowest dose (0.1 mg) employed. Also DHEA treatment was more effective in increasing the contents of cytochrome aa_3 in liver

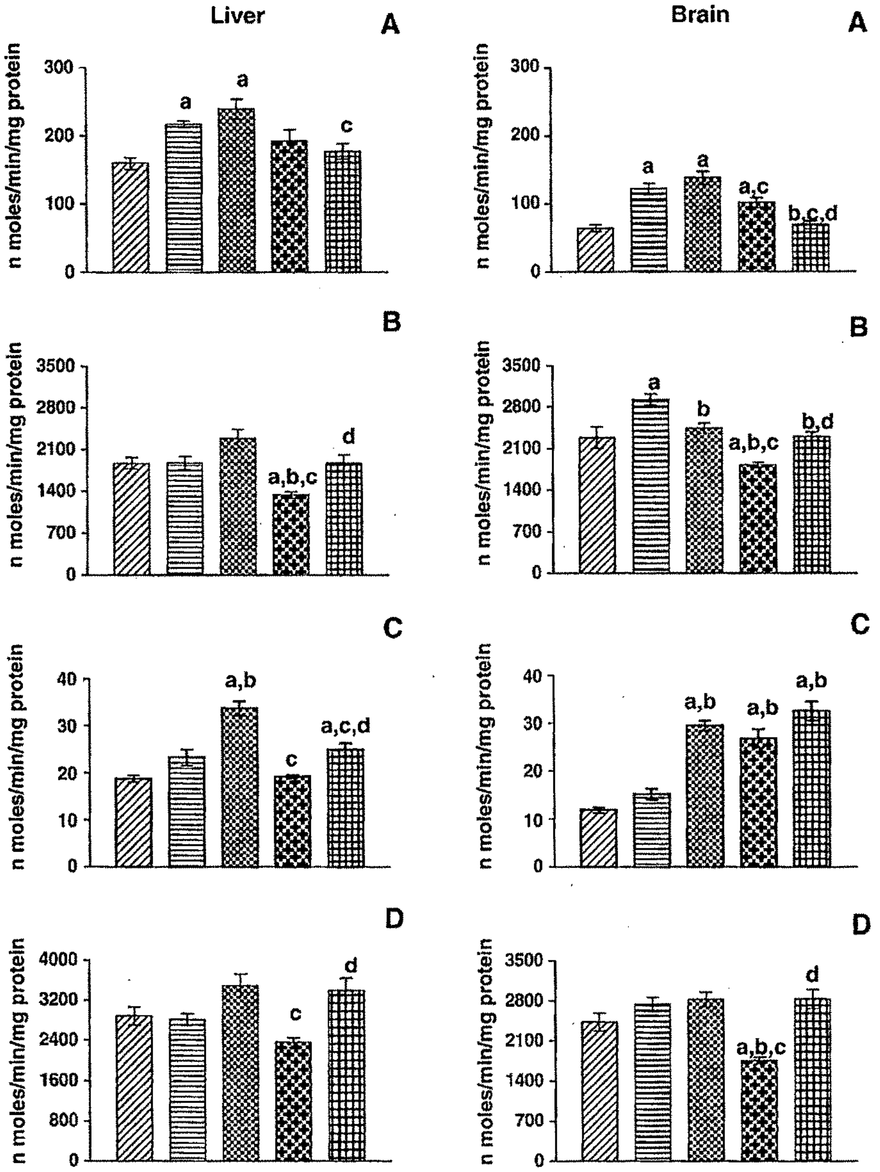

Fig. 1. Effect of DHEA treatment on mitochondrial and cytosolic dehydrogenases activities in rat liver and brain. The results are given as mean+SEM of 10–12 independent observations. (A) Glutamate dehydrogenase; (B) malate dehydrogenase (mitochondrial); (C) succinate DCIP reductase; and (D) malate dehydrogenase (cytosolic); ▨, Untreated, ▤, 0.1 mg DHEA, ▩, 0.2 mg DHEA, ▩, 1.0 mg DHEA and ▦, 2.0 mg DHEA. a, $p < 0.05$ compared with control; b, $p < 0.05$ compared with 0.1 mg DHEA; c, $p < 0.05$ compared with 0.2 mg DHEA and d, $p < 0.05$ compared with 1 mg DHEA groups. The p value between the groups in liver as well as brain were: GDH, $p = 0.001$; MDH (Mitochondrial), $p < 0.001$; SDR, $p < 0.001$ and MDH (cytosolic), $p < 0.001$.

mitochondria, by contrast in the brain mitochondria more pronounced effect was seen on the content of cytochrome b (Table 5).

Likewise the DHEA treatment differentially influenced GDH and SDR activities in the liver and brain mitochondria. Paradoxically, however, a negative effect on mitochondrial and cytosolic MDH was seen after treatment with 1.0 mg DHEA (Fig. 1). Stimulation of malic enzyme following treatment with DHEA has been reported by other workers [8,14]. However, in our studies we obtained an opposite effect. The reason for this discrepancy remains unclear at this stage. It is possible that this may be related to the dose, route of administration as well as duration of treatment [8,14]. DHEA treatment also differentially stimulated the ATPase activities in liver and brain mitochondria (Table 6). The increased respiratory activities in mitochondria correlate well with observed increases in the contents of the aa_3 and b cytochromes and the dehydrogenases activities.

Although DHEA and DHEA-S are known to be synthesized in the highest quantity by adrenal gland, there are no known receptors demonstrated for these two steroids [28]. The mechanism for action of DHEA therefore remains unclear. Based on its stimulatory effect on malic enzyme synthesis, it has been suggested that DHEA action is mediated via thyroid hormones, which are known to stimulate MDH synthesis [8,14]. Other suggested mechanism is the direct interaction of DHEA with macromolecules [28]. However, inhibition of state 3 respiration rate and FoF_1 ATPase activity in mitochondria by addition of DHEA and DHEA-S *in vitro* [29,30,31] would argue against this possibility. Alternative mechanism that has been proposed is that secondary metabolites, steroid in nature, may mediate DHEA action [32]. More recently, it has been demonstrated that DHEA is metabolized to 7α-hydroxy-DHEA and δ5-androstene-3β,17β-diol. The former is considered to be the active metabolite [33,34].

In the present studies, we observed that the exposure to DHEA resulted in increase in the contents of cytochrome aa_3, b and ATPase activity but not of content of cytochrome $c+c_1$. These observations deserve some comments. It is well established that crucial polypeptide of cytochrome oxidase, cytochrome b and FoF_1 ATPase are coded by mitochondrial DNA [35]. By contrast, cytochrome $c+c_1$ is a nuclear gene product [35]. It may hence be suggested that DHEA action may be mediated by activating specific mitochondrial genes coding for polypeptide subunits of cytochrome aa_3, b and FoF1 ATPase. In this connection, it is interesting to note that presence of dexamethasone binding site in COX II region of mitochondrial genome has been demonstrated [36–38]. It would be interesting to know if similar DHEA binding site(s) exists on mitochondrial genome. Likewise, increase in the GDH and SDR activities suggests that DHEA action may also be specific for activating these nuclear genes.

It is possible that DHEA treatment-induced changes in the respiratory activities in mitochondria from the two tissues could influence the cellular reactive oxygen species (ROS) parameters. This interesting possibility needs to be examined by separate experiments

In conclusion, our present studies have shown that treatment with DHEA resulted in stimulation of respiratory activity and increase in the energy potential of the liver and the brain mitochondria. However, the effects were dose dependent and tissue specific for a given parameter. Also, higher dose (2.0 mg) of DHEA had adverse effects. Thus, our results point out that although exogenous DHEA can stimulate the energy related functions in the tissues such as liver and brain which can have beneficial effects in the elderly population [5], one has to exercise caution in therapeutic application of DHEA. Especially one has to take into consideration the fact that overdosing with DHEA can have adverse effects.

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Thyroid Hormone-Induced Alterations in Membrane Structure-Function Relationships: Studies on Kinetic Properties of Rat Kidney Microsomal Na^+,K^+-ATPase and Lipid/Phospholipid Profiles

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Abstract The effects of thyroidectomy (Tx) and subsequent treatment with 3,5,3′-triiodothyronine (T_3) or combined replacement therapy (T_R) with T_3 and thyroxine (T_4) on the substrate and temperature kinetics properties of Na^+,K^+-ATPase and lipid/phospholipid makeup of rat kidney microsomes were examined. Enzyme activity was somewhat high in the hypothyroid (Tx) animals and increased significantly following T_3 treatment, while T_R treatment caused a decrease. In the Tx and T_3 groups enzyme activity resolved in two kinetic components, while in the T_R group the enzyme showed allosteric behavior up to 0.5 mM ATP concentration. The K_m and V_{max} values of both the components decreased in Tx animals without affecting the catalytic efficiency. T_3 treatment caused a significant increase in the V_{max} of both the components, with a significant increase in the catalytic efficiency, while the K_m values were not upregulated. The T_R regimen lowered the K_m and V_{max} of component II but improved the catalytic efficiency. Thyroid status-dependent changes were also noted in the temperature kinetics of the enzyme. Regression analysis revealed that changes in the substrate and temperature kinetics parameters correlated with specific phospholipid components.

Keywords Thyroid hormone · Triiodothyronine · Thyroxine · Microsomal Na^+,K^+-ATPase · Substrate and temperature kinetics · Lipid/phospholipid profile

Introduction

Thyroid hormones affect the function of several tissues and organs, including the kidneys. Thus, it has been reported that in hypothyroid rats renal plasma flow, glomerular filtration rate and filtered Na^+ load decreased (Holmes & Di Scala, 1970; Katz & Lindheimer, 1973; Katz, Emmanouel & Lindheimer, 1975; Kinsella & Sacktor, 1985; Lo et al., 1976; Michael et al., 1972). These changes have been correlated with the decrease in levels of Na^+,K^+-ATPase in the kidneys (Lo et al., 1976). In animals made hypothyroid by feeding aminotriazole in the diet, the Na^+,K^+-ATPase activity in the proximal convoluted tubules decreased by 57%, which was corrected by simultaneous treatment with L-thyroxine (T_4) (Garg & Tisher, 1985). In surgically thyroidectomized (Tx) rats the Na^+,K^+-ATPase activity in the kidney cortex decreased by about 50% and treatment with 50 μg 3,3′,5-triiodo-L-thyronine (T_3) on 3 alternate days caused 67% stimulation of the enzyme activity over the hypothyroid value (Lo et al., 1976). The observed increase was correlated directly with the proportionate upregulation of the mRNA encoding the α- and β-subunits of Na^+,K^+-ATPase (Chaudhury et al., 1987; Gick, Ismail-Beigi & Edelman, 1988; McDonough et al., 1988). It is well documented that Na^+,K^+-ATPase in the kidneys represents a pure α_1 and β_1 combination (Blanco & Mercer, 1998).

While the Na^+,K^+-ATPase in the whole kidney cortex represents predominantly a pure α_1 and β_1 combination (Blanco & Mercer, 1998), recently it has been demonstrated that the microsomal enzyme in several tissues comprises a combination of α- and β_3-subunits (Arystarkhova & Sweadner, 1997). Also, the stoichiometry of α- and β_3-subunits is tissue-specific (Arystarkhova & Sweadner, 1997). Thus, in the kidney microsomal enzyme α- and β_3-subunits occur in proportion of about 3:1

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(Arystarkhova & Sweadner, 1997). In earlier studies from our laboratory we demonstrated that the liver microsomal Na^+,K^+-ATPase shows significant differences from the plasma membrane Na^+,K^+-ATPase with respect to its K_m for ATP, Na^+ and K^+ (Katewa & Katyare, 2003a). Based on these observations, we suggested that the microsomal enzyme may be involved in the temperature homeostasis of the internal organs (Katewa & Katyare, 2003a).

The requirement of acidic phospholipids for Na^+,K^+-ATPase has been well documented (Robinson & Flashner, 1979), and the thyroid hormones are known to regulate lipid metabolism in responsive tissues (Hoch, 1988). That the microsomes are the major sites of lipid biosynthesis is also well recognized.

In light of the above, microsomal Na^+,K^+-ATPase offers an interesting and unique system to evaluate the regulation of the structure-function relationships of membrane-bound enzymes such as Na^+,K^+-ATPase by thyroid hormones.

Although treatment with T_3 is known to elicit maximum stimulatory response at the end of 48 h (Satav & Katyare, 1991; Tata, 1964), it has now been pointed out that tissue euthyroid status with respect to the contents of thyroid hormones is reached only after a combined treatment with T_3 and T_4: (0.9 μg T_4 + 0.15 μg T_3)/100 g body weight for 18 consecutive days (Escobar-Morreale et al., 1996).

In view of the above, it was of interest to find out the effect of thyroid hormone deficiency and treatment with the two thyroid hormone regimens cited (Escobar-Morreale et al., 1996; Satav & Katyare, 1991; Tata, 1964) on kidney microsomal lipid/phospholipid profiles and how the possible changes could influence the microsomal structure-function relationships. More importantly, it was of interest to examine if the combined T_4 and T_3 treatment (Escobar-Morreale et al., 1996) was able to restore membrane structure-function relationships to the normal euthyroid state.

To illustrate this point, we determined the substrate and temperature kinetics properties of microsomal Na^+,K^+-ATPase together with the lipid/phospholipid profile of kidney microsomes and their correlation with the kinetics properties of Na^+,K^+-ATPase.

Materials and Methods

Chemicals

T_3, T_4, bovine serum albumin (BSA) fraction V and 1,6-diphenyl-1,3,5-hexatriene were purchased from Sigma (St. Louis, MO). ATP was from SRL (Mumbai, India). Silica gel G was from E. Merck (Darmstadt, Germany). All other chemicals were of analytical reagent-grade and purchased locally.

Animals and Treatment with Thyroid Hormones

Weanling male albino rats (3–4 weeks old, 30–35 g body weight) of Charles-Foster strain underwent Tx and were allowed to grow for 8–10 weeks (Katyare & Rajan, 2005). Only those animals showing considerable decrease in body weight (50–60%) were used for further studies. The controls were sham-operated. Tx rats were randomly divided in three groups. One group of Tx animals received 25 μg T_3/100 g of body weight subcutaneously (s.c.) and were killed after 48 h (Modi et al., 2007; Satav & Katyare, 1991; Tata, 1964). This group is referred to as "T_3." The second group of animals received a replacement therapy with (0.9 μg T_4 + 0.15 μg T_3)/100 g body weight s.c. for 18 consecutive days (Escobar-Morreale et al., 1996). The animals were killed on day 19. This group is hereafter referred to as "T_R." The third group (designated "Tx") received an equivalent volume of vehicle (0.9% saline containing 5 mM NaOH) in which thyroid hormone solutions were prepared. Thyroid hormone solutions were prepared freshly prior to use. Sham-operated controls received an equivalent volume of saline/NaOH vehicle.

The experimental protocol was approved by the Departmental Ethical Committee.

Isolation of Microsomes

The animals were killed by decapitation. The kidneys were removed quickly and kept in chilled (0–4°C) isolation medium (0.25 M sucrose containing 10 mM Tris-HCl buffer [pH 7.4] and 1 mM ethylenediaminetetraacetic acid [EDTA] and 25 μg BSA/ml) (Patel & Katyare, 2006a). After removing the kidney capsule, the medulla was carefully scraped off and the cortex homogenized using a Potter Elvehjem-type glass-Teflon homogenizer to obtain 10% (w/v) homogenate. After sedimenting mitochondria, the postmitochondrial supernatant was subjected to a further centrifugation at 12,000 x *g* for 10 min to sediment the light mitochondrial fraction. The resulting supernatant was then centrifuged at 100,000 x *g* for 1 h to sediment the microsomal fraction. The pellet was washed once by resuspending and resedimenting (Kaushal, Dave & Katyare, 1999; Patel & Katyare, 2006a).

ATPase Assay

After preincubating the microsomal protein (30–50 μg) in the assay medium containing 50 mM Tris-HCl buffer (pH 7.4), 120 mM NaCl, 10 mM KCl and 5 mM $MgCl_2$ at 37°C for 1 min, the reaction was initiated by addition of ATP at a final concentration of 5 mM (Katewa & Katyare, 2003a, 2003b). The reaction was terminated after 10 min by addition of 0.1 ml of 5% (w/v) sodium dodecyl sulfate (SDS) solution, and the amount of liberated inorganic phosphate (Pi) was estimated by the method of Katewa & Katyare (2003b).

For the substrate kinetics studies, concentration of ATP was varied in the range 0.1–5 mM.

For temperature kinetics studies, experiments were carried out with a fixed ATP concentration (5 mM) and the temperature was varied from 5°C to 53°C with an increment of 4°C at each step.

Reaction velocity, v, is expressed as micromoles of Pi liberated per hour per milligram protein.

Lipid Analysis

Microsomal lipids/phospholipids were extracted with freshly prepared chloroform:methanol (2:1vol/vol) according to procedures described previously (Folch, Lees & Sloane Stanley, 1957; Pandya, Dave & Katyare, 2004).

Separation of phospholipid classes by thin layer chromatography (Pandya et al., 2004; Skipski, Peterson & Barclay, 1964), estimations of cholesterol (Zlatkis, Zak & Boyle, 1953) and phospholipid phosphorus (Bartlett, 1959) and determination of membrane fluidity were carried out as described (Pandya et al., 2004). These procedures have been described in detail (Modi et al., 2007; Pandya et al., 2004).

The content of individual phospholipid classes was calculated by multiplying the values of total phospholipid (TPL) with percent composition of the individual phospholipid classes (Pandya et al., 2004).

Data Analysis

The data for substrate kinetics were computer-analyzed using Sigma Plot version 6.1 (Jandel Corporation, San Rafael, California, USA) by three methods: Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden plots for the determination of K_m and V_{max} (Dixon & Webb, 1979; Patel et al., 2000). The values of K_m and V_{max} obtained by the three methods were in close agreement and were averaged for the final presentation of results.

The values of Kcat (turnover number) were calculated from the corresponding V_{max} values using the equation

$$\mathrm{Kcat} = \frac{V_{max}\,(\mathrm{moles}) \times N}{3600 \times 15.13 \times 10^4}$$

where N is Avogadro's number and 15.13×10^4 is the molecular weight of Na^+,K^+-ATPase (Blanco & Mercer, 1998; Wetzel, Arystarkhova & Sweadner, 1999). Since it is not possible to know the number of the ATPase molecules, the values of Kcat/K_m was normalized to ^{App}Kcat/K_m (Patel & Katyare, 2006b).

Table 1 Effect of Tx and subsequent treatment with thyroid hormones on Na^+,K^+-ATPase activity in rat kidney microsomes

Animals	Activity (μmoles of Pi liberated/h/mg protein)		Activity ratio
	25°C	37°C	
Control (8)	9.50 ± 0.51	18.11 ± 0.73	1.92 ± 0.042
Tx (6)	12.36 ± 0.63[a]	22.60 ± 1.11[a]	1.83 ± 0.029
T_3 (6)	34.54 ± 0.92[c,*]	75.72 ± 2.15[c,*]	2.19 ± 0.056
T_R (6)	7.05 ± 0.27[b,*]	15.04 ± 0.59[a,*]	2.14 ± 0.070

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses. Activity ratios = activity at 37°C/activity at 25°C.

[a] $p < 0.01$, [b] $p < 0.002$, [c] $p < 0.001$ compared to euthyroid control; *$p < 0.001$ compared to Tx

$$^{App}\mathrm{Kcat}/K_m = \frac{\mathrm{Kcat}}{K_m \times V(37°C)}$$

where V (37°C) refers to the values given in Table 1.

The regression analysis across the groups was carried out using Jandel (Corte Madera, CA) Sigmastat Statistical Software, version 2.0. Estimation of protein was by the method of Lowry et al. (1951) using BSA as the standard. Statistical evaluation of the data was by Student's t-test.

Results

In the preliminary experiments, we measured Na^+,K^+-ATPase activity at 25°C and 37°C. Measurements at 25°C revealed that Tx resulted in a 30% increase in the activity and treatment with a single dose of T_3 resulted in a further 3.6-fold increase compared to euthyroid controls. The T_R regimen, on the other hand, resulted in a 26% decrease. A similar trend was seen even for measurements made at 37°C. Thus, the activity ratios were more or less comparable for all groups (Table 1).

In the next set of experiments, we determined the dependence of the enzyme activity on substrate concentration. Typical substrate saturation curves and corresponding Eadie-Hofstee plots are shown in Figure 1. As can be noted in the control, Tx and T_3 groups, the enzymes displayed a normal pattern of substrate saturation kinetics (Fig. 1a–c), whereas in the T_R group up to the ATP concentration of 1 mM the enzyme displayed allosteric characteristics (e.g., *see* Fig. 1d, inset), beyond which the normal substrate saturation pattern was followed (Fig. 1d).

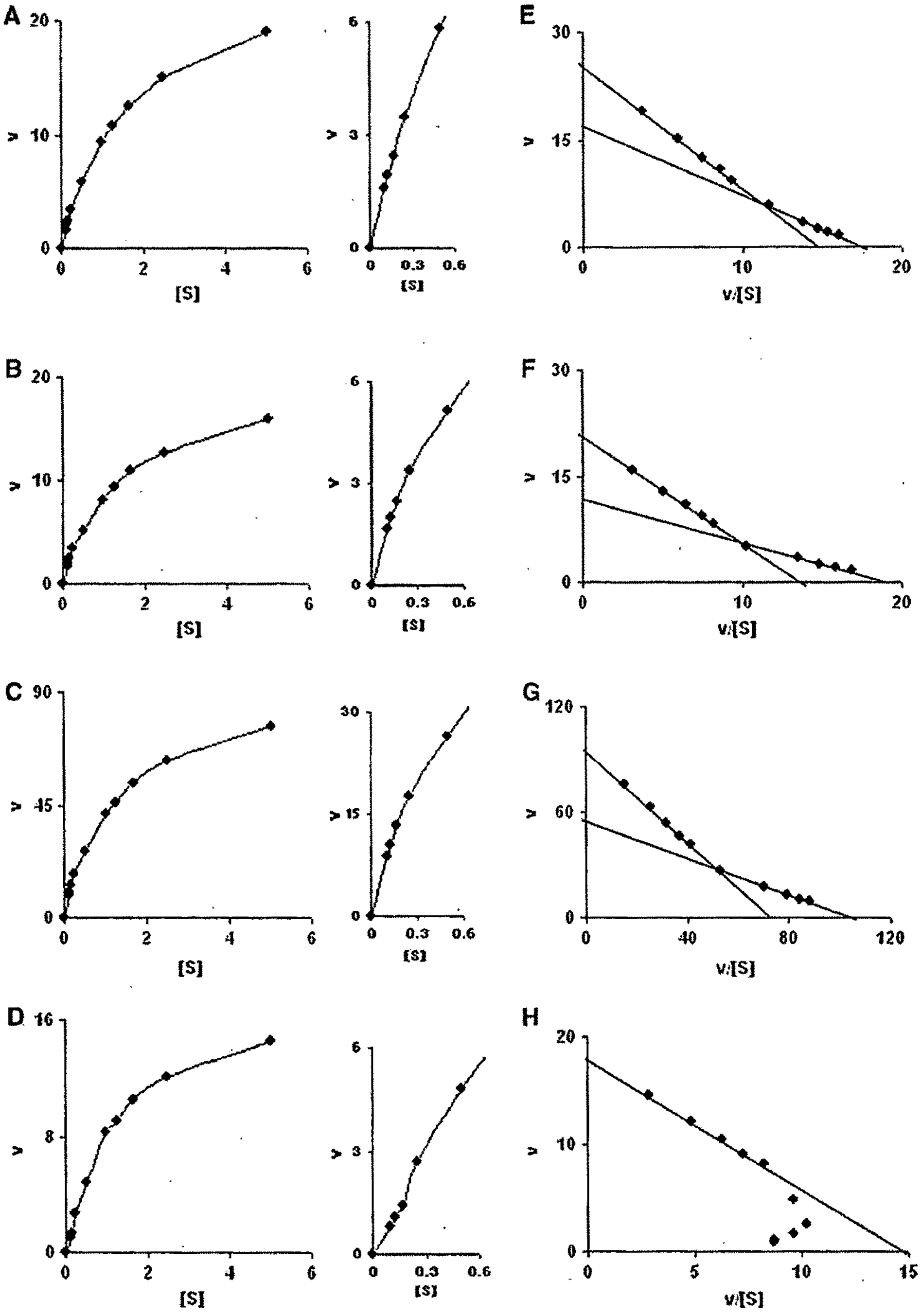

Fig. 1 Typical substrate saturation plots for rat kidney microsomal Na^+,K^+-ATPase. The enzyme activity, *v*, on the abscissa is plotted *vs.* substrate (ATP) concentration (S) on the ordinate. **a–d** Control, Tx, T_3 and T_R groups, respectively. *Insets* in **a–d** show magnified views of substrate saturation curve up to ATP concentration of 0.5 mM. Note especially the allosteric pattern in inset for **d**. Corresponding Eadie-Hofstee plots are shown in **e–h**. The enzyme activity, *v*, on the abscissa is plotted *vs.* v/S on the ordinate. The plots are typical of four to eight independent experiments, as indicated in Table 2

These differences were also illustrated when the data were transformed into Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden plots. For the sake of brevity, only Eadie-Hofstee plots are shown in Figure 1e–h. Thus, in the control, Tx and T_3 groups the enzyme activity resolved in two kinetically differentiable components (Fig. 1e–g); in the T_R animals only the low-affinity component II was present (Fig. 1h).

In the control group the K_m values for the two components were 0.90 and 1.90 mM, respectively, and the

Table 2 Effect of Tx and subsequent treatment with thyroid hormones on substrate kinetics properties of rat kidney microsomal Na^+,K^+-ATPase

Animals	Component I		Component II	
	K_m	V_{max}	K_m	V_{max}
Control (8)	0.90 ± 0.080	14.23 ± 0.87	1.90 ± 0.16	23.62 ± 1.00
Tx (6)	0.55 ± 0.018^c	10.79 ± 0.44^b	1.52 ± 0.11	21.39 ± 1.47
T_3 (6)	0.57 ± 0.022^c	$54.77 \pm 2.10^{d,**}$	$1.22 \pm 0.04^{d,*}$	$90.80 \pm 2.98^{d,**}$
T_R (4)	— Allosteric —		1.42 ± 0.11^a	19.04 ± 0.78^b

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses. The kinetic components represent the potential and the response of the enzyme to increasing concentrations of the substrate. K_m, mM; V_{max}, µmole of Pi liberated/h/mg protein.

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.002$, [d] $p < 0.001$ compared to euthyroid control; $*p < 0.05$, $**p < 0.001$ compared to Tx

Table 3 Effect of Tx and subsequent treatment with thyroid hormones on $^{App}Kcat/K_m$ values for the kinetic components of microsomal Na^+,K^+-ATPase in rat kidney microsomes

Animals	$^{App}Kcat/K_m \times 10^{11}$	
	Component I	Component II
Control (8)	9.92 ± 0.45	7.86 ± 0.47
Tx (6)	9.73 ± 0.46	7.03 ± 0.42
T_3 (6)	$14.12 \pm 0.47^{b,**}$	$10.90 \pm 0.46^{b,**}$
T_R (4)	-	$10.58 \pm 0.86^{a,*}$

Values of $Kcat/K_m$ were computed as described in the text. The results are given as mean ± SEM of the number indicated in parentheses.

[a] $p < 0.02$, [b] $p < 0.001$ compared to euthyroid control; $*p < 0.01$, $**p < 0.001$ compared to Tx

corresponding values for V_{max} were 14.23 and 23.62 units (µmole of Pi liberated/h/mg protein). In the Tx group the K_m of component I decreased appreciably. A similar tendency toward decrease was noted even for the K_m of component II. However, the decrease was not statistically significant. Even in T_3-treated animals, the K_m of component I remained low and comparable to that of Tx animals. However, the V_{max} increased significantly (3.35- and 5.03-fold increase compared to control or Tx group) (Table 2). The T_R regimen was unable to upregulate the K_m of component II, and the V_{max} decreased by 19%. Component I was not discernible in the T_R group.

In view of the observed changes in the K_m and V_{max} values under the different experimental conditions (Table 2), it was of interest to find out if the catalytic efficiency of the enzyme was influenced by thyroid hormone status. Catalytic efficiency was computed in terms of $^{App}Kcat/K_m$ values, as described above in "Materials and Methods." As is evident, the hypothyroid state had no effect on catalytic efficiency of the enzyme (Table 3). T_3 treatment, on the other hand, resulted in a 1.4-fold increase in the efficiency of components I and II. A similar 1.4-fold increased was seen even in the T_R group for component II (Table 3).

Table 4 Effect of Tx and subsequent treatment with thyroid hormones on Hill plot analysis on rat kidney microsomal Na^+,K^+-ATPase

Animals	Hill coefficient		Transition concentration (mM)
	n_1	n_2	
Control (8)	0.94 ± 0.013	1.33 ± 0.016	0.50 ± 0.031
Tx (6)	0.88 ± 0.014	1.35 ± 0.035	0.53 ± 0.050
T_3 (6)	0.89 ± 0.010	1.36 ± 0.009	0.55 ± 0.027
T_R (4)	–	1.33 ± 0.026	–

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses

Hill plot analysis of the substrate kinetics data indicated that for the control, Tx and T_3 groups at up to 0.5–0.55 mM concentration of ATP one ATP molecule was bound, while beyond this concentration two molecules of ATP were bound (Table 4). By contrast, for the T_R group two ATP molecules were bound throughout the substrate concentration range. Typical Hill plots depicting these substrate binding characteristics are shown in Figure 2.

We then examined the influence of thyroid status on the temperature kinetics of the enzyme. The typical activity *vs.* temperature curves and the corresponding Arrhenius plots are shown in Figure 3. As can be noted, in the Tx, T_3 and T_R groups optimal temperature decreased to 45°C; in the control group the optimal temperature of the enzyme was 49°C (Fig. 3a–d).

The Arrhenius plots in general followed a biphasic pattern (Fig. 3) in the control, Tx and T_3 groups, and the values of energy of activation in the low temperature range (E_L) were higher than those of energy of activation in the high temperature range (E_H). However, the T_R group proved to be an exception, where the pattern was reversed (Fig. 3h); i.e., the value of E_H was high and that of E_L was substantially low.

The data on values of E_H, E_L and phase transition temperature (Tt) are summarized in Table 5. Thus, in the control group the values of E_H and E_L were 39.9 and 65.7 KJ/mole, respectively, with Tt occurring at 23.5°C. In the

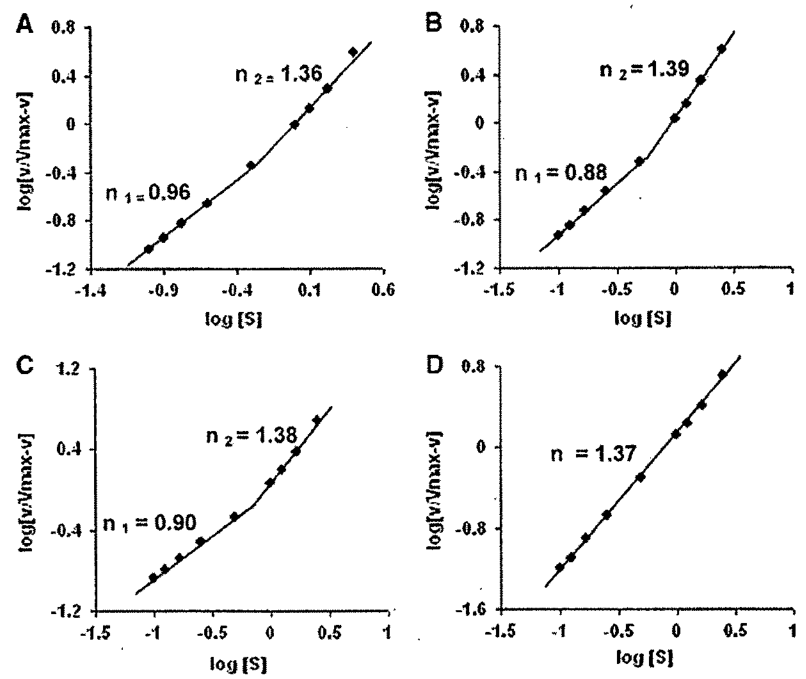

Fig. 2 Typical Hill plots for rat kidney microsomal Na^+,K^+-ATPase. Log (v/V_{max} - v) on the abscissa is plotted *vs.* log (S) on the ordinate. The Hill coefficients n_1 and n_2 represent the number of ATP molecules bound over the given concentration range of ATP. **a** Control, **b** Tx, **c** T_3 and **d** T_R. The plots are typical of four to eight independent experiments, as indicated in Table 4

Tx group the value of E_H increased by 44% while that of E_L increased by 14%; Tt decreased by 4°C. T_3 treatment resulted in further increases in the values of E_H and E_L (52% and 46%, respectively) while returning Tt to normal. The T_R group was characterized by a 27% increase in E_H but a 45% decrease in E_L; Tt was comparable to the euthyroid control (Table 5).

In light of these differential effects, it was of interest to find out how the thyroid status affected the lipid milieu and to seek its possible correlation with the substrate and temperature kinetics parameters. The data on the TPL and cholesterol (CHL) contents as influenced by thyroid status are given in Table 6; values of fluorescence polarization, p, are also included. Tx resulted in no appreciable change in TPL content but CHL content became almost half compared to the euthyroid control. This was also reflected in the doubling of the TPL/CHL (mole:mole) ratio in the Tx group. However, the membrane fluidity was unchanged. T_3 treatment increased TPL content without affecting CHL content. As a consequence, the TPL/CHL (mole:mole) ratio increased in the Tx group together with a decrease in membrane fluidity. The T_R regimen resulted in a significant increase in TPL content and tended to increase CHL to the euthyroid level. The TPL/CHL (mole:mole) ratio was still comparable to the Tx group. The T_R regimen also resulted in a significant decrease in membrane fluidity.

The effects of thyroid status on phospholipid composition are summarized in Table 7. As can be noted, Tx resulted in an almost two fold increase in the phosphatidylserine (PS) and phosphatidic acid (PA) components, while the phosphatidylethanolamine (PE) component decreased by 27%. The composition of other phospholipid components was not affected. Treatment with T_3 brought about a significant reduction in phosphatidylinositol (PI), but T_3 was unable to restore to normal PS and PA, which remained elevated. Likewise, PE remained low as in the Tx animals. The T_R regimen resulted in a two fold increase in the lysophospholipid (Lyso) component, while the effects on PI and PS were opposite. PI decreased compared to the control and Tx groups, and PS decreased compared to Tx animals, thus bringing its value close to that of the euthyroid control. PE was restored to normal, and PA became more or less comparable to the euthyroid value. The phosphatidylcholine (PC) component was unchanged under all experimental conditions. These changes were also reflected in terms of the content of individual phospholipid classes (Table 8).

Discussion

From the data presented it is clear that the microsomal enzyme differed in several respects from the predominant Na^+,K^+-ATPase in the kidneys, which comprises the pure α_1 and β_1 combination. Thus, the activity of the microsomal enzyme in hypothyroid animals increased beyond the value in euthyroid controls (Table 1). Hypothermia is a well-recognized feature of hypothyroidism (Tata, 1964). It may hence be suggested that the increase in enzyme activity may represent a compensatory mechanism to

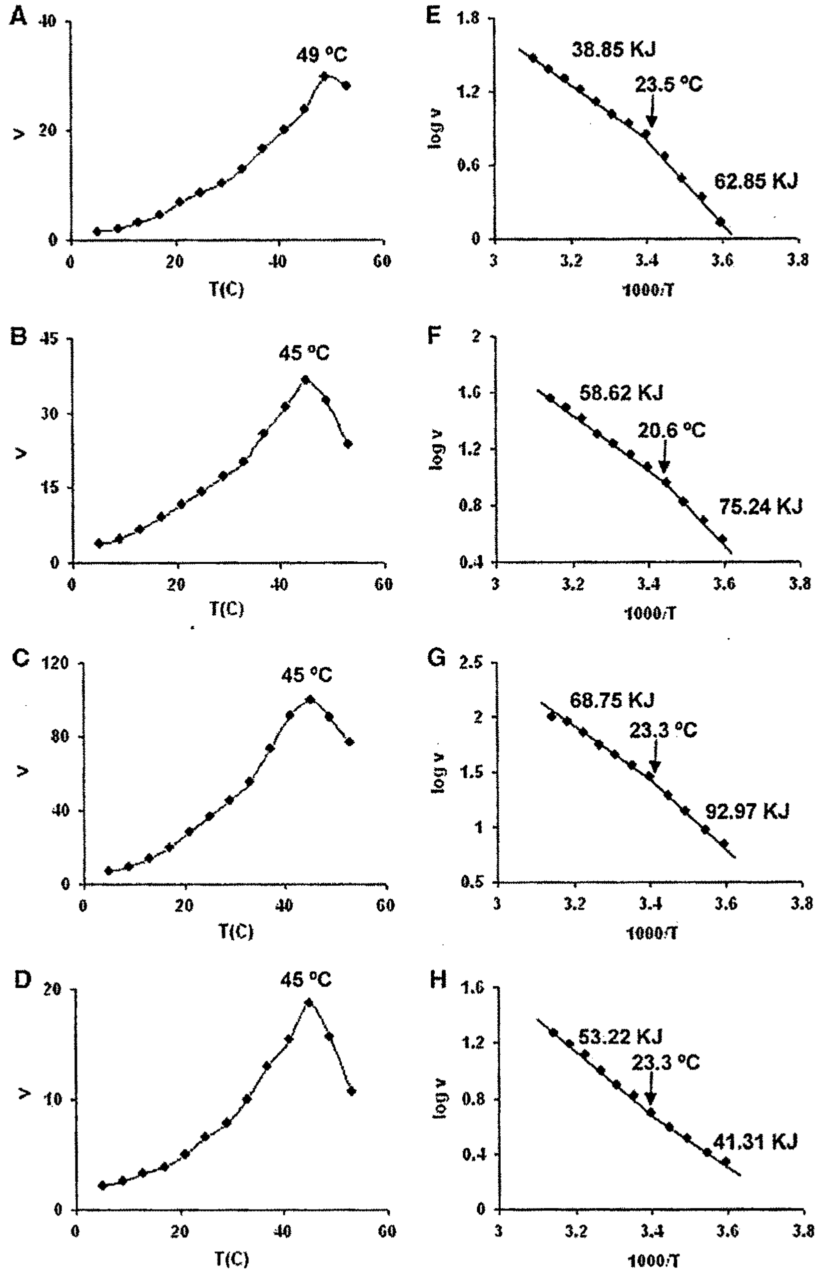

Fig. 3 Typical plots showing dependence of Na^+,K^+-ATPase activity on temperature and the corresponding Arrhenius plots. In temperature curves, enzyme activity, *v*, on the abscissa is plotted *vs.* temperature on the ordinate. **a** Control, **b** Tx, **c** T_3 and **d** T_R. The corresponding Arrhenius plots are shown in **e**–**h**. In Arrhenius plots log *v* on the abscissa is plotted *vs.* 1,000/*T* on the ordinate, where *v* and *T* represent, respectively, the activity at the corresponding absolute temperature (temperature in °C + 273.2). The plots are typical of six to eight independent experiments, as indicated in Table 5

combat hypothermia. Such an assumption would agree with our earlier suggestion that the microsomal enzyme may play a role in temperature homeostasis of the internal organs rather than in translocation of Na^+ and K^+ (Katewa & Katyare, 2003a, 2003b). The decreased K_m value especially of component I in the Tx animals (Table 2) may also represent an additional compensatory mechanism for combating hypothermia, which agrees well with the decreased energy potential of kidney mitochondria in hypothyroidism (Katyare et al., 1977; Satav & Katyare, 1991). The hypothyroid state, however, did not influence the catalytic efficiency ($^{App}Kcat/K_m$) of the enzyme (Table 3).

The microsomal enzyme also differed from the predominant $\alpha_1\beta_1$ Na^+,K^+-ATPase of the kidneys with respect to the exaggerated response to treatment with a single injection of T_3 (Table 1). It has been reported that the activity of $\alpha_1\beta_1$ Na^+,K^+-ATPase increased by about 70% above the hypothyroid value following treatment with T_3 on 3 alternate days; the dose of T_3 used in these studies was 50 μg/100 g body weight, i.e., two times higher than the single-dose regimen employed in our present studies (Lo et al., 1976). These authors also reported that the K_m of the enzyme was unchanged by T_3 treatment. However, their studies were restricted by use of a substrate (ATP) concentration range of 0.25–1 mM (Lo et al., 1976). Against this we find that the K_m of both the kinetic components of the microsomal enzyme was significantly lowered. As is to be expected, the catalytic efficiency of both kinetic components increased significantly under these conditions (Table 3). The T_R regimen, on the other hand, presented a rather interesting feature. While the activity decreased below the euthyroid value (Table 1), the enzyme displayed allosteric characteristics up to the ATP concentration of 0.5 mM; the $S_{0.5}$ ($S_{0.5}$ is the concentration of substrate required for half-maximal activity) for the allosteric component was around 0.9 mM (*data not shown*). However, the T_R regimen was not able to upregulate either the activity or the V_{max} of component II. However, in animals receiving T_R treatment, the catalytic efficiency of component II increased significantly (Table 3).

It has been reported that increased activity of kidney cortex Na^+,K^+-ATPase following treatment with T_3 directly correlated with increased transcripts of α- and β-subunits and their respective mRNAs in equal proportion (Chaudhury et al., 1987; Gick et al., 1988; McDonough et al., 1988). Therefore, one wonders if a similar situation would prevail in the case of the microsomal enzyme which comprises the α_1,β_3 isoform.

Table 5 Effect of Tx and subsequent treatment with thyroid hormones on Arrhenius kinetics properties of rat kidney microsomal Na^+,K^+-ATPase

Animals	Energy of activation (KJ/mole)		Phase transition temperature (Tt, °C)
	E_H	E_L	
Control (8)	39.9 ± 1.46	65.7 ± 2.02	23.5 ± 0.84
Tx (6)	57.6 ± 0.57^b	75.0 ± 1.60^a	19.4 ± 0.57^b
T_3 (6)	$64.5 \pm 1.83^{b,*}$	$96.0 \pm 3.15^{b,**}$	$24.4 \pm 0.65^{**}$
T_R (4)	$50.7 \pm 1.54^{b,*}$	$35.4 \pm 3.28^{b,**}$	$23.1 \pm 0.96^{*}$

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses.

[a] $p < 0.01$, [b] $p < 0.001$ compared to euthyroid control; $^*p < 0.01$, $^{**}p < 0.001$ compared to Tx

It is well recognized that α is the catalytic subunit while β is the regulatory subunit of Na^+,K^+-ATPase (Blanco & Mercer, 1998; Wetzel et al., 1999). That the enzyme activity did not change in hypothyroid animals (Table 1) suggests that thyroid hormones may regulate the synthesis of the predominant $\alpha_1\beta_1$ isoform without affecting the microsomal enzyme, which may be a compensatory mechanism for combating hypothermia, as pointed out above. By the same consideration, exaggerated response to T_3 treatment suggests that this experimental condition may specifically stimulate the synthesis of the catalytic α_1-subunit without stimulating the synthesis of the regulatory β_3-subunit. It has been reported that in control rat kidney the stoichiometry of β_3:α_1 is about 1:3. Therefore, it is possible that a disproportionate increase in the α_1-subunit may lead to hyperstimulation of the microsomal enzyme. This interesting possibility, however, needs to be verified and confirmed by more direct experiments. By similar logic, it may be suggested that sustained long-term combined treatment with T_3 and T_4 might introduce another kind of disproportionate combination of $\alpha_1\beta_3$ isoforms, thereby lowering the activity. The results also suggest that the synthesis of α_1 and β_3 subunits may be differentially regulated by T_3 and T_4, respectively. Once again, this interesting possibility needs further confirmation by direct experimentation.

Thyroid status also introduced subtle changes in the temperature kinetics of the enzyme, which included lowering of the optimal temperature by 4°C in all the experimental groups. However, the most important change was an increase in the energies of activation in Tx animals, which was not corrected by T_3 treatment (Fig. 1, Table 5). The T_R group presented a reversed Arrhenius pattern, where the value of E_H increased and that of E_L became almost half. Clearly, the T_3 and T_R regimens did not restore the membrane characteristics to normal, although both treatments restored the value of Tt, which had decreased in the Tx animals.

Table 6 Effect of Tx and subsequent treatment with thyroid hormones on total phospholipids (TPL), cholesterol (CHL) and fluorescence polarization (p) in rat kidney microsomes

Animals	TPL (μg/mg protein)	CHL (μg/mg protein)	TPL/CHL (mole:mole)	p
Control (8)	235.1 ± 6.72	204.2 ± 2.86	0.58 ± 0.02 0.	0.190 ± 0.001
Tx (4)	250.4 ± 7.94	97.3 ± 2.87^b	1.29 ± 0.02^b	0.192 ± 0.002
T_3 (4)	$276.6 \pm 5.66^{b,*}$	93.0 ± 6.27^b	$1.57 \pm 0.09^{b,***}$	$0.267 \pm 0.001^{b,***}$
T_R (4)	$396.5 \pm 6.33^{b,***}$	$166.5 \pm 9.14^{a,***}$	$1.21 \pm 0.06^{b,**}$	$0.247 \pm 0.001^{b,***}$

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses.

[a] $p < 0.01$, [b] $p < 0.001$ compared to euthyroid control; $^*p < 0.05$; $^{**}p < 0.02$, $^{***}p < 0.001$ compared to Tx

Table 7 Effects of Tx and subsequent treatment with thyroid hormones on phospholipid composition in rat kidney microsomes

Phospholipid class	Composition (% of total)			
	Control (8)	Tx (4)	T_3 (4)	T_R (4)
Lyso	2.91 ± 0.14	2.86 ± 0.36	2.86 ± 0.15	$6.62 \pm 0.20^{c,****}$
SPM	24.15 ± 0.36	23.60 ± 0.63	25.19 ± 0.35	24.00 ± 0.31
PC	36.22 ± 1.47	35.91 ± 1.05	38.07 ± 1.20	34.25 ± 1.19
PI	3.82 ± 0.12	3.25 ± 0.24	$2.08 \pm 0.13^{c,***}$	2.71 ± 0.09^{c}
PS	4.91 ± 0.12	8.22 ± 0.27^{c}	8.00 ± 0.11^{c}	$4.21 \pm 0.14^{b,****}$
PE	22.59 ± 1.52	16.58 ± 1.34^{a}	$15.32 \pm 1.02^{b,*}$	$22.12 \pm 0.96^{*}$
PA	5.41 ± 0.11	9.59 ± 0.35^{c}	$8.48 \pm 0.10^{c,**}$	$6.10 \pm 0.07^{c,****}$

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses.

[a] $p < 0.02$, [b] $p < 0.01$, [c] $p < 0.001$ compared to euthyroid control; $*p < 0.05$, $**p < 0.02$, $***p < 0.002$, $****\ p < 0.001$ compared to Tx

Table 8 Effects of Tx and subsequent treatment with thyroid hormones on phospholipid content in rat kidney microsomes

Phospholipid class	Content (μg /mg of microsomal protein)			
	Control (8)	Tx (4)	T_3 (4)	T_R (4)
Lyso	6.8 ± 0.38	7.1 ± 0.44	7.9 ± 0.28^{a}	$26.3 \pm 0.87^{d,****}$
SPM	56.8 ± 1.95	58.8 ± 1.12	$69.7 \pm 1.98^{d,***}$	$95.1 \pm 1.32^{d,***}$
PC	85.4 ± 3.10	90.5 ± 5.39	105.3 ± 2.74^{d}	$135.8 \pm 3.45^{d,****}$
PI	9.0 ± 0.45	8.2 ± 0.65	$5.8 \pm 0.24^{d,**}$	$10.8 \pm 0.41^{c,**}$
PS	11.6 ± 0.48	20.7 ± 1.27^{d}	22.1 ± 0.57^{d}	$16.7 \pm 0.52^{d,*}$
PE	52.8 ± 2.53	41.3 ± 0.86^{b}	42.4 ± 1.85^{b}	$87.9 \pm 2.61^{d,****}$
PA	12.8 ± 0.50	23.8 ± 2.33^{d}	23.5 ± 0.55^{c}	24.2 ± 0.37^{d}

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses.

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.02$; [d] $p < 0.001$ compared to euthyroid control; $*p < 0.05$, $**p < 0.02$, $***p < 0.002$, $****p < 0.001$ compared to hypothyroid

The analysis of lipid/phospholipid profiles revealed thyroid status-dependent changes. Thus, both the T_3 and T_R regimens tended to increase TPL but were unable to bring back to euthyroid level the decreased CHL content. Thyroid status-dependent changes were also evident in the composition and contents of individual phospholipids. These changes, one may anticipate, could cause differences in charge distribution across the membranes, thereby influencing the substrate and temperature kinetics parameters of the enzyme. We explored this possibility by seeking correlation between compositional changes in the lipid/phospholipid components and various kinetic parameters. These data from regression analysis are given in Tables 9 and 10.

Table 9 Correlation of substrate kinetics parameters of microsomal ATPase with membrane lipid/phospholipid composition across all the groups

Parameter	Correlation	
	Positive	Negative
K_m 1	PS (+0.597) PE (+0.635) CHL (+0.728)	PI (−0.711)
V_{max} 1	TPL (+0.605)	PS (−0.760) PE (−0.587)
K_m 2	–	–
V_{max} 2	PI (+0.550)	PS (−0.641) PE (−0.630)

The experimental details are given in the text. Values given in parentheses indicate the regression coefficient, *r*, which is based on four to eight independent experiments, indicated in Tables 6 and 7

Thus, analysis across the groups revealed that K_m 1 correlated positively with PS, PE and CHL, while PI showed a negative correlation. K_m 2 was not influenced by any lipid component. V_{max} 1 and V_{max} 2 showed positive correlations, respectively, with TPL and PI, while both V_{max} 1 and V_{max} 2 correlated negatively with PS and PE (Table 9). This is somewhat paradoxical since kidney cortex Na^+,K^+-ATPase has been shown to be dependent on acidic phospholipid PS and PI for its activity (Robinson & Flashner, 1979). The results thus suggest that the

Table 10 Correlation of temperature kinetics parameters of microsomal ATPase with membrane lipid/phospholipid composition

Parameter	Control, Tx, T_3		Control, T_R		Tx, T_R	
	Positive	Negative	Positive	Negative	Positive	Negative
E_H	PI (+0.863)	PS (−0.742)	Lyso (+0.654)	PS (−0.721)	PI (+0.746)	Lyso (−.868)
	PA (+0.795)	PE (−0.858)	TPL (+0.665)	PI (−0.599)	PA (+0.698)	PE (−0.656)
		CHL (−0.917)		CHL (−0.783)	Fluidity (+0.761)	TPL (−0.773)
		Fluidity (−0.696)		Fluidity (−0.799)		CHL (−0.943)
E_L	PI (+0.633)	PS (−0.845)	PC (+0.681)	Lyso (−0.891)	PS (+0.604)	Lyso (−0.867)
	PA (+0.577)	PE (−0.694)	PS (+0.571)	PA (−0.706)	PI (+0.955)	PE (−0.911)
		CHL (−0.721)	CHL (+0.668)		PA (+0.939)	TPL (−0.977)
		Fluidity (−0.880)			Fluidity (+0.985)	CHL (−0.944)
Tt	-	-	SPM (+0.669)	PE (−0.601)	Lyso (+0.648)	PI (−0.859)
					PE (+0.803)	PA (−0.929)
					TPL (+0.932)	Fluidity (−0.896)
					CHL (+0.822)	

The experimental details are given in the text. Values given in parentheses indicate the regression coefficient, *r*, which is based on four to eight independent experiments, as indicated in Tables 6 and 7

microsomal $\alpha_1\beta_3$ isoform also differs from its $\alpha_1\beta_1$ counterpart with respect to the specific lipid/phospholipid requirements.

Correlation studies for temperature kinetics were found to be a bit more complex since the Arrhenius pattern reversed in the T_R group (Fig. 3, Table 5). Hence, we carried out the regression analysis for the control, Tx and T_3; for the control and T_R; and for the Tx and T_R groups. These data are given in Table 10.

In the control, Tx and T_3 groups both E_H and E_L correlated positively with PI and PA and negatively with PS, PE, CHL and membrane fluidity.

Analysis of the control and T_R groups revealed that Lyso and TPL were major positive modulators of E_H, whereas PS, PI, CHL and membrane fluidity showed a negative correlation. E_L correlated positively with PC, PS and CHL and negatively with Lyso and PA.

Correlation studies for the Tx and T_R group show that PI, PA and membrane fluidity correlated positively with E_H, while PS, PI, PA and fluidity correlated positively with E_L. Lyso, PE, TPL and CHL were negative modulators of E_H and E_L.

In the control and T_R groups, Lyso and PE were positive and PI and PA were negative modulators of Tt. For the Tx and T_R groups the correlation with Tt was more complex: Lyso, PE, TPL and CHL played a positive role and PI, PA and membrane fluidity had a negative influence.

The present investigations were undertaken to find out if treatments with combined replacement therapy with T_3 and T_4 (Escobar-Morreale et al., 1996) could restore the microsomal function to the euthyroid state. Our present results show that neither treatment was able to restore the kinetics properties of microsomal Na$^+$,K$^+$-ATPase or the lipid/phospholipid profile to the normal euthyroid state. Therefore, it may be suggested that while replacement therapy is able to restore the thyroid hormone status of a given tissue to the euthyroid state (Escobar-Morreale et al., 1996), more complex and intricate mechanisms may be involved in restoring the membrane structure-function relationship and enzyme kinetics parameters to the euthyroid state.

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Improved method for estimation of inorganic phosphate: Implications for its application in enzyme assays

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The conventional method of Fiske and Subba Row for the estimation of inorganic phosphate (Pi) is although rapid, but suffers from the disadvantage that the color is unstable and hence the optical density (OD) measurements have to be carried out within a short time span of 8-12 min. This poses a restriction on the number of samples, which can be handled in a batch. Although, modified procedures involving use of alternate reducing agents/or increasing the concentration of H_2SO_4 in conventional method have been subsequently developed, but the problem of color stability could not be solved. In addition, the use of higher concentrations H_2SO_4 has rendered the methods unsuitable in enzyme assays, especially if the acid labile phosphate containing substrates have been used. In the present study, attempts have been made to suitably modify the method to improve the stability of the color and sensitivity and also for its applicability in enzyme assays, especially when acid labile phosphate containing substrates such as ATP is used. We used the higher concentrations (0.625, 0.8 and 1.0 *N*) of H_2SO_4 rather than 0.5 *N* used in the conventional assay procedures. Under these conditions, the reagent blanks do not develop color for up to 24 h, whereas the intensity of the molybdenum blue color in the standard and/or experimental tubes increased with time reaching optimum value at 24 h. Simultaneously, the absorption maximum shifts from 660 nm to 820 nm. The highest concentration of H_2SO_4 (1.0 *N*) is found to be the most effective in the process of color development. The sensitivity of the method is from 1.7 to 2.1 times higher, as compared to the conventional Fiske and Subba Row method for the measurements carried out at the end of 15 min at 820 nm and with the highest concentration of H_2SO_4 (1.0 *N*); the sensitivity increased 4.8-fold at the end of 24 h. Presence of glucose and sucrose (1-10 m*M*), NaCl and KCl (5-100 m*M*), $MgCl_2$ (1-10 m*M*) and BSA (10 to 500 µg per assay tube) do not interfere either with color development or with OD measurements. The extent of ATP hydrolysis is 1.6 to 3.4% for up to 1 h, depending upon the concentration of H_2SO_4 used. Only negligible hydrolysis of G6P is observed under these conditions. These results suggest that the presently modified method is suitable for Pi analysis in the enzyme assays, in the presence of labile phosphate containing substrates.

Keywords: Pi determination, Stabilization, Molybdenum blue color, Improved sensitivity

Fiske & Subba Row[1] first described the quantitative method, widely used for colorimetric estimation of phosphate. The method was based on conversion of inorganic orthophosphate (Pi) to phosphomolybdic acid, followed by the reduction of molybdenum to produce the molybdenum blue species, which had ill-defined absorption characteristics. This method used a mixture of sodium sulfite, sodium bisulfite and 1, 2, 4-aminonaphthol sulfonic acid (ANSA) as the reducing agent. Although, the method was rapid, it suffered from the disadvantage that the color was unstable and hence the optical density (OD) measurements had to be carried out within a short time span of 8-12 min. Subsequently, modifications using reducing agents such as ascorbic acid, hydroquinone, 2, 4-aminophenol, thiosulfate, stannous chloride, hydrazine sulfate etc. were described, however, the problem of color instability persisted[2,3]. Using a mixture of hydrazine sulfate and stannous chloride as reducing agent, although the sensitivity improved for measurements made at 700 nm, the color which developed maximally in 3 min was stable only up to 40 min[3]. Use of high concentration of H_2SO_4 (1.0 *N*) posed additional problem[3].

In a recently described method[4], excess molybdate was removed by adding oxalic acid and the color was extracted in isobutyl alcohol that stabilized the color for 7 h. In another method, phosphomolydic acid was extracted with methyl isobutyl ketone and quantification of molybdenum was done by the

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atomic absorption spectrophotometry[5], rather than by colorimetric method[1]. Methods for determination of phosphorous in the presence of pyrophosphate in soybean and of phosphate released by hydrolysis of nucleic acids using HNO_3 were also reported[6,7], however, no modifications were introduced for Pi determination carried out by the conventional method[1]. In the procedure described by Bartlett[8], stability of the color and sensitivity improved significantly. However, as the color was developed by boiling the samples at 100°C for 7 min in the presence of 1.2 *N* H_2SO_4, the method could not be used in the enzyme assays, especially when a substrate such as ATP which contains labile phosphate groups was used.

It was desirable to develop a method that produced a stable molybdenum blue species and at the same time was rapid, had higher sensitivity and had applicability for the enzyme assays. Keeping these requirements in mind, in the present communication, attempts were made to optimize the assay conditions for the estimation of Pi by developing the color in the presence of higher concentrations of H_2SO_4 (0.625, 0.8 and 1.0 *N*) and monitoring the OD changes over a period of up to 24 h. Corresponding absorption spectra at fixed time intervals were recorded and the values of extinction coefficient (E_M) were computed.

Materials and Methods

Chemicals

D-Glucose 6-phosphate (G6P) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Bovine serum albumin (BSA) fraction V and sodium salt of adenosine 5'-triphosphate (ATP) were obtained from SRL, *Mumbai*, India and 1, 2, 4-aminonaphthol sulfonic acid (ANSA) was purchased from Glaxo Laboratories, (India) Ltd., Mumbai. Sodium dodecyl sulfate (SDS) was obtained from Koch-Light, Colnbrook, England. All other chemicals were of analytical reagent grade and purchased locally.

Procedure for phosphate estimation with varying concentrations of H_2SO_4

Aliquots of standard solution (40 μg Pi/ml) containing 1-8 μg Pi were taken and the volume was made up to 3.2 ml with distilled water. To this, 0.5 ml of stock solution of H_2SO_4 was added to achieve the final concentration of 0.5, 0.625, 0.8 or 1.0 *N* i.e. to get the final concentration of 0.5 *N*, concentration of stock solution was 4.0 *N*. This was followed by the addition of 0.2 ml of 5% ammonium molybdate solution (freshly prepared in distilled water). The color was developed by the addition of 0.1 ml of reducing agent solution (details given below) with vigorous shaking. OD measurements and recording of absorption spectra were carried out in a Shimadzu model UV 160 A or JASCO model V 530 UV/VIS spectrophotometers.

The triturate of reducing agent was prepared by grinding 1.2 g sodium sulfite, 1.2 g sodium bisulfite and 0.4 g ANSA using a porcelain mortar and pestle and stored in an amber-colored bottle[9]. Reducing reagent solution was prepared fresh prior to use and contained 40 mg triturate/ml of distilled water.

Acid hydrolysis of ATP and G6P

For determination of extent of acid hydrolysis, two phosphate esters were incubated under phosphate estimation conditions in the presence of varying concentrations (0.5, 0.625, 0.8 or 1.0 *N*) of H_2SO_4. In incubation mixture, the final concentrations of ATP were 2.0 and 5.0 m*M*, and that of G6P were 1.0 and 5.0 m*M*. Release of Pi was monitored over a 24 h period. Instability of the color posed a problem, when determinations were carried out in the presence of 0.5 *N* H_2SO_4 by the conventional Fiske and Subba Row method[1]. Thus, G6P and ATP at two above-mentioned concentrations were incubated with 0.5 *N* H_2SO_4 and aliquots were taken at specified time intervals to monitor the amount of Pi released using conventional procedure[1]. The extent of substrate hydrolysis at different time intervals was determined from the amount of Pi released, for which the corresponding values of slope were used (see Fig. 4 and 5).

Effect of sugars, salts and proteins

To examine, if sugars, salts or proteins interfered in the development of color and/or determination of the OD, experiments were carried in the presence of glucose and sucrose (1-10 m*M*), NaCl and KCl (5-100 m*M*), and $MgCl_2$ (1-10 m*M*) in the presence of varying concentrations of H_2SO_4 as detailed above. The interference by proteins was determined by including 10-500 μg BSA/assay tube in the presence of SDS (final concentration, 0.25% w/v). These experiments were performed using 4 μg Pi, which represented the middle of the assay range. Color development was monitored spectrophotometricaly for up to 24 h

Results

Effect of varying H_2SO_4 concentration

In the initial experiments, estimation of Pi was carried out by the conventional Fiske and Subba Row method[1]. The final concentration of H_2SO_4 was 0.5 *N*. Under these conditions, OD readings between 8-15 min were linear with the Pi concentration. However, at the end of 30 min, linearity was lost and the standards as well as reagent blank developed intense blue color, making measurements of the OD of the sample tubes difficult. Thus, further experiments were carried out only with the three higher concentrations (0.625, 0.8 or 1.0 *N*) of H_2SO_4 and the time course of color development was followed for up to 24 h. These data are shown in Fig. 1.

In the presence of higher concentrations (0.625, 0.8 or 1.0 *N*) of H_2SO_4, the color in the reagent blank as well standards were stable even up to 24 h. The time course of color development determined by measurements at 660 and 820 nm is shown in Fig. 1. The intensity of the color increased with time in all the sets of experiments and optimum color development was observed in the presence of 1.0 *N* H_2SO_4. Also, magnitude of increase in the OD was higher, when monitored at 820 nm. This suggested that with progress of time there was a shift towards formation of molybdenum blue species which absorbed at 820 nm. To verify this, absorption spectra of standard Pi (4 µg) solution at different time intervals were recorded. It was evident from the absorption spectra (Fig. 2) that although the absorption at 660 and 820 nm increased with the time, increase of absorbance at 820 nm was of greater magnitude. The spectra also confirmed that 1.0 *N* H_2SO_4 was most efficient both in the color development and shift in the absorption maxima.

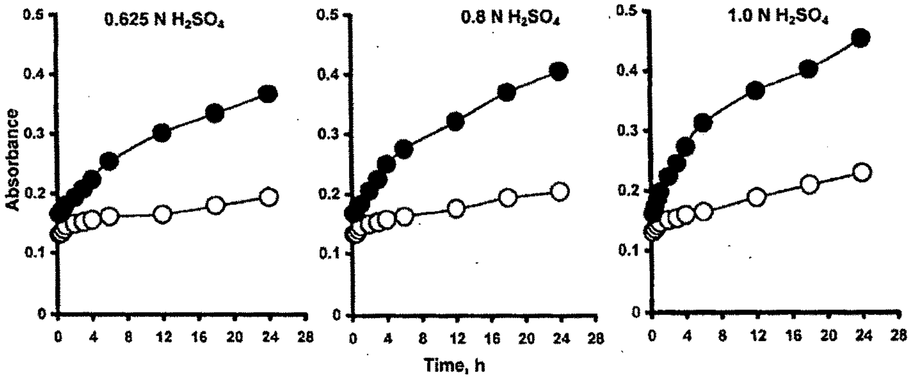

Fig. 1—Time course of color development in Pi assay [Experiments were performed using 4 µg Pi. Concentrations of H_2SO_4 were 0.625, 0.8 and 1.0 *N*. OD measurements at 660 nm and at 820 nm were carried out at specified time periods as indicated. Each data point represented average of 3 independent observations. Variation ranged from 2-3%. OD measurements at 660 nm (-○-) and at 820 nm (-●-)]

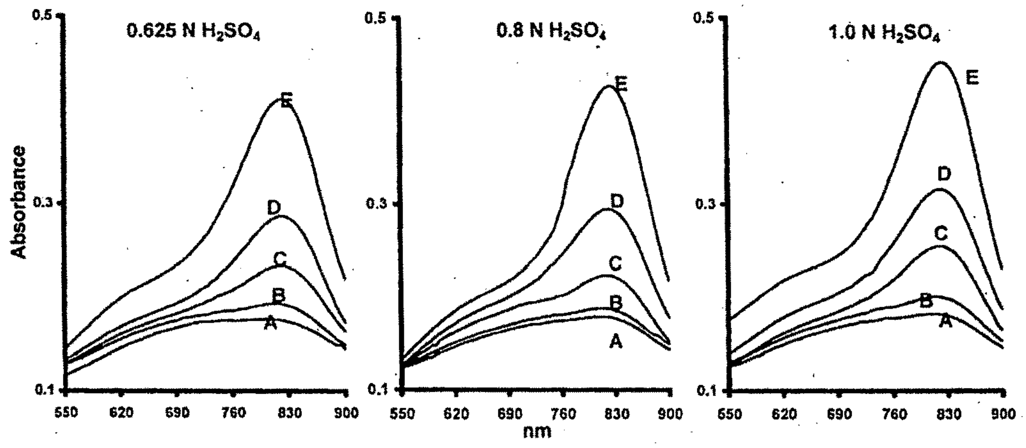

Fig. 2—Absorption spectra depicting the time course of color development in Pi assay [Experiments were performed using 4 µg Pi. Concentrations of H_2SO_4 were 0.625, 0.8 and 1.0 *N*. Absorption spectra were recorded at: A, 15 min; B, 1 h; C, 6 h; D, 12 h; and E, 24 h]

Also, under these conditions, there was a gradual shift in absorption maxima around 820-830 nm region towards higher wavelength. Typical plots depicting this shift in the absorption maxima in the presence of 0.625 and 1.0 *N* H_2SO_4 are shown in Fig. 3; the picture was intermediate for 0.8 *N* H_2SO_4 (data not shown).

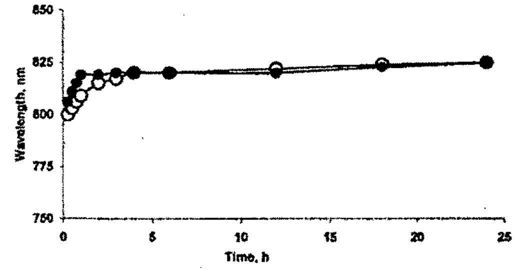

Fig. 3—Time course of shift in absorption maxima in Pi assay [Experiments were performed using 4 µg Pi. Concentrations of H_2SO_4 were 0.625 and 1.0 *N*. Each data point represented average of 3 independent observations. Variation ranged from 2-3%. (-o-), 0.625 *N*; and (-●-) 1.0 *N* H_2SO_4]

The relative improvement in sensitivity of Pi estimation, in terms of a standard curve was monitored at different time intervals. For this purpose, the OD readings were recorded at 660 and 820 nm. These standard curves are shown in Fig. 4. It may be noted that the plots were linear up to the Pi

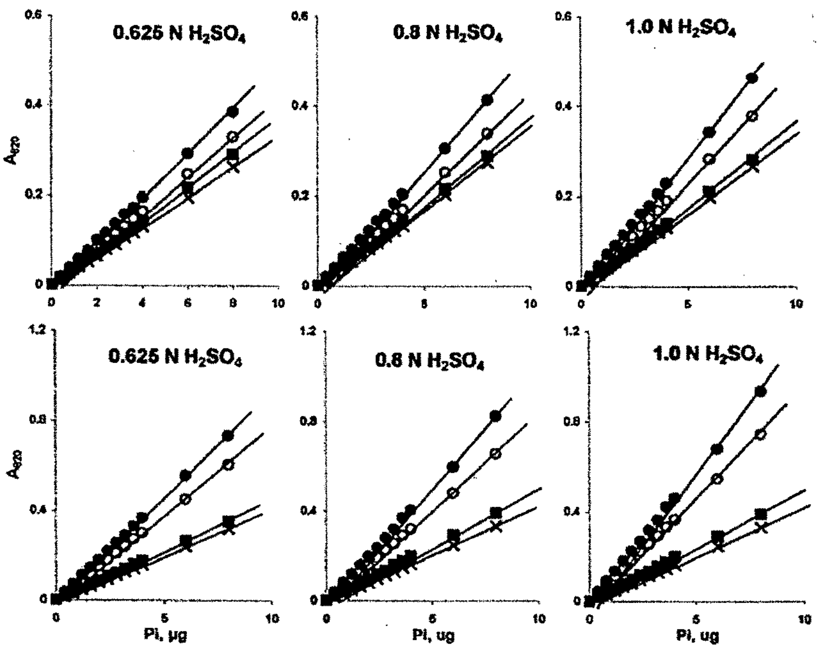

Fig. 4—Standard curves showing Pi concentration-dependent changes in absorbance at different time intervals [Pi concentration was 0-8 µg. Concentrations of H_2SO_4 were 0.625, 0.8 and 1.0 *N*. OD measurements were carried out at 660 and at 820 nm. Each data point represented average of 3 independent observations. Variations ranged from 2-3%. The scales are different for OD at 660 and 820 nm. (-x-), 15 min; (-■-), 1 h; (-o-), 12 h; and (-●-), 24 h]

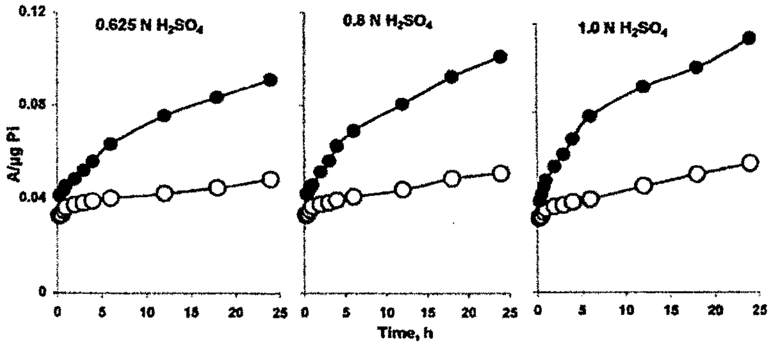

Fig. 5—Plots depicting changes in the slope (OD/µg Pi) with time [Color was developed using 4 µg Pi. Concentrations of H_2SO_4 were 0.625, 0.8 and 1.0 *N*. OD measurements at 660 nm and at 820 nm were carried out at specified time periods as indicated. Each data point represented average of 3 independent observations. Variation ranged from 2-3%. OD measurements at: 660 nm (-o-) and at 820 nm (-●-)]

concentration of 8 μg, under all the experimental conditions. However, the slopes increased with time. The changes in slope with time, monitored at 660 and 820 nm are shown in Fig. 5, which once again emphasized the efficacy of 1.0 *N* H_2SO_4.

The values of molar extinction coefficients (E_M) at different time intervals were computed based on the changes in the slope (Fig. 5) and the results are given in Table 1. Under the experimental conditions outlined above (Fig. 5), the E_M values at 660 or 820 nm did not change appreciably up to 1 h after development of the color and thereafter, the values increased steadily up to 24 h. The values of E_M determined at 660 nm for the three concentrations (0.625, 0.8 or 1.0 *N*) of H_2SO_4 were 1.46, 1.53 and 1.75-times higher, compared to that obtained in the conventional Fiske Subba Row method[1] ($E_M = 2914$) (Table 1). For measurements at 820 nm, these values (24 h) were 2.23, 2.42 and 2.81-times higher. This was also reflected in about 2-fold increase in the E_M at 820 nm at the end of 24 h, as compared to the corresponding values at 660 nm. Once again, 1 *N* H_2SO_4 was found to be more efficient in the color development.

Effect of H_2SO_4 concentration on substrate hydrolysis

Although the color development in the presence of 1.0 *N* H_2SO_4 (final conc.) was the most efficient in improving the sensitivity, it raised a concern with respect to applicability of the method in enzyme assays, where substrates such as ATP that contain acid labile phosphate was used. Therefore, it was pertinent to check the acid hydrolysis of the substrates under the assay conditions. Hence, the experiments were carried out to monitor hydrolysis of ATP (2 and 5 m*M*) and G6P (1 and 5 m*M*) in the presence of H_2SO_4 (0.5, 0.625, 0.8 and 1.0 *N*). The data for extent of hydrolysis of ATP with 0.5, 0.625 and 1.0 *N* H_2SO_4 are shown in Fig. 6. Results with 0.8 *N* H_2SO_4 showed an intermediate pattern (data not shown). The extent of ATP hydrolysis ranged from 0.08 to 1.51% at the end of 30 min for the three H_2SO_4 concentrations and at the end of 1 and 2 h, these values ranged from 0.4 to 3.4%, and 0.7 to 6.5% respectively. By 12 h, almost an equilibrium point was reached and the extent of hydrolysis ranged from 5.3 to 18.6%, depending upon the concentration of H_2SO_4 (Fig. 6). Hydrolysis of G6P was negligible (0.01-0.29%) under these conditions (data not shown).

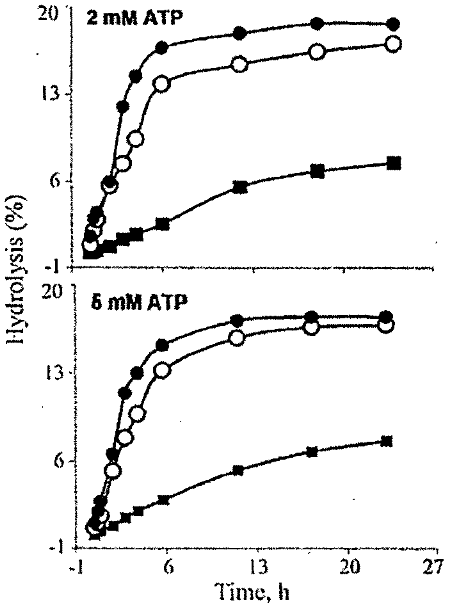

Fig. 6—Time course of ATP hydrolysis [Concentration of ATP was either 2.0 m*M* or 5.0 m*M*. Each data point represented average of 3 independent observations. Variation ranged from 2-3%. H_2SO_4 concentration was (-■-), 0.5; (-○-), 0.625 and (-●-), 1.0 *N*]

Table 1—Comparison of the sensitivity with three different concentrations of H_2SO_4 for determination of phosphorous by the molybdenum blue method

[Experimental details are as given in the text. The values of molar extinction coefficient (E_M) were computed from the data in Fig. 5. Each value represented the average of 3 independent observations. The value of E_M at 660 nm in conventional Fiske and Subba Row method[1] using 0.5 *N* H_2SO_4 at 0.25 h was 2914]

H_2SO_4 conc.	Wavelength (nm)	Molar extinction coefficient (E_M) at different time intervals, h										
		0.25	0.5	0.75	1	2	3	4	6	12	18	24
0.625 *N*	660	4030	4129	4302	4464	4600	4700	4811	4960	5232	5555	6002
	820	5109	5258	5406	5617	5989	6436	6907	7862	9350	10354	11358
0.8 *N*	660	4117	4166	4340	4526	4650	4737	4873	5047	5444	6026	6312
	820	5196	5282	5592	5679	6316	6956	7762	8544	9982	11470	12561
1.0 *N*	660	4067	4204	4402	4551	4687	4786	4948	5096	5853	6510	7105
	820	4997	5344	5754	6138	6932	7614	8469	9697	11371	12474	14049

Effect of sugars, salts and BSA

Inclusion of sugars (1-10 m*M*), salts (1-100 m*M*) or BSA (10-500 μg BSA/assay tube) at the concentrations indicated did not interfere with the color development or measurement of OD. Under these conditions, intensity of color development ranged from 96-103%, compared to the controls, where there was no addition of sugars, salts or BSA (data not shown).

Discussion

With the view of stabilizing the molybdenum blue color in the conventional phosphorus assay and improving the sensitivity, in the present study, we employed higher concentrations of H_2SO_4 than that was used in the conventional procedure[1]. Using the conventional procedure, we got the slope in the range of 0.022-0.025 OD units/μg Pi at 660 nm (averaged 0.0235 OD units/μg Pi; E_M 2914) and the color was stable maximally up to 15 min (data not shown). However, increased concentrations of H_2SO_4 stabilized the color as well as increased the sensitivity (Figs 1-5, Table 1). The sensitivity at 660 nm in the presence of 0.625 *N* H_2SO_4 was from 1.4-1.5 times higher between 15 min to 1 h period, compared to conventional value, and more than doubled at the end of 24 h. In terms of E_M values at 820 nm, the sensitivity was 1.8-1.9 times higher between 15 min to 1 h and increased to a 4 fold higher value at the end of 24 h (Fig. 5, Table 1). With 0.8 *N* H_2SO_4, sensitivity at 660 nm was from 1.4-1.6 times higher from 15 min up to 1 h period and increased to 2.2-fold at the end of the 24 h. For measurements at 820 nm, the sensitivity was 1.8-2.0 times higher between 15 min to 1 h and increased to 4.3-times higher value at the end of 24 h (Fig. 5, Table 1). In the presence of 1.0 *N* H_2SO_4, the sensitivity at 660 nm was 1.4-1.6 times higher between 15 min to 1 h and increased to 2.4-fold higher value at the end of 24 h. When the comparison was made with E_M values at 820 nm, the sensitivity was 1.7-2.1 times higher between 15 min to 1 h period and increased to a 4.8-fold higher at the end of 24 h (Fig. 5, Table 1).

The above results suggested that a further improvement in the sensitivity was achieved, if the measurements were carried out at 820 nm. Besides, the presence of 1.0 *N* H_2SO_4 had a marginal beneficial effect over 0.625 *N* H_2SO_4 at short time intervals i.e. up to 1 h. However, the use of higher concentrations of H_2SO_4 also raised a concern for applicability of the method in enzyme assays, especially where substrates such as ATP was used. Thus, we monitored the acid hydrolysis of ATP under the Pi assay conditions (Fig. 6). As can be noted, the extent of acid hydrolysis of ATP in the presence of 0.625 *N* H_2SO_4 during 15 min to 1 h period ranged from 0.7 to 3.4%. If the enzyme assay was carried out in 0.1 ml system and after terminating the reaction, if one used the entire assay medium for Pi determination in 4.0 ml assay, the background OD due to acid hydrolysis of ATP would get diluted 40-times and thus the substrate blank would increase only negligibly. Thus, the corresponding slope (Fig. 5) could be used to compute the amount of Pi released.

In Chen's[4] method, the color stability was achieved by removing excess molybdate by adding oxalic acid and extracting the color complex in isobutyl alcohol[4]. In the method of Linden *et al*[5], phophomolybdic acid was extracted with methyl isobutyl ketone and the measurement of molybdenum was carried out by atomic absorption spectrophotometry[5]. These procedures rendered the method cumbersome and time-consuming. Compared to these procedures, our method was simple, since no additional steps or extraction of the color complex was required for stabilizing the color and improving sensitivity. Our results also suggested that molybdenum blue absorbing in the region of 820-830 nm is the only stable species of the color.

In conclusion, the results of the present study demonstrated that Pi assays carried out in the presence of 0.625 *N* H_2SO_4 with OD measurements made at 820 nm increased the sensitivity. Further, it was also found that the inclusion of sugars, salts or protein such as BSA did not interfere with color development.

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Treatment with dehydroepiandrosterone (DHEA) stimulates oxidative energy metabolism in the liver mitochondria from developing rats

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Abstract

Effects of treatment with DHEA (0.2 mg or 1.0 mg / kg body weight for 7 days) on oxidative energy metabolism on liver mitochondria from developing and young adult rats were examined. Treatment with DHEA resulted in a progressive dose-dependent increase in the liver weights of the developing animals without change in the body weight. In the young adult rats treatment with 1.0 mg DHEA showed increase only in the body weight. Treatment with DHEA stimulated state 3 and state 4 respiration rates in developing as well as young adult rats in dose-dependent manner with all the substrates used; magnitude of stimulation was age-dependent. In young adults the extent of simulation of state 3 respiration rates declined at higher dose (1.0 mg) of DHEA with glutamate and succinate as substrates. Stimulation of state 3 respiration rates was accompanied by increase in contents of cytochrome aa_3, b and $c + c_1$ and stimulation of ATPase and dehydrogenases activities in dose- and age-dependent manner. (Mol Cell Biochem **293:** 193–201, 2006)

Key words: Dehydroepiandrosterone (DHEA); oxidative energy metabolism; cytochrome; ATPase activity; dehydrogenases; liver mitochondria.

Abbreviations: ADP adenosine diphosphate; DCIP dicholrophenolindophenol; DHEA dehydroepiandrosterone; EDTA disodium salt of ethylenediaminetetraacetic acid; MOPS 4-morpholinopropanesulfonic acid; TMPD N,N,N',N'-tetra methyl-*p*-phenylenediamine;

Introduction

The steroids dehydroepiandrosterone (DHEA) and its sulfated conjugate DHEA-S are secreted in highest amounts by the adrenal cortex in the adult humans [1, 2]. The plasma levels of DHEA are low in infancy and reach the highest value around the age of 20–35 years. However, the rate of synthesis and the plasma levels decline thereafter. In the elderly population the plasma levels of DHEA fall drastically to about 10% of the adult values. Based on this characteristic pattern, DHEA is considered to be a youth hormone [3, 4]. Effects of exogenous DHEA on several energy-linked functions in mitochondria have been reported [5–7]. Additionally, beneficial effects of DHEA in elderly population have been claimed [3, 4, 8]. However, since there are no known receptors for DHEA or DHEA-S the mechanism of action remains unclear [9].

Earlier studies from our laboratory had demonstrated that treatment of young adult male rats with DHEA for seven days significantly stimulated oxidative energy metabolism related parameters in the liver and brain mitochondria. The effects were dose-dependent and tissue- and substrates-specific [10].

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These results prompted us to examine the possibility as to whether the age-related pattern of DHEA levels relate to the development of mitochondrial function. To achieve this aim we treated the rats belonging to different age group viz. 2 week, 4 week and young adults with DHEA for one week and evaluated the effects on energy-linked functions. Our results show that treatment with DHEA stimulated the respiratory activity and/or accelerated the developmental process of maturation of mitochondrial function. These results are summarized in the present communication.

Materials and methods

Chemicals

Dehydroepiandrosterone (DHEA) (3β-Hydroxy-5-androsten-17-one (+)-dehydroisoandrosterone) was purchased from Sigma–Aldrich, USA. Sodium salt of L-glutamic acid was purchased from E Merck, Germany. Sodium salts of succinic acid, pyruvic acid, L-malic acid, ADP and rotenone,, bovine serum albumin fraction V (BSA), 4-morpholinopropanesulfonic acid (MOPS), dichlorophenolindophenol (DCIP), NAD$^+$, NADH, oxaloacetic acid and disodium salt of ethylenediaminetetraacetic acid (EDTA), were obtained from Sigma Chemical Co. USA. N,N,N',N'-Tetra methyl-*p*-phenylenediamine (TMPD) was purchased from British Drug Houses, Dorset, Poole, England. Ascorbic acid was from Sarabhai Chemicals, Vadodara. All other chemicals were of analytical-reagent grade and were purchased locally.

Animals and treatment with DHEA

Male albino rats of Charles–Foster strain, 2 week, 4 week, and young adults (8–10 week old) were used. At the start of experiments the initial body weight of the animals in the three aforementioned groups were 22.6 ± 0.22 g (range 22.2–23.2 g), 51.2 ± 0.49 g (range 46.6–52.3 g) and 236.6 ± 1.14 g (range 233.5–238.6 g), respectively. The animals were injected subcutaneously (s.c.) with 0.2 or 1.0 mg DHEA per kg body weight for seven consecutive days [10]. The controls received equivalent volume of saline vehicle. DHEA suspensions were prepared fresh in saline prior to use. The animals were sacrificed on the 8th day for isolation of mitochondria. Thus at the time of presentation of the results the ages of the developing animals were 3 and 5 weeks respectively.

Isolation of mitochondria

Isolation of liver mitochondria was essentially according to the procedure described previously with some modifications [11, 12]. Thus briefly, the animals were killed by decapitation and the liver was quickly removed and placed in beakers containing chilled (0–4°C) isolation medium which consisted of 250 mM sucrose containing 5 mM MOPS and 1 mM EDTA all at pH 7.4; 0.25 mg BSA /ml of isolation medium was included. Tissue was minced with a pair of scissors. The tissue was washed repeatedly with the isolation medium to remove adhering blood. The tissue was then homogenized using a Potter–Elvehjem type glass–Teflon homogenizer to obtain 10% (w/v) homogenate. Nuclei and cell debris were removed by centrifugation at 650 × g for 10 min in a Sorvall RC 5B plus centrifuge. The supernatant was subjected to further centrifugation at 7500 × g for 10 min to sediment the mitochondria. The mitochondria were washed once by gently suspending in the isolation medium and resedimenting and were finally suspended in the isolation medium to give a protein concentration in the range of 25–30 mg/ml . The post mitochondrial supernatant (S1) was subjected to further centrifugation at 12,000 × g for 10 min to sediment light mitochondria. The resultant supernatant (S2) was then centrifuged at 100,000 × g for 1 h to sediment the microsomal fraction which was discarded and the supernatant (S3) was used as the cytosolic fraction [13]. All the steps in the isolation procedure were performed at 0–4°C.

Oxidative phosphorylation

Measurements of oxidative phosphorylation were carried out at 25°C using a Clark-type oxygen electrode as described previously [11, 12, 14, 16]. The respiration medium (total volume 1.6 ml) consisted of 225 mM sucrose, 20 mM KCl, 10 mM MOPS pH 7.4, 5 mM potassium phosphate buffer pH 7.4, 0.2 mM EDTA and 160 μg of BSA (i.e. 0.1 mg BSA /ml). After adding mitochondria (4–8 mg protein depending on the substrate used) in the electrode chamber, respiration was induced by addition of substrates. Final concentrations of the substrates used were: glutamate (10 mM), pyruvate + malate (10 mM + 1 mM), succinate (10 mM) and ascorbate + TMPD (10 mM +0.1 mM). For measurements with the latter two substrates 1 μM rotenone was included. State 3 respiration rates initiated by the addition of 80–200 n moles of ADP and state 4 resipration rates ensuing after its depletion were recorded. Calculations of ADP/O ratio (n mole of ADP phosphorylated / n atom of oxygen consumed), ADP phosphorylation rates and Respiratory Control Ratio (RCR) were as described previously [14–17]. RCR is defined as the ratio of state 3 respiration rate / state 4 respiration rate [14–17].

Cytochrome content

The contents of cytochromes were estimated from the difference spectra as described previously [11, 12]. Briefly, 6–8 mg

Table 1. Effect of DHEA treatment on body weight and liver weight

		Body weight, g	Liver weight	
Age Group	Treatment	Final	g	% of body wt.
3 week	Untreated (24)	39.00 ± 0.87	1.33 ± 0.03	3.42 ± 0.04
	0.2 mg DHEA (24)	39.83 ± 0.60	1.44 ± 0.03^{a}	3.64 ± 0.08^{a}
	1.0 mg DHEA (24)	38.81 ± 0.71	1.58 ± 0.05^{b}	4.08 ± 0.11^{b}
5 week	Untreated (18)	79.59 ± 2.24	3.54 ± 0.10	4.47 ± 0.10
	0.2 mg DHEA (18)	79.57 ± 3.76	3.83 ± 0.12	4.92 ± 0.24
	1.0 mg DHEA (18)	79.96 ± 4.08	4.30 ± 0.15^{b}	5.38 ± 0.16^{b}
Young Adult	Untreated (12)	254.6 ± 5.28	8.90 ± 0.46	3.45 ± 0.07
	0.2 mg DHEA (12)	263.6 ± 8.03	9.10 ± 0.51	3.45 ± 0.06
	1.0 mg DHEA (12)	281.9 ± 7.93^{a}	9.60 ± 0.56	3.41 ± 0.06

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.

a, $p < 0.02$; and b, $p < 0.001$ compared with the corresponding untreated group.

mitochondrial protein (in potassium phosphate buffered sucrose) was solubilized by adding 0.25 ml of 10% Triton X 100. The total volume was made up to 2.5 ml. The sample was then transferred to two 1 ml cuvettes. The sample in the reference cuvette was oxidized by adding small amount of potassium ferricyanide and the sample in the experimental cuvette was reduced by adding a few mg of sodium dithionite. The difference spectra of reduced versus oxidized samples were recorded in a JASCO UV/VIS spectrophotometer model V-530. The contents of cytochromes aa_3, b and $c + c_1$ were calculated using the wavelength pairs 604–624, 559–580 and 535–552 nm and millimolar extinction coefficients 24, 23.4 and 18.7 respectively [18].

Assay of dehydrogenases

Measurements of glutamate dehydrogenase (GDH) activity were carried out spectrophotometrically at 25°C in the assay system (1 ml) consisting of 125 mM potassium phosphate buffer pH 7.4, 10 mM sodium glutamate, 0.1% Triton X 100 and 100–200 μg of mitochondrial protein as the source of the enzyme. After incubating at 25°C for 1 min the reaction was initiated by the addition of 1.5 mM NAD^+ and the linear rate of reaction was recorded at 5 sec interval by monitoring the increase in the absorbance at 340 nm [19].

Malate dehydrogenase (MDH) activity was measured in assay system (1 ml) containing 125 mM potassium phosphate buffer pH 7.4, 2.5 mM oxaloacetate, 0.1% Triton X 100 and 5–15 μg of mitochondrial or cytosolic protein. After preincubating at 25°C for 1 min the reaction was initiated by the addition of 1.5 mM NADH and the linear rate of reaction was recorded at 5 sec interval by monitoring the decrease in the absorbance at 340 nm [20].

Measurements of succinate DCIP reductase (SDR) activity were carried out employing the assay system (1 ml) containing 125 mM potassium phosphate buffer pH 7.4, 0.1 mM sodium azide, 15 mM sodium succinate and 100–300 μg of mitochondrial protein as the source of the enzyme. After incubating at 25°C for 1 min the reaction was initiated by the addition of 10 μM DCIP and the decrease in the absorbance at 600 nm was recorded at 5 sec interval [21].

Assay of ATPase

The ATPase activity was measured in 0.1 ml assay medium containing 50 mM MOPS pH 7.4, 75 mM KCl and 0.4 mM EDTA. The assays were performed in the absence and presence of 6 mM $MgCl_2$ and 100 μM DNP, or a combination of both. After pre-incubating the mitochondrial protein (40–60 μg) in the assay medium at 37°C for 1 min, the reaction was initiated by the addition of ATP at the final concentration of 5 mM. The reaction was terminated after 15 min by the addition of 0.1 ml of 5% (w/v) sodium dodecyl sulfate (SDS) [11, 12, 16, 22]. Estimation of released inorganic phosphate was according to the procedure described previously [22].

Protein estimation was according to the method of Lowry *et al.* using bovine serum albumin as the standard [23].

Results are given as mean ± SEM.

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

Results

Data in Table 1 show that in the 3 week group at the end of the treatment period the final body weight of the DHEA treated animals was comparable to untreated controls. However, the liver weight showed a dose-dependent 8% and 19% increase. In the 5 week animals also the body weight was unchanged by DHEA treatment but the liver weight showed a dose-dependent 8 and 22% increase. In the young adult

Table 2. Effect of DHEA treatment on oxidative phosphorylation in rat liver mitochondria using glutamate as the substrate

Age Group	Treatment	ADP/O ratio	Respiration rate (n mole O_2/ min / mg protein) + ADP	Respiration rate (n mole O_2/ min / mg protein) –ADP	Respiratory Control Ratio	ADP phosphorylation rate (n mole / min / mg protein)
3 week	Untreated (6)	3.12 ± 0.16	13.46 ± 0.77	5.68 ± 0.37	2.41 ± 0.21	84.07 ± 6.72
	0.2 mg DHEA (9)	3.11 ± 0.09	16.18 ± 0.89^a	7.60 ± 0.49^b	2.20 ± 0.18	101.17 ± 7.00
	1.0 mg DHEA (8)	3.06 ± 0.18	18.23 ± 0.35^d	9.16 ± 0.56^d	2.00 ± 0.11	111.90 ± 8.32^a
5 week	Untreated (9)	3.11 ± 0.16	17.63 ± 0.94	6.87 ± 0.34	2.59 ± 0.13	110.80 ± 9.23
	0.2 mg DHEA (14)	3.09 ± 0.13	21.09 ± 1.38	8.74 ± 0.74^a	2.52 ± 0.14	128.30 ± 7.40
	1.0 mg DHEA (9)	3.22 ± 0.11	24.99 ± 1.40^d	9.24 ± 0.57^b	2.77 ± 0.19	160.90 ± 10.30^b
Young Adult	Untreated (12)	3.11 ± 0.10	28.87 ± 1.24	10.77 ± 0.43	2.70 ± 0.10	179.70 ± 9.39
	0.2 mg DHEA (12)	3.22 ± 0. 14	37.00 ± 1.84^c	13.60 ± 0.73^b	2.73 ± 0.05	236.10 ± 11.13^b
	1.0 mg DHEA (12)	3.18 ± 0.11	33.10 ± 0.81^b	18.08 ± 1.13^d	1.91 ± 0.12^d	210.20 ± 7.78

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.
a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.002$ and d, $p < 0.001$ compared with the corresponding untreated group.

animals treatment with 1.0 mg DHEA resulted in about 19% increase in body weight. However, DHEA treatment had practically no effect on the liver weight. These observations are consistent with our earlier reported observations [10].

Oxidative phosphorylation

General

In the untreated controls the state 3 respiration rates with glutamate increased progressively with age. Thus compared to the 3 week animals in the young adults the state 3 respiration rate had almost doubled. A similar trend was seen for state 4 respiration rates. The state 3 respiration rate with pyruvate + malate was relatively very low in 3 week pups, increased 3.6 fold by the 5th week of life, and in the young adults the increase amounted to 4.4 folds. Generally a similar trend was seen for state 4 respiration rates. With succinate as the substrate, compared to the three week group, the increase in state 3 respiration rate was about 1.63 and 2.1 fold respectively in the 5week old and the young adult animals. State 4 respiration rate had already reached young adult value by 5th week. For ascorbate + TMPD as the electron donor system the state 3 respiration rate increased marginally (19% increase) by the 5th week; in the young adults the rate had tripled. A similar trend was seen for state 4 respiration rate (Tables 2–5). We have noted similar trend earlier for rat and mouse liver and rat brain mitochondria [12, 18, 24–26].

The contents of cytochrome aa_3, b and $c+c_1$ increased progressively up to the young adult stage (Table 6). A similar trend was seen even for the ATPase activities (Table 7). The GDH activity also increased progressively with age whereas mitochondrial MDH activity increased only by 59% in the

Table 3. Effect of DHEA treatment on oxidative phosphorylation in rat liver mitochondria using pyruvate + malate as the substrate

Age Group	Treatment	ADP/O ratio	Respiration rate (n mole O_2/ min / mg protein) + ADP	Respiration rate (n mole O_2/ min / mg protein) –ADP	Respiratory Control Ratio	ADP phosphorylation rate (n mole / min / mg protein)
3 week	Untreated (7)	3.01 ± 0.14	3.56 ± 0.21	2.67 ± 0.19	1.32 ± 0.06	21.29 ± 1.94
	0.2 mg DHEA (8)	3.02 ± 0.11	4.80 ± 0.26^c	3.07 ± 0.26	1.63 ± 0.09^b	28.47 ± 1.40^b
	1.0 mg DHEA (9)	3.25 ± 0.17	5.98 ± 0.34^c	3.52 ± 0.26^b	1.76 ± 0.14^b	39.09 ± 2.45^e
5 week	Untreated (9)	3.22 ± 0.13	12.69 ± 0.41	6.59 ± 0.58	2.05 ± 0.11	81.43 ± 3.72
	0.2 mg DHEA (7)	3.05 ± 0.10	14.47 ± 0.95	7.52 ± 0.43	1.95 ± 0.09	87.82 ± 5.87
	1.0 mg DHEA (9)	3.07 ± 0.15	16.97 ± 1.09^d	10.29 ± 0.96^c	1.75 ± 0.08^a	102.90 ± 6.29^c
Young Adult	Untreated (12)	3.19 ± 0.07	15.82 ± 0.59	7.33 ± 0.21	2.17 ± 0.07	101.00 ± 4.36
	0.2 mg DHEA (12)	3.05 ± 0.08	17.74 ± 0.60^a	9.51 ± 0.32^c	1.87 ± 0.03^c	107.90 ± 4.39
	1.0 mg DHEA (12)	3.16 ± 0.06	30.97 ± 1.93^d	16.37 ± 1.11^e	1.94 ± 0.09	195.40 ± 12.19^e

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.
a, $p < 0.05$; b, $p < 0.02$; c, $p < 0.01$, d, $p < 0.002$ and e, $P < 0.001$ compared with the corresponding untreated group.

Table 4. Effect of DHEA treatment on oxidative phosphorylation in rat liver mitochondria using succinate as the substrate

			Respiration rate (n mole O_2/ min / mg protein)			
Age Group	Treatment	ADP/O ratio	+ ADP	–ADP	Respiratory Control Ratio	ADP phosphorylation rate (n mole / min / mg protein)
3 week	Untreated (8)	2.26 ± 0.12	27.09 ± 1.20	13.98 ± 1.06	1.96 ± 0.12	120.2 ± 6.64
	0.2 mg DHEA (9)	2.24 ± 0.13	30.00 ± 1.06	16.36 ± 0.62	1.85 ± 0.08	133.5 ± 6.54
	1.0 mg DHEA (6)	2.19 ± 0.19	32.95 ± 2.33^{a}	17.71 ± 0.88^{b}	1.87 ± 0.13	143.8 ± 5.01^{b}
5 week	Untreated (9)	2.21 ± 0.11	44.18 ± 2.01	21.59 ± 1.57	2.13 ± 1.43	183.3 ± 7.90
	0.2 mg DHEA (8)	2.14 ± 0.17	44.22 ± 1.87	23.36 ± 1.62	1.96 ± 0.12	187.5 ± 10.90
	1.0 mg DHEA (9)	2.23 ± 0.16	52.07 ± 3.05^{a}	29.09 ± 1.29^{d}	1.84 ± 0.09	230.2 ± 12.02^{c}
Young Adult	Untreated (12)	2.38 ± 0.08	56.43 ± 2.00	21.64 ± 1.05	2.67 ± 0.11	267.1 ± 11.71
	0.2 mg DHEA (12)	2.39 ± 0.09	71.31 ± 2.15^{e}	36.99 ± 2.51^{e}	2.08 ± 0.15^{c}	340.7 ± 16.77^{d}
	1.0 mg DHEA (12)	2.35 ± 0.09	55.23 ± 2.34	33.79 ± 1.91^{e}	1.69 ± 0.08^{e}	259.7 ± 15.01

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.
a, $p < 0.05$; b, $p < 0.02$; c, $p < 0.01$, d, $p < 0.002$ and e, $P < 0.001$ compared with the corresponding untreated group.

Table 5. Effect of DHEA treatment on oxidative phosphorylation in rat liver mitochondria using ascorbate + TMPD as the substrate

			Respiration rate (n mole O_2/ min / mg protein)			
Age Group	Treatment	ADP/O ratio	+ ADP	–ADP	Respiratory Control Ratio	ADP phosphorylation rate (n mole / min / mg protein)
3 week	Untreated (11)	0.42 ± 0.02	9.52 ± 0.31	6.05 ± 0.43	1.55 ± 0.03	8.10 ± 0.62
	0.2 mg DHEA (12)	0.41 ± 0.03	16.26 ± 1.19^{d}	11.43 ± 0.75^{d}	1.42 ± 0.05^{a}	13.23 ± 1.15^{d}
	1.0 mg DHEA (12)	0.41 ± 0.02	26.15 ± 0.99^{d}	17.18 ± 0.91^{d}	1.81 ± 0.06^{a}	21.14 ± 1.46^{d}
5 week	Untreated (11)	0.42 ± 0.03	11.33 ± 0.81	8.20 ± 0.75	1.42 ± 0.04	9.12 ± 0.45
	0.2 mg DHEA (11)	0.41 ± 0.02	30.80 ± 1.93^{d}	21.24 ± 1.54^{d}	1.47 ± 0.05	24.34 ± 1.33^{d}
	1.0 mg DHEA (11)	0.43 ± 0.03	39.65 ± 2.96^{d}	25.37 ± 1.06^{d}	1.61 ± 0.11	31.05 ± 1.52^{d}
Young Adult	Untreated (12)	0.43 ± 0.03	28.34 ± 1.38	22.04 ± 1.16	1.30 ± 0.03	23.72 ± 1.41
	0.2 mg DHEA (12)	0.41 ± 0.02	36.51 ± 1.15^{d}	27.34 ± 1.38^{c}	1.39 ± 0.06	29.74 ± 1.58^{c}
	1.0 mg DHEA (12)	0.43 ± 0.02	44.88 ± 1.44^{d}	30.50 ± 1.33^{d}	1.52 ± 0.08^{b}	38.94 ± 2.40^{d}

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.
a, $p < 0.05$; b, $p < 0.02$; c, $p < 0.01$ and d, $p < 0.001$ compared with the corresponding untreated group.

Table 6. Effect of DHEA treatment on the cytochrome content of rat liver mitochondria

			Cytochrome content (pmoles / mg protein)		
Age Group	Treatment		aa_3	b	$c + c_1$
3 week	Untreated	(12)	102.7 ± 3.02	154.2 ± 4.51	202.9 ± 5.91
	0.2 mg DHEA	(12)	153.1 ± 5.36^{c}	176.6 ± 7.55^{a}	362.2 ± 9.07^{c}
	1.0 mg DHEA	(12)	138.8 ± 6.19^{c}	169.4 ± 3.26^{a}	283.6 ± 3.99^{c}
5 week	Untreated	(18)	138.8 ± 6.19	199.8 ± 8.60	281.7 ± 11.70
	0.2 mg DHEA	(18)	162.6 ± 11.21	303.9 ± 14.35^{c}	442.2 ± 21.87^{c}
	1.0 mg DHEA	(15)	177.1 ± 9.89^{b}	291.1 ± 15.74^{c}	397.4 ± 18.28^{c}
Young Adult	Untreated	(10)	136.0 ± 2.46	278.7 ± 7.77	320.0 ± 14.21
	0.2 mg DHEA	(10)	166.3 ± 3.88^{c}	372.7 ± 11.83^{c}	394.0 ± 16.73^{b}
	1.0 mg DHEA	(10)	153.4 ± 5.83^{a}	339.9 ± 20.70^{c}	327.4 ± 16.00

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.
a, $p < 0.02$; b, $p < 0.01$; and c, $p < 0.001$ compared with the corresponding untreated group.

Table 7. Effect of DHEA treatment on ATPase activity in rat liver mitochondria

		Activity (μmole Pi liberated/hr/mg protein)			
Age Group	Treatment	Basal	$+Mg^{2+}$	+DNP	$+Mg^{2+}$ +DNP
3 week	Untreated (12)	0.96 ± 0.24	2.16 ± 0.18	7.42 ± 0.22	7.78 ± 0.19
	0.2 mg DHEA (12)	2.41 ± 0.20^d	2.96 ± 0.11^c	13.37 ± 0.74^d	12.21 ± 0.50^d
	1.0 mg DHEA (12)	3.83 ± 0.26^d	3.44 ± 0.21^d	15.18 ± 0.66^d	14.01 ± 0.72^d
5 week	Untreated (12)	1.56 ± 0.09	2.67 ± 0.11	9.31 ± 0.74	9.44 ± 0.36
	0.2 mg DHEA (12)	3.80 ± 0.34^d	3.09 ± 0.16^a	10.27 ± 0.52	9.59 ± 0.58
	1.0 mg DHEA (12)	2.21 ± 0.12^d	3.26 ± 0.19^b	14.94 ± 0.62^d	11.56 ± 0.71^b
Young Adult	Untreated (12)	1.94 ± 0.08	5.43 ± 0.26	17.89 ± 0.87	20.08 ± 0.71
	0.2 mg DHEA (12)	4.63 ± 0.04^d	8.92 ± 0.65^d	31.32 ± 1.18^d	32.48 ± 1.09^d
	1.0 mg DHEA (12)	4.15 ± 0.22^d	8.54 ± 0.66^d	24.93 ± 0.58^d	21.83 ± 0.94

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses

a, $p < 0.05$; b, $p < 0.02$; c, $p < 0.01$ and d, $p < 0.001$ compared with the corresponding untreated group

young adults. As against this the cytosolic MDH activity increased progressively with age. Increase in SDR activity amounted to 1.75 and 2.36 folds under these conditions (Fig 1).

Effects of DHEA treatment

Treatment with DHEA resulted in progressive increase in state 3 respiration rate with glutamate in a dose-dependent manner. Thus in 3 week pups after treatment with DHEA state 3 respiration rate became comparable to that in the 5 week untreated animals. Likewise when the 5 week animals were treated with DHEA, the state 3 respiration rate was stimulated by 20% and 42% respectively by the two dose regimens employed. Interestingly, at higher dose (1.0 mg) of DHEA the value was very close to that in the untreated young adults. A parallel trend was seen even for state 4 respiration rates. In the young adult rats 0.2 mg dose proved to be more efficacious (Table 2) which is consistent with our earlier observation [10]. DHEA treatment was less effective

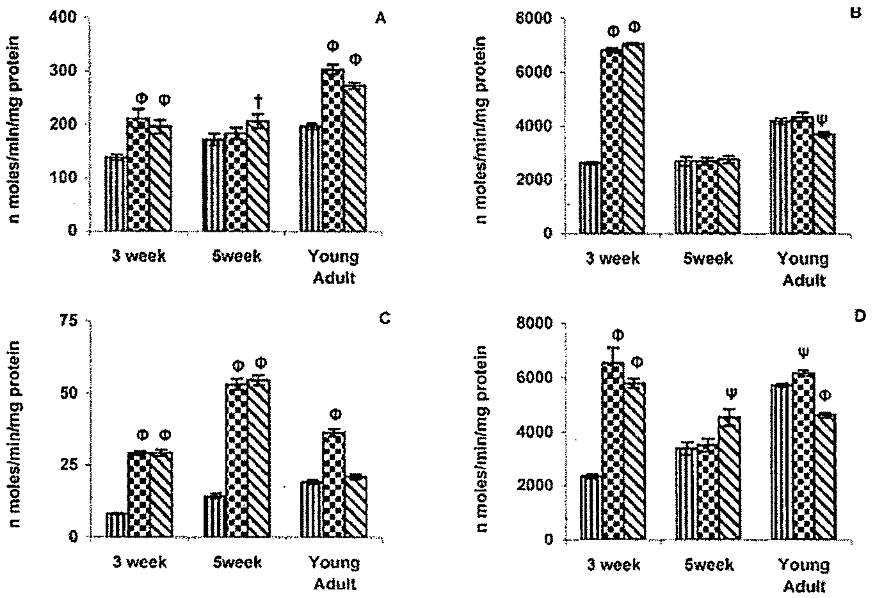

Fig. 1. Effect of DHEA treatment on mitochondrial and cytosolic dehydrogenases activities in rat liver. The results are given as mean ± SEM of 12 independent observations. A, Glutamate dehydrogenase; B, Malate dehydrogenase (Mitochondrial); C, Succinate DCIP reductase and D, Malate dehydrogenase (cytosolic); ▥, Untreated; ▩, 0.2 mg DHEA and ▧, 1.0 mg DHEA; †, p < 0.05; ψ, p < 0.01 and Φ, p < 0.001 compared with the corresponding untreated group.

in improving state 3 or state 4 respiration rates with pyruvate + malate in 3 week pups. Although the respiratory activity increased significantly, the values were substantially low compared to those in 5 week untreated controls. By contrast, in the 5 week group treatment with both the doses of DHEA increased the state 3 as well as state 4 respiration rates near or above the values for untreated young adults. In the case of the young adults the state 3 as well as state 4 respiration rates almost doubled only by 1.0 mg dose of DHEA (Table 3). For succinate, only 1.0 mg DHEA treatment was effective in stimulating the state 3 and state 4 respiration rates. Especially, in the 5 week animals the values became comparable to untreated control. In the young adults, 0.2 mg DHEA treatment only had stimulatory effect on state 3 respiration rate (Table 4) which is consistent with our earlier observations [10]. With ascorbate + TMPD system, treatment with DHEA caused a progressive dose-dependent stimulation of respiratory activities. Interestingly, the magnitude of increase was always higher in developing animals. Thus in 3 week group the state 3 respiration rates increased by 1.71 and 2.75 fold while state 4 respiration rates increased by 1.89 and 2.84 fold. In 5 week group the increase amounted to 2.73 and 3.5 fold and 2.59 and 3.10 fold respectively for the state 3 and state 4 respiration rates. Under these experimental conditions the increase in state 3 and state 4 respiration rates in young adult group ranged from 24–58% (Table 5).

DHEA treatment also resulted in significant increase in the contents of all the cytochrome. The lower dose of DHEA (0.2 mg) was more effective in 3 week group. In the 5 week animals both the doses were more or less equally effective. In the young adults only 0.2 mg dose had a greater stimulatory effect (Table 6). DHEA treatment in general resulted in increase in the basal and Mg^{2+}-dependent ATPase activities in the developing animals. Under these conditions the DNP- and Mg^{2+}+DNP-dependent ATPase activities were also stimulated maximally with the effect being more pronounced in 3 week group. In the young adults also all the ATPase activities increased significantly (Table 7).

DHEA treatment brought the GDH values in developing animals close to untreated young adults, whereas the mitochondrial MDH activity increased by 2.6 and 2.9 fold only in the 3 week animals. In the 3 week pups DHEA treatment caused 3.6 fold increases in SDR activity. A similar 3.73 and 3.83 fold increase was seen even in the 5 week group. In the young adults only 0.2 mg DHEA treatment caused about 2.4 fold increase in SDR activity. The DHEA treatment also significantly stimulated cytosolic MDH activity in 3 week group; only 1.0 mg dose was effective in increasing the cytosolic MDH activity in 5 week animals. By contrast, this dose had a negative effect in the young adults where only a marginal increase of 11% was seen with 0.2 mg dose (Fig. 1).

Discussion

The present studies were initiated to find out if treatment with exogenous DHEA can accelerate the process of mitochondrial maturation and development in the developing animals. From the data presented this seems to be indeed the case. Thus treatment with DHEA regimen was able to stimulate the respiration rates with all the substrates employed bringing them closer to or beyond the next higher age group. Under these conditions the ADP/O ratios were not altered (Tables 2–5) which is consistent with our earlier observations [10] In this connection it is interesting to note that earlier we have observed that treatment with exogenous corticosterone or synthetic glucocorticoid, dexamethasone resulted in significant uncoupling of oxidative phosphorylation in rat liver and brain mitochondria [12, 24, 25]. Therefore the action of DHEA should be considered as unique because it stimulated respiration rates without affecting the ADP/O ratios thereby increasing the bioenergetics potential of mitochondria. This is very well reflected in the increase in the ADP-phosphorylation rates (Tables 2–5). As is well recognized, all the developmental processes are energy dependent [27–29]. Viewed in this context, the effects of DHEA treatment seem to be beneficial and conducive to development and maturation of mitochondrial functions.

The enhancement of respiratory functions seems to be aided by increase in the contents of cytochromes. It may be pointed out here that DHEA treatments were more effective in increasing the contents of cytochromes aa_3 and $c + c_1$ in the 3 week animals. In the 5 week animals the effect was more pronounced on the increases in the content of cytochromes b and $c + c_1$. The most important aspect was that the levels of cytochromes became comparable to untreated young adults or increased beyond these values. In the young adults, although contents of cytochromes increased, the increase was of lesser magnitude (Table 6). The observed enhancement in respiration rates was also accompanied by significant increases in the dehydrogenase activities (Fig. 1). Interestingly, DHEA treatments also stimulated the ATPase activity in the developing animals which became somewhat comparable to the untreated young adults values (Table 7). However, most substantial stimulatory effect of DHEA treatment was evident only in the young adults.

It is well recognized that the mitochondrial dehydrogenases and cytochrome $c + c_1$ are coded by the nuclear genes whereas crucial peptides of cytochromes aa_3 and b, as well as ATPase are coded by mitochondrial DNA [30]. The differential stimulatory effects of DHEA treatment on rates of substrate oxidation and on increase in the contents of cytochromes which we observe here seem to be consistent with age-related profile of DHEA synthesis and plasma levels of DHEA [3, 31]. In other words the results of our present studies suggest that DHEA may play crucial role in activating specific mitochondrial and nuclear genes during mitochondrial development and maturation.

The mechanism of action of DHEA still remains unclear since there are no known receptors of DHEA [9]. However, recently it has been shown that DHEA is metabolized to 7α hydroxy DHEA and δ 5 androstene 3 β, 17 β diol [32, 33]; the former is considered to be active metabolite [32, 33]. Thus based on our studies it may be suggested that DHEA and or its active metabolite may influence the process of development and/or aging by activation or silencing specific nuclear and/or mitochondrial genes in an age-dependent manner. It has been shown that in aging mice in the initial stages there is up-regulation of genes encoding mitochondria complex I, III, IV and V which is followed by down-regulation at the later stage [34]. It may hence be suggested that DHEA may play an age-dependent role in gene regulation in development and aging. However, further experiments are needed to verify and substantiate this possibility.

It has been shown that DHEA increases synthesis of nitric oxide (NO) by stimulating inducible NO synthase ($_i$NOS) especially in the endothelial cells [35] although inhibitory effects in BV-2 microglia have also been reported [36]. It is unlikely that the enhanced respiration rates together with increased contents of cytochromes and increased levels of dehydrogenases as well as ATPase activities which we observe here could have been mediated via NO. Especially of interest to note here is the recent finding that NO binds at the active site of cytochrome oxidase and modulates its activity [37, 38]. DHEA has been shown to afford protection from oxidative stress [39]. However, implication of the same in the process of maturation of mitochondrial function during development is unclear at this stage.

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Dehydroepiandrosterone (DHEA) treatment stimulates oxidative energy metabolism in the cerebral mitochondria from developing rats

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Abstract

Effects of treatment with dehydroepiandrosterone (DHEA) (0.2 or 1.0 mg/kg body weight for 7 days) on oxidative energy metabolism in cerebral mitochondria from developing and young adult rats were examined. Treatment with DHEA did not change the body weight of developing rats but resulted in increase in the brain weight in 5 week group. In young adult rats the body weight increased following treatment with 1.0 mg DHEA. State 3 and state 4 respiration rates with all the substrates increased following DHEA treatment, the effect being more pronounced in the developing rats. State 4 respiration rates were stimulated to variable extents. Contents of cytochromes aa_3 and b increased following DHEA treatment and once again the effect was more pronounced in the developing rats. DHEA treatment marginally changed the content of cytochromes $c + c_1$. In the developing rats the ATPase activity and the levels of dehydrogenases increased significantly by DHEA treatment.

Results of our studies have shown that treatment with exogenous DHEA accelerates the process of maturation of cerebral mitochondria thus emphasizing the role of DHEA in brain development in postnatal life.

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Keywords: Dehydroepiandrosterone (DHEA); Oxidative energy metabolism; Cytochromes; ATPase activity; Dehydrogenases; Brain mitochondria

1. Introduction

Dehydroepiandrosterone (DHEA) and its sulfated ester DHEAS are synthesized in the highest quantities by the young adult human adrenals (Milgrom, 1990). The plasma levels of DHEA show a characteristic age-dependent pattern. The levels are low in infancy, start increasing in adolescence and reach maximum values around the age of 20–30 years (Hinson and Raven, 1999; Buvat, 2003; Celec and Starka, 2003). The levels decline thereafter and in the elderly individuals the levels fall to about 10% of the young adult values (Hinson and Raven, 1999; Parker, 1999). Based on this characteristic pattern DHEA is considered to be a youth hormone (Hinson and Raven, 1999; Buvat, 2003; Celec and Starka, 2003). Significant quantities of DHEA are also present in the human as well as the rat brain although the levels are lower for the rat (Corpechot et al., 1981; Vallee et al., 2000; Steckelbroeck et al., 2002; Racchi et al., 2003; Ren and Hou, 2005). Also, age-dependent decline in the content of DHEA in the brain has been reported (Weill-Engerer et al., 2002; Kazihnitkova et al., 2004). While the brain derives its DHEA partly from the adrenals, the brain itself is capable of synthesizing DHEA and DHEAS as well as pregnenolone (Racchi et al., 2003). Hence, DHEA, DHEAS and pregnenolone are considered to be neurosteroids (Racchi et al., 2003).

Although DHEA and DHEAS are synthesized in the highest quantities, there are no known receptors for DHEA (Milgrom, 1990). The role of DHEA is thus generally unclear except for its being a precursor of sex steroids (Milgrom, 1990). Interestingly, however, it has been shown that when the experimental animals are fed diets supplemented with DHEA, this results in proliferation of mitochondria in the liver and stimulation of mitochondrial functional parameters (Min Kyung et al., 1989; Bellei et al., 1992).

In the rat the respiratory activity of cerebral mitochondria shows a characteristic pattern of maturation. Thus, the respiratory activity is low in the early neonatal life but increases significantly in the postnatal period up to the age of 5 weeks where the values reach those of young adults (Milstein et al., 1968; Holtzman and Moore, 1975; Rajan and Katyare, 1991; Katyare et al., 2003). This offers an opportunity to evaluate the role of DHEA in regulating postnatal development and maturation of the cerebral mitochondria using a rat model. With this objective in mind we carried out experiments to examine in details if exogenous supplementation with DHEA in

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the neonatal rat can accelerate the process of maturation of cerebral mitochondria. To achieve this objective we treated developing rats with DHEA and examined the effects on the respiratory parameters, in comparison with young adult rats treated in the similar manner. Our results show that treatment with DHEA significantly stimulated the process of functional maturation of the cerebral mitochondria in the developing animals. The results of these investigations are summarized in the present communication.

2. Materials and methods

2.1. Chemicals

Dehydroepiandosterone (3β-hydroxy-5-androsten-17 one (+) dehyroisoandrosterone) was obtained from Sigma–Aldrich, USA. Sodium salts of succinic acid, pyruvic acid, L-malic acid, ADP and rotenone, bovine serum albumin fraction V (BSA), 4-morpholinopropanesulfonic acid (MOPS), dichlorophenolindophenol (DCIP), NAD^+, NADH, oxaloacetic acid, disodium salt of ethylenediaminetetraacetic acid (EDTA), were from Sigma Chemical Co., USA. Sodium salt of L-glutamic acid was obtained from E Merck, Germany. *N,N,N′,N′*-Tetra methyl-*p*-phenylenediamine (TMPD) was purchased from British Drug Houses, Dorset, Poole, England. Ascorbic acid was from Sarabhai Chemicals, Vadodara. All other chemicals were of analytical-reagent grade and were purchased locally.

2.2. Animals and treatment with DHEA

Male albino rats of Charles–Foster strain, 2 and 4 weeks and young adults (8–10 weeks old) were used. At the start of the experiments the initial body weight of the animals in the individual age group were matched (e.g. see Table 1). The animals were injected subcutaneously (s.c.) with 0.2 or 1.0 mg DHEA/kg body weight for 7 consecutive days This dose regimen is based on our earlier dose–response studies in young adult rats where it was observed that the optimum effects on mitochondria were elicited at these doses in a parameter-dependent manner (Patel and Katyare, submitted for publication). Suspensions of DHEA were prepared fresh in saline prior to use. The controls received equivalent volume of saline vehicle. The animals were sacrificed on the 8th day for isolation of mitochondria. Thus, at the time of presentation of the results the age of the developing animals was 3 and 5 weeks, respectively. In a given batch, depending on the age group 12–24 animals were used.

2.3. Isolation of mitochondria

Isolation of brain mitochondria was essentially according to the procedure described previously (Katyare et al., 1977; Swegert et al., 1999) with some modifications employing the isolation medium consisting of 250 mM sucrose containing 5 mM MOPS and 1 mM EDTA all at pH 7.4; 0.25 mg BSA/ml of isolation medium was included. The mitochondria were washed once by gently suspending in the isolation medium and resedimenting and were finally suspended in the isolation medium to give a protein concentration in the range of 10–15 mg/ml. All the steps in the isolation procedure were performed at 0–4 °C. Tissues from two animals from 3 and 5 week groups were pooled to obtain a single preparation whereas brain tissue from one young adult animal provided sufficient mitochondrial material.

2.4. Oxidative phosphorylation

Measurements of oxidative phosphorylation were carried out at 25 °C using a Clark-type oxygen electrode employing the respiration medium (total volume 1.6 ml) comprising 225 mM sucrose, 20 mM KCl, 10 mM MOPS, pH 7.4, 5 mM potassium phosphate buffer, pH 7.4, 0.2 mM EDTA and 160 μg of BSA (i.e. 0.1 mg BSA/ml) as described previously (Swegert et al., 1999). After introducing the required quantity of mitochondria (4–8 mg protein depending on the substrate used) in the electrode chamber, respiration was initiated by the addition of the substrates. State 3 respiration rates induced by the addition of 80–200 *n* moles of ADP and state 4 rates ensuing after its depletion were recorded. Calculations of ADP/O ratio and ADP phosphorylation rates were as described previously (Katewa and Katyare, 2004; Pandya et al., 2004).

2.5. Cytochrome content

The contents of cytochromes were quantified from the difference spectra of sodium dithionite reduced versus potassium ferricyanide oxidized samples as described previously (Katewa and Katyare, 2004; Pandya et al., 2004). The difference spectra were recorded in a JASCO UV/VIS spectrophotometer model V-530. The contents of cytochromes aa_3, b and $c + c_1$ were calculated using the wavelength pairs 604–624, 559–580 and 535–552 nm and millimolar extinction coefficients 24, 23.4 and 18.7, respectively (Subramaniam and Katyare, 1990).

2.6. Assay of dehydrogenases

Glutamate dehydrogenase (GDH), malate dehydrogenase (MDH) and succinate DCIP reductase (SDR) activities were determined according to the procedures described (Ochoa, 1955; King, 1967; Leighton et al., 1968).

2.7. Assay of ATPase

The ATPase activity was measured in the assay medium (total volume 0.1 ml) containing 350 mM sucrose, 10 mM MOPS, pH 7.4, 10 mM KCl and 0.2 mM EDTA. The assays were performed in the absence and presence of 2 mM $MgCl_2$ and 50 μM DNP or a combination of both. After preincubating the mitochondrial protein (40–60 μg) in the assay medium at 37 °C for 1 min the reaction was initiated by the addition of ATP at the final concentration of

Table 1
Effect of DHEA treatment on body weight and brain weight

Age group	Treatment	Body weight (g)		Brain weight	
		Initial	Final	g	Percentage of body weight
3 week	Untreated (24)	21.82 ± 0.77	37.35 ± 0.97	1.24 ± 0.02	3.33 ± 0.07
	0.2 mg DHEA (24)	22.79 ± 0.52	38.84 ± 0.59	1.26 ± 0.01	3.25 ± 0.04
	1.0 mg DHEA (24)	24.19 ± 0.50	37.75 ± 0.82	1.28 ± 0.02	3.41 ± 0.07
5 week	Untreated (18)	51.77 ± 2.26	80.50 ± 2.54	1.34 ± 0.01	1.69 ± 0.05
	0.2 mg DHEA (18)	49.17 ± 1.95	79.44 ± 2.39	1.38 ± 0.02	1.78 ± 0.07
	1.0 mg DHEA (18)	53.31 ± 2.61	80.79 ± 4.84	1.45 ± 0.01^c	1.89 ± 0.03^b
Young adult	Untreated (12)	256.06 ± 6.72	261.10 ± 9.13	1.71 ± 0.09	0.65 ± 0.04
	0.2 mg DHEA (12)	255.06 ± 5.79	273.10 ± 10.40	1.71 ± 0.06	0.63 ± 0.02
	1.0 mg DHEA (12)	266.78 ± 6.81	296.30 ± 9.03^a	1.80 ± 0.08	0.61 ± 0.02

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses. Superscript letters: a, $p < 0.02$; b, $p < 0.002$; c, $p < 0.001$ compared with the corresponding untreated group.

2 mM. The reaction was carried out for 15 min. At the end of the incubation period, the reaction was terminated by the addition of 1.1 ml of 5% (w/v) TCA (Katyare et al., 1977; Swegert et al., 1999). The tubes were allowed to stand on ice and then centrifuged at 3000 rpm for 10 min and 0.8 ml aliquots of supernatant fluid were taken up for the estimation of liberated inorganic phosphate. Estimation of inorganic phosphate was according to the procedure described by Katewa and Katyare (2003).

Protein estimation was according to the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Results are given as mean ± S.E.M.

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

3. Results

3.1. General

Treatment with DHEA had no effect on the body weight of animals in the 3 and 5 week groups. However, in the 5 week group, treatment with 1.0 mg DHEA brought about a small but significant increase (11% on body weight basis) in brain weight. In the young adult animals treatment with 1.0 mg DHEA resulted in 14% increase in the body weight (Table 1).

3.2. Effects on oxidative phosphorylation

In the untreated animals the state 3 respiration rates increased progressively up to the adult stage. The contents of the cytochromes also increased progressively up to the adult stage. Also, the ATPase activity and the levels of dehydrogenases showed increase with development. The observed pattern (Tables 2–7 and Fig. 1) is consistent with our earlier observations and those reported by other researchers (Milstein et al., 1968; Holtzman and Moore, 1975; Rajan and Katyare, 1991; Katyare et al., 2003). Treatment with the two doses (0.2 and 1.0 mg) of DHEA resulted in 2.7 and 3.1-fold increase in state 3 respiration rate with glutamate in the 3 week group thus making these values comparable to untreated young adults. In the 5 week group the increase amounted to 2.6–2.8-fold, respectively, and the values became comparable to those of young adult animals receiving DHEA treatment. In the young adult animals the increase was of smaller magnitude ranging from 53 to 69%. Under these conditions, in the developing animals the effect on state 4 respiration rate was generally of lesser magnitude and the mitochondria were tightly coupled as is evident from the respiratory control ratios. By contrast, in the young adult rats receiving 1.0 mg DHEA an opposite effect was seen where increase in state 4 respiration rate was significantly high which resulted in lowering of respiratory control ratio. ADP/O ratios were unchanged following DHEA treatments (Table 2). Practically similar trend was seen in the 3 and 5 week group with pyruvate + malate as the respiratory substrates whereas in the case of the young adult rats, treatment with DHEA had only marginal effect on state 3 respiration rate (Table 3). With succinate as the substrate, treatment of developing rats with 0.2 DHEA resulted in bringing the state 3 respiration rates to the level noted for untreated young adults. Higher dose, i.e. 1.0 mg had no further beneficial effect. Thus, the increase in the state 3 respiration rates ranged from 2.47–2.90-fold. In the young adult rats treatment with 0.2 mg DHEA caused 35% increase in the state 3 respiration rate; higher dose of 1.0 mg had adverse effect in that the increase amounted to only about 15%. The pattern for effects on state 4 respiration rate was similar to that noted for state 3 respiration rates. As the consequence, the respiratory control ratios were generally unchanged (Table 4). When ascorbate + TMPD was used as a electron donor system, in the 3 week group state 3 and state 4 respiration rates increased by 2.2–2.4-fold. In the 5 week group the increase ranged from 45 to 122%. In the young adults, once again the corresponding increase ranged only from 16 to 37%. The trend for state 4 respiration rates was comparable to that noted above for state 3 respiration rates. Consequently, the respiratory control ratios were generally unchanged (Table 5).

The contents of cytochrome aa_3 and b increased after DHEA treatment in a dose-dependent manner in the 3 week group; in the 5 week group 0.2 mg DHEA seemed to elicit maximum response. In the young adults also there was a dose-dependent increase in the content of both the cytochrome classes. Content of cytochrome $c + c_1$ seemed to be marginally influenced by treatment with 0.2 or 1.0 mg dose of DHEA and the observed changes were not statistically significant (Table 6).

Table 2
Effect of DHEA treatment on oxidative phosphorylation in rat brain mitochondria using glutamate as the substrate

Age group	Treatment	ADP/O ratio	Respiration rate (n moles of O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (n moles/min/mg protein)
			+ADP	−ADP		
3 week	Untreated (9)	3.00 ± 0.23	8.15 ± 0.67	4.89 ± 0.41	1.69 ± 0.09	47.94 ± 4.49
	0.2 mg DHEA (9)	3.05 ± 0.13	22.29 ± 1.18[a]	8.06 ± 0.44[a]	2.78 ± 0.11	134.87 ± 7.57[a]
	1.0 mg DHEA (9)	3.06 ± 0.17	25.06 ± 1.80[a]	9.48 ± 0.55[a]	2.63 ± 0.07	150.33 ± 9.13[a]
5 week	Untreated (9)	3.01 ± 0.17	11.09 ± 0.93	5.51 ± 0.26	1.99 ± 0.07	64.57 ± 3.44
	0.2 mg DHEA (9)	3.14 ± 0.21	28.97 ± 1.24[a]	8.38 ± 0.20[a]	3.62 ± 0.19	172.50 ± 9.98[a]
	1.0 mg DHEA (6)	3.09 ± 0.18	30.79 ± 2.17[a]	8.08 ± 0.15[a]	4.07 ± 0.27	186.00 ± 9.01[a]
Young adult	Untreated (12)	3.09 ± 0.08	19.15 ± 0.85	4.08 ± 0.22	3.92 ± 0.13	117.60 ± 5.53
	0.2 mg DHEA (12)	3.10 ± 0.08	29.08 ± 1.60[a]	7.53 ± 0.31[a]	3.85 ± 0.11	180.80 ± 10.89[a]
	1.0 mg DHEA (12)	3.08 ± 0.09	32.28 ± 0.60[a]	18.12 ± 0.57[a]	1.81 ± 0.06	198.50 ± 5.69[a]

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses. Superscript letter: a, $p < 0.001$ compared with the corresponding untreated group.

Table 3
Effect of DHEA treatment on oxidative phosphorylation in rat brain mitochondria using pyruvate + malate as the substrate

Age group	Treatment	ADP/O ratio	Respiration rate (n moles of O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (n moles/min/mg protein)
			+ADP	−ADP		
3 week	Untreated (7)	3.07 ± 0.23	7.10 ± 0.60	3.03 ± 0.15	2.14 ± 0.12	42.41 ± 2.65
	0.2 mg DHEA (9)	3.08 ± 0.21	19.03 ± 1.07^{a}	9.68 ± 0.41^{a}	1.96 ± 0.05	114.60 ± 5.70^{a}
	1.0 mg DHEA (8)	3.06 ± 0.23	21.56 ± 1.51^{a}	9.10 ± 0.66^{a}	2.38 ± 0.06	128.10 ± 7.28^{a}
5 week	Untreated (9)	3.08 ± 0.20	8.45 ± 0.52	3.36 ± 0.27	2.44 ± 0.15	51.29 ± 3.36
	0.2 mg DHEA (7)	2.96 ± 0.24	22.83 ± 2.04^{a}	8.19 ± 0.52^{a}	3.00 ± 0.24	87.82 ± 5.87^{a}
	1.0 mg DHEA (6)	3.01 ± 0.29	27.36 ± 2.20^{a}	7.51 ± 0.44^{a}	3.98 ± 0.20	159.10 ± 7.88^{a}
Young adult	Untreated (12)	3.08 ± 0.08	21.36 ± 1.09	4.83 ± 0.21	4.47 ± 0.19	129.90 ± 5.71
	0.2 mg DHEA (12)	3.09 ± 0.06	22.64 ± 0.69	7.76 ± 0.29^{a}	2.98 ± 0.14	139.80 ± 4.57
	1.0 mg DHEA (12)	3.07 ± 0.08	26.48 ± 0.81^{a}	7.78 ± 0.18^{a}	3.53 ± 0.21	162.80 ± 7.34^{a}

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses. Superscript letter: a, $p < 0.001$ compared with the corresponding untreated group.

Table 4
Effect of DHEA treatment on oxidative phosphorylation in rat brain mitochondria using succinate as the substrate

Age group	Treatment	ADP/O ratio	Respiration rate (n moles of O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (n moles/min/mg protein)
			+ADP	−ADP		
3 week	Untreated (9)	2.07 ± 0.11	7.98 ± 0.52	5.76 ± 0.41	1.40 ± 0.06	31.13 ± 2.75
	0.2 mg DHEA (9)	2.09 ± 0.15	22.73 ± 0.54^{c}	17.05 ± 0.49^{c}	1.34 ± 0.03	93.91 ± 5.33^{c}
	1.0 mg DHEA (9)	2.06 ± 0.14	23.18 ± 1.19^{c}	16.35 ± 0.91^{c}	1.43 ± 0.06	93.82 ± 4.93^{c}
5 week	Untreated (9)	2.08 ± 0.14	8.86 ± 0.50	6.00 ± 0.32	1.52 ± 0.06	34.87 ± 1.66
	0.2 mg DHEA (9)	2.03 ± 0.08	22.80 ± 1.88^{c}	14.14 ± 0.99^{c}	1.60 ± 0.04	91.50 ± 6.39^{c}
	1.0 mg DHEA (9)	2.03 ± 0.14	21.91 ± 1.16^{c}	14.09 ± 0.95^{c}	1.58 ± 0.06	87.57 ± 4.95^{c}
Young adult	Untreated (12)	2.07 ± 0.06	24.86 ± 1.01	13.52 ± 0.85	1.97 ± 0.15	102.60 ± 4.73
	0.2 mg DHEA (12)	2.09 ± 0.08	33.64 ± 0.77^{c}	18.12 ± 0.69^{c}	1.89 ± 0.07	140.20 ± 5.77^{c}
	1.0 mg DHEA (12)	2.06 ± 0.07	28.58 ± 0.85^{b}	17.25 ± 0.67^{b}	1.68 ± 0.05	116.70 ± 3.04^{a}

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses. Superscript letters: a, $p < 0.02$; b, $p < 0.01$; c, $p < 0.001$ compared with the corresponding untreated group.

DHEA treatment increased the levels of GDH in all the groups which ranged from 19 to 90% with maximum effect being seen in the young adult animals. By contrast, the mitochondrial MDH activity increased by 3.6–3.9-fold only in the 3 week group; in the young adults 1.0 mg DHEA had adverse effect. DHEA treatment caused substantial 3.3–6.8-fold increase in the SDR activity with the effect being more pronounced in the 5 week group. In the young adult rats, treatment with 1.0 mg DHEA had adverse effect. The effects on cytosolic MDH were marginal and 26 and 38% increase could be noted in the developing animals (Fig. 1).

Table 5
Effect of DHEA treatment on oxidative phosphorylation in rat brain mitochondria using ascorbate + TMPD as the substrate

Age group	Treatment	ADP/O ratio	Respiration rate (n moles of O_2/min/mg protein)		Respiratory control ratio	ADP phosphorylation rate (n moles/min/mg protein)
			+ADP	−ADP		
3 week	Untreated (10)	0.69 ± 0.04	7.25 ± 0.61	5.18 ± 0.62	1.48 ± 0.09	9.69 ± 0.59
	0.2 mg DHEA (12)	0.71 ± 0.03	15.74 ± 0.87^{d}	11.91 ± 0.90^{d}	1.38 ± 0.13	22.43 ± 1.58^{d}
	1.0 mg DHEA (12)	0.71 ± 0.06	17.30 ± 1.01^{d}	12.55 ± 0.76^{d}	1.38 ± 0.03	23.39 ± 1.27^{d}
5 week	Untreated (11)	0.72 ± 0.03	14.70 ± 1.14	8.72 ± 0.64	1.68 ± 0.06	20.96 ± 1.79
	0.2 mg DHEA (12)	0.73 ± 0.05	21.24 ± 1.93^{d}	13.27 ± 1.04^{d}	1.60 ± 0.04	29.19 ± 1.80^{b}
	1.0 mg DHEA (12)	0.74 ± 0.05	29.45 ± 1.78^{d}	19.38 ± 1.21^{d}	1.55 ± 0.08	43.00 ± 2.09^{d}
Young adult	Untreated (12)	0.75 ± 0.03	23.80 ± 1.43	14.38 ± 0.91	1.67 ± 0.03	34.62 ± 1.87
	0.2 mg DHEA (12)	0.74 ± 0.02	27.71 ± 1.21^{a}	19.71 ± 0.89^{d}	1.42 ± 0.04	41.14 ± 2.14^{a}
	1.0 mg DHEA (12)	0.73 ± 0.03	29.81 ± 0.74^{c}	19.67 ± 0.66^{d}	1.53 ± 0.04	43.83 ± 1.80^{c}

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses. Superscript letters: a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.002$; d, $p < 0.001$ compared with the corresponding untreated group.

Table 6
Effect of DHEA treatment on the cytochrome content of rat brain mitochondria

Age group	Treatment	Cytochrome content (pmoles/mg protein)		
		aa_3	b	$c + c_1$
3 week	Untreated (12)	126.0 ± 6.35	152.2 ± 6.65	192.2 ± 9.18
	0.2 mg DHEA (11)	165.6 ± 7.41[b]	176.4 ± 6.67[a]	191.4 ± 11.06
	1.0 mg DHEA (12)	213.2 ± 7.71[b]	222.0 ± 9.74[b]	169.5 ± 12.42
5 week	Untreated (18)	143.8 ± 4.04	168.7 ± 5.94	231.5 ± 9.23
	0.2 mg DHEA (18)	209.1 ± 11.00[b]	259.8 ± 11.90[b]	264.8 ± 14.21
	1.0 mg DHEA (12)	187.1 ± 4.83[b]	222.6 ± 8.09[b]	238.0 ± 8.20
Young adult	Untreated (12)	156.1 ± 5.16	175.8 ± 6.37	221.3 ± 8.21
	0.2 mg DHEA (12)	180.4 ± 3.80[b]	196.9 ± 5.25[a]	200.3 ± 6.60
	1.0 mg DHEA (12)	269.1 ± 5.07[b]	354.4 ± 6.57[b]	196.4 ± 9.04

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses. Superscript letters: a, $p < 0.02$; b, $p < 0.001$ compared with the corresponding untreated group.

Table 7
Effect of DHEA treatment on ATPase activity in rat brain mitochondria

Age group	Treatment	Activity (μmoles of Pi liberated/h/mg protein)			
		Basal	+Mg^{2+}	+DNP	+Mg^{2+}, +DNP
3 week	Untreated (12)	0.18 ± 0.01	1.79 ± 0.07	0.19 ± 0.01	1.80 ± 0.08
	0.2 mg DHEA (12)	0.32 ± 0.03[e]	3.92 ± 0.24[e]	0.39 ± 0.02[c]	4.07 ± 0.16[e]
	1.0 mg DHEA (12)	0.35 ± 0.02[e]	5.59 ± 0.22[e]	0.38 ± 0.02[c]	5.68 ± 0.18[e]
5 week	Untreated (12)	0.23 ± 0.02	2.25 ± 0.16	0.35 ± 0.02	2.65 ± 0.16
	0.2 mg DHEA (12)	0.35 ± 0.03[a]	5.07 ± 0.29[e]	0.46 ± 0.03[c]	5.25 ± 0.35[e]
	1.0 mg DHEA (12)	0.44 ± 0.02[e]	4.76 ± 0.20[e]	0.47 ± 0.02[e]	5.54 ± 0.24[e]
Young adult	Untreated (12)	0.31 ± 0.04	5.64 ± 0.16	0.43 ± 0.02	6.49 ± 0.21
	0.2 mg DHEA (12)	0.74 ± 0.06[e]	7.58 ± 0.18[e]	0.78 ± 0.04[c]	7.48 ± 0.16[d]
	1.0 mg DHEA (12)	0.67 ± 0.03[e]	6.26 ± 0.16[b]	0.60 ± 0.04[c]	8.79 ± 0.17[e]

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses. Superscript letters: a, $p < 0.05$; b, $p < 0.02$; c, $p < 0.01$; d, $p < 0.002$; e, $p < 0.001$ compared with the corresponding untreated group.

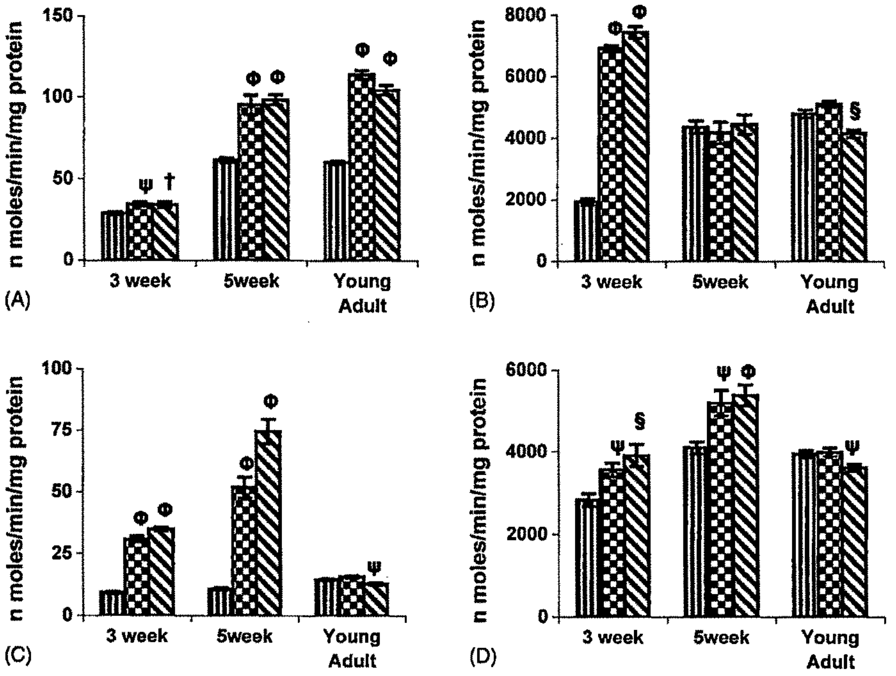

Fig. 1. Effect of DHEA treatment on mitochondrial and cytosolic dehydrogenases activities in rat brain. The results are given as mean ± S.E.M. of 15 independent observations. (A) Glutamate dehydrogenase, (B) malate dehydrogenase (mitochondrial), (C) succinate DCIP reductase and (D) malate dehydrogenase (cytosolic); ▥, untreated; ▦, 0.2 mg DHEA; ▧, 1.0 mg DHEA. (†) $p < 0.02$; (ψ) $p < 0.01$; (§) $p < 0.002$; (Φ) $p < 0.001$ compared with the corresponding untreated group.

DHEA treatments significantly increased the ATPase activity in developing animals bringing it close to untreated young adult values. Increments in the ATPase activity in the young adult group were comparatively of lower magnitude, except for the basal ATPase activity (Table 7).

4. Discussion

Present investigations were undertaken to find out if treatment with exogenous DHEA would accelerate the process of maturation and development of cerebral mitochondria in the developing rats. This aspect assumes importance in view of the fact that the brain contains significant amounts of DHEA and the fact that the brain itself synthesizes DHEA (Corpechot et al., 1981; Vallee et al., 2000; Steckelbroeck et al., 2002; Racchi et al., 2003; Ren and Hou, 2005). Besides, it has been shown that the concentration of DHEA in the brain declines with aging (Weill-Engerer et al., 2002; Kazihnitkova et al., 2004). This parallels the known decline in the respiratory activity of mitochondria from different tissues including the brain (Deshmukh and Patel, 1980; Ferrandiz et al., 1994; Cocco et al., 2005; Navarro et al., 2005).

The data of our present studies indeed suggest that DHEA may play a significant role in regulating the respiratory activity in the brain mitochondria especially during developmental stages. This assumption is substantiated by the fact that treatment with DHEA significantly stimulated the state 3 respiration rates in the mitochondria from the developing animals with glutamate, pyruvate + malate and ascorbate + TMPD while the effect on succinate-linked respiratory activity was of lesser magnitude. The ADP/O ratios were not affected by treatment with DHEA and the respiratory control ratios improved indicating that the mitochondria were structurally intact. The possibility that DHEA by itself improved the respiratory activity and/or stability of mitochondria seems unlikely. It has been shown that incubation in vitro with DHEA resulted in deterioration of all respiratory parameters in the brain mitochondria (Morin et al., 2002). In the present studies, the animals received DHEA treatment for 7 consecutive days. The route of administration of DHEA was s.c. which ensures slow and sustained release of the steroids (Jani et al., 1991; Katyare et al., 2003; Pandya et al., 2004). Therefore, it is likely that the observed effects could be attributed to activation of specific nuclear and mitochondrial genes following chronic exposure to DHEA. This indeed seems to be the case when one considers the significant increases in the contents of cytochromes aa_3 and b, ATPase and dehydrogenases activities (Tables 6 and 7 and Fig. 1). However, DHEA treatment, had practically no effect on the content of cytochrome $c + c_1$ (Table 6). It is now well recognized that the crucial peptides of cytochromes aa_3 and b, and of ATPase are mitochondrial gene products whereas cytochrome $c + c_1$ and the dehydrogenases are coded by the nuclear genes (Poyton and Mc Ewen, 1996). In view of this, it may be suggested that DHEA action may be mediated by activating specific mitochondrial and nuclear genes. It may also be suggested that DHEA may not have a role in regulating expression of cytochrome $c + c_1$ components. The increases in the contents of cytochromes aa_3 and b and of the dehydrogenases agree well with observed increase in the respiratory activities in the cerebral mitochondria isolated from the developing rats. The net consequence was increase in the ADP phosphorylation rates which is an index of energy potential of the mitochondria (Katewa and Katyare, 2004). It may be pointed out here that the contents of dehydrogenases and cytochrome aa_3 were lower in the developing animals. Lower contents of dehydrogenases and cytochrome aa_3 could have been the rate limiting steps in electron transport and oxidative capacity in the developing animals thereby restricting the energy potential, i.e. ADP phosphorylation rates (Tables 2–5). This indeed seems to be the case when one considers the substantial increase in state 3 respiration rates and ADP phosphorylation rates as pointed out above.

It may also be pointed out that the effects of DHEA treatments on the mitochondria from young adult animals were either of the lower magnitude or seemed to have adverse effects. Thus, treatment with 0.2 mg DHEA had marginal stimulatory effects whereas higher dose, i.e. 1.0 mg had occasionally adverse effects. These observations deserve some comments.

It has been demonstrated that DHEA is rapidly converted to 7α hydroxy DHEA and δ5-androstene-3β, 17β-diol (Steckelbroeck et al., 2002; Weill-Engerer et al., 2003) The former is considered to be the active metabolite which mediates the effects of DHEA (Steckelbroeck et al., 2002; Weill-Engrer et al., 2003). Presence of 7α hydroxylase in the brain tissue has been demonstrated (Akwa et al., 1992) The enzyme activity is low in newborn rats, reaches a value of about 1.5 pmol/min/mg microsomal protein in the weaning rats (i.e. 3 weeks old), increases about two-fold by the 5th week and reaches optimum level thereafter (Akwa et al., 1992). These authors also reported that the enzyme has K_m of 13.8 μM and V_{max} of 332 pmol/min/mg microsomal protein (Akwa et al., 1992). However, the activity measurements were carried out using suboptimal concentrations of the substrate DHEA. It may hence be suggested that when DHEA is supplied in excess exogenously, it may be able to saturate the enzyme and thereby there may be increased conversion of DHEA to 7α hydroxy DHEA in the developing rat brain. This could then be responsible for observed stimulatory effect. By contrast, in the young adult animals, the enzyme may be generating sufficient quantites of 7α hydroxy DHEA to maintain homeostasis and exogenously supplied DHEA may not have extra beneficial effects. However, the possibility that the effects are indeed mediated by increased amount of 7α hydroxy DHEA need to verified by more direct experiment using 7α hydroxy DHEA. Our results also show that in the young adult animals the higher dose (1.0 mg) of DHEA had at times adverse effect. This is not really surprising since catabolic effects of higher doses of thyroid hormones in intact euthyroid and hypothyroid rats have been demonstrated (Satav and Katyare, 1982; Katyare and Rajan, 2005).

As is well recognized, development is an energy-dependent process (Golovachev and Nadal'yak, 1975; Okada, 1994; Rust, 1994). As is evident from the data presented, oxidative energy potential, i.e. ADP phosphorylation rates increased substantially in the developing animals following DHEA treatment. The results thus clearly demonstrate a positive correlation and role of DHEA in the process of development and maturation of cerebral mitochondria.

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Treatment with dehydroepiandrosterone (DHEA) stimulates oxidative energy metabolism in the cerebral mitochondria
A comparative study of effects in old and young adult rats

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Abstract

The content of the neurosteroids, dehydroepiandrosterone (DHEA) in the brain decreases with aging. Also the oxidative energy metabolism is known to decrease with aging. Hence we examined the effects of treatment with DHEA (0.2 or 1.0 mg/kg body weight for 7 days) on oxidative energy metabolism in brain mitochondria from old and young adult rats. State 3 respiration rates in brain mitochondria from old animals were considerably lower than those in young adults. Treatment with DHEA stimulated state 3 and state 4 respiration rates in both the groups of the animals in a dose-dependent manner. In the old rats following DHEA treatment, the state 3 respiration rates became comparable to or increased beyond those of untreated young adults. In contrast to the old rats, stimulatory effect of DHEA treatment was of greater magnitude in the young adults. However, at higher dose (1.0 mg) the effect declined. Cytochrome aa_3 content in the brain mitochondria from old rats was significantly low but the content of cytochrome *b* was unchanged while the content of cytochromes $c+c_1$ had increased. Treatment with DHEA increased the content of cytochrome aa_3 and *b* in old as well as in young adult animals. Higher dose of DHEA (1.0 mg) had adverse effect on the content of cytochrome $c+c_1$. DHEA treatment stimulated ATPase activity in a dose-dependent manner in young adult rats whereas in the old rats the effect on ATPase activity was marginal. Dehydrogenases activities were somewhat lower in the old rats. DHEA treatment stimulated mitochondrial dehydrogenases activities in both the groups. Results of our studies suggest that judicious use of DHEA treatment can improve oxidative energy metabolism parameters in brain mitochondria from young adult as well as old rats.

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Keywords: Dehydroepiandrosterone (DHEA); Oxidative energy metabolism; ATPase activity; Cytochromes; Dehydrogenases; Brain mitochondria

Dehydroepiandrosterone (DHEA), a steroid secreted by adrenal cortex, has a characteristic age-related pattern. The concentration of DHEA in the serum is low in infancy, starts increasing in adolescence, and reaches a peak in adult life around 20–30 year. The level starts declining thereafter around the age of 35–40 years and in the elderly the levels decrease substantially [8,13,27]. Based on this characteristic profile, DHEA is considered to be the youth hormone [5,13]. Interestingly, DHEA and its sulfated conjugate DHEAS, and pregnenolone are known to be synthesized by the brain and are considered as neurosteroids [29]. The concentration of DHEA is in ng range/g tissue with the highest concentration being seen in the anterior pituitary [7,30,34,35]. The concentration of DHEAS is about 10 times higher [7]. An age-dependent decline in the content of DHEAS in the brain has been reported [17]. Presence of DHEA and DHEAS in human brain and decreased contents of DHEA and DHEAS in brain in Alzheimer's disease and dementia have been reported [35]. There are reports to indicate that the exogenous supplementation with DHEA helps to improve memory and behavior in elderly population [4]. In rats fed diet supplemented with DHEA significant stimulation of mitochondrial function and proliferation of mitochondria have been reported [3,23].

Of the total oxygen consumption by the body about 20% oxygen is utilized in the brain [10] which is consistent with the fact that the electrophysiological functions of the brain are known to be energy dependent [1,2].

Therefore, it is of interest to know if treatment with exogenous DHEA has beneficial effect on improving memory and behavior in elderly population due to improvement of cerebral mitochondrial energy transduction functions.

We tested this possibility by treating old male rats with DHEA. Simultaneously the effects were also examined in young

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adults. Our results indicate that DHEA treatment stimulates the respiratory activity parameters in old rats as well as in young adults in a dose-dependent manner. In the old rats DHEA treatment was able to restore respiratory parameters close to untreated young adults or beyond this level.

Dehydroepiandrosterone (DHEA) was obtained from Sigma–Aldrich, USA. Sodium salts of succinic acid, pyruvic acid, L-malic acid, ADP, rotenone, bovine serum albumin fraction V (BSA), 4-morpholinopropanesulfonic acid (MOPS), dicholrophenolindophenol (DCIP), NAD$^+$, NADH, oxaloacetic acid, disodium salt of ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. Sodium salt of L-glutamic acid was purchased from E Merck Germany. *N,N,N′,N′*-tetra-methyl-*p*-phenylenediamine (TMPD) was obtained from British Drug Houses, Dorset, Poole, England. Ascorbic acid was from Sarabhai Chemicals, Vadodara. All other chemicals were of analytical-reagent grade and were obtained locally.

Male young adult (8–10 week old) and old (18–24 month old) albino rats of Charles–Foster strain were used. At the start of experiment the body weight of young adult rats was in the range of 235–240 g while that of old rats was in the range of 358–375 g. The animals were injected subcutaneously (s.c.) with 0.2 mg or 1.0 mg DHEA per kg body weight for seven consecutive days. The number of animals in the different groups ranged from 12 to 20. DHEA suspension was prepared fresh in saline prior to use. The controls received equivalent volume of saline vehicle. The animals were sacrificed on the 8th day for isolation of mitochondria. The experimental protocol was approved by the Departmental Animal Ethics Committee.

Isolation of brain mitochondria was essentially according to the procedure described previously with some modifications [33] using isolation medium consisting of 250 mM sucrose containing 5 mM MOPS and 1 mM EDTA all at pH 7.4; 0.25 mg BSA/ml of isolation medium was included.

Measurements of oxidative phosphorylation were carried out at 25 °C using a Clark-type oxygen electrode as described previously [15]. The respiration medium (total volume 1.6 ml) consisted of 225 mM sucrose, 20 mM KCl, 10 mM MOPS pH 7.4, 5 mM potassium phosphate buffer pH 7.4, 0.2 mM EDTA and 160 mg of BSA (i.e. 0.1 mg BSA/ml). Calculations of ADP/O ratio and ADP phosphorylation rates were as described previously [15].

The contents of cytochromes were quantified from the difference spectra as described previously [15]. The difference spectra of reduced versus oxidized samples were recorded in a JASCO UV/VIS spectrophotometer model V-530. The contents of aa_3, b and $c+c_1$ cytochromes were calculated using the wavelength pairs 604–624, 559–580 and 535–552 nm and millimolar extinction coefficients 24, 23.4 and 18.7, respectively [32].

Glutamate dehydrogenase (GDH), mitochondrial and cytosolic malate dehydrogenase (MDH) and succinate DCIP reductase (SDR) activities were measured spectrophotometrically at 25 °C [18,19,26].

The ATPase activity was determined in the assay medium (total volume 0.1 ml) containing 350 mM sucrose, 10 mM MOPS pH 7.4, 10 mM KCl and 0.2 mM EDTA. The assays were performed in the absence and presence of $MgCl_2$ (2 mM) and 50 μM DNP, or a combination of both [16,33]. Estimation of released inorganic phosphate was by the method of Katewa and Katyare [14].

Protein estimation was by the method of Lowry et al. using bovine serum albumin as the standard [20].

Results are given as mean ± S.E.M.

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

Treatment with 1.0 mg DHEA resulted in 17% increase in body weight of young adult rats without having any effect on the brain weight. By contrast, DHEA treatment had no appreciable effect on body weight in the old rats. However, treatment with 1.0 mg DHEA resulted in a small but reproducible increase (4% increase) in brain weight (data not given). Possibly, the increased respiratory metabolism (detailed below) may have induced the increase in the brain weight.

The effects of DHEA treatment on oxidative energy metabolism are summarized in Tables 1 and 2. In untreated old rats the state 3 respiration rates and ADP-phosphorylation rates were about 16–38% lower compared to those in the corresponding young adult rats for the different substrate used. The state 4 respiration rate increased with pyruvate + malate with the opposite seen for succinate. Consequently, the respiratory control ratios were low for all the substrate which is indicative of mitochondrial fragility; fragility of cerebral mitochondria in old mice has been reported [25]. Our observations are consistent with those reported by other researchers [6,7,11,25]. The results thus emphasize decreased energy potential of brain with aging.

DHEA treatment of young animals brought about significant stimulation of respiratory activities with glutamate, pyruvate + malate, succinate and ascorbate + TMPD in a dose-dependent manner. These changes were also reflected in terms of corresponding increase in the ADP-phosphorylation rates (Tables 1 and 2). The state 4 respiration rates also showed parallel increase. DHEA treatment also resulted in increase in the contents of cytochrome aa_3 and b in a dose-dependent manner. Interestingly the content of cytochrome $c+c_1$ was unchanged (Table 3). Likewise, the ATPase activities also increased after DHEA treatment (Table 4). A similar picture was obtained for GDH, mitochondrial MDH and SDR with the effect being more pronounced with 0.2 mg DHEA. The cytosolic MDH was not affected (Fig. 1).

In the old rats, treatment with DHEA stimulated the state 3 respiration rates and brought them to the levels comparable to untreated young adults (Tables 1 and 2). Parallel increase in ADP-phosphorylation rates and state 4 respiration rates were also evident (Tables 1 and 2). The old rats were characterized by significant decrease in cytochrome aa_3 content (Table 3) which agrees well with the low respiration rate that we report here. Decreased complex IV activity in cerebral mitochondria from old mice has been reported [12]. The content of cytochrome b was unchanged but that of $c+c_1$ increased substantially which could be a compensatory mechanism. Increased transcription of cytochrome c gene in old mice has been reported by other researchers [21]. Treatment with DHEA resulted in increasing the contents of cytochrome aa_3 and b in a dose-dependent

Table 1
Effect of DHEA treatment on oxidative phosphorylation in rat brain mitochondria using glutamate and pyruvate + malate as the substrate

Substrate	Age group	Treatment	ADP/O ratio	Respiration rate (nmol O_2/min/mg protein)		Respiratory control ratio	ADP-phosphorylation rate (nmol/min/mg protein)
				+ADP	−ADP		
Glutamate	Young Adult	Untreated (12)	3.09 ± 0.10	18.23 ± 0.79	4.56 ± 0.21	4.04 ± 0.15	111.80 ± 4.68
		0.2 mg DHEA (12)	3.03 ± 0.09	29.73 ± 2.01^c	7.88 ± 0.36^c	3.75 ± 0.14	180.00 ± 10.17^c
		1.0 mg DHEA (12)	3.01 ± 0.12	33.38 ± 0.75^c	18.53 ± 0.67^c	1.88 ± 0.08	193.70 ± 6.40^c
	Old	Untreated (20)	3.03 ± 0.17	13.06 ± 0.63***	4.81 ± 0.24	2.88 ± 0.13***	76.79 ± 4.54***
		0.2 mg DHEA (12)	3.01 ± 0.13	23.31 ± 1.78^c	8.71 ± 0.61^c	2.74 ± 0.13	139.50 ± 11.10^c
		1.0 mg DHEA (12)	3.00 ± 0.12	20.70 ± 0.45^c	8.38 ± 0.41^c	2.51 ± 0.11	124.00 ± 5.38^c
Pyruvate + Malate	Young Adult	Untreated (12)	3.10 ± 0.10	21.49 ± 1.14	4.71 ± 0.27	4.64 ± 0.23	132.30 ± 4.37
		0.2 mg DHEA (12)	3.13 ± 0.08	22.96 ± 0.76	7.92 ± 0.39^c	2.97 ± 0.16	143.20 ± 4.87
		1.0 mg DHEA (12)	3.13 ± 0.10	26.92 ± 0.92^b	8.01 ± 0.36^c	3.34 ± 0.28	169.10 ± 9.05^b
	Old	Untreated (16)	3.09 ± 0.14	18.00 ± 0.72*	7.28 ± 0.40***	2.61 ± 0.17***	109.90 ± 5.43**
		0.2 mg DHEA (12)	3.14 ± 0.17	20.35 ± 1.00	8.96 ± 0.25^b	2.29 ± 0.12	126.00 ± 6.61
		1.0 mg DHEA (9)	3.12 ± 0.13	23.40 ± 1.03^c	8.97 ± 0.40^a	2.97 ± 0.17	145.80 ± 8.07^b

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses.
[a] $p < 0.01$ compared with the corresponding untreated group.
[b] $p < 0.002$ compared with the corresponding untreated group.
[c] $p < 0.001$ compared with the corresponding untreated group.
[*] $p < 0.02$ compared with the untreated young adult group.
[**] $p < 0.01$ compared with the untreated young adult group.
[***] $p < 0.001$ compared with the untreated young adult group.

manner. However, higher dose (1.0 mg DHEA) had adverse effect on the content of cytochrome $c + c_1$ (Table 3). The ATPase activities were generally low in old animals and DHEA treatment had only marginal effect (Table 4). However, following treatment with DHEA the levels of GDH, mitochondrial MDH, and SDR increased substantially reaching values close to or higher than the young adult controls in a dose-dependent manner. A similar trend was evident even for cytosolic MDH activity (Fig. 1).

It has been reported that in the old animals initially there is up-regulation of the genes encoding peptides in complex I, III, IV and V of the respiratory chain which is followed by down-regulation at later stage [21]. Our results on the respiratory

Table 2
Effect of DHEA treatment on oxidative phosphorylation in rat brain mitochondria using succinate and ascorbate + TMPD as the substrate

Substrate	Age group	Treatment	ADP/O ratio	Respiration rate (nmol O_2/min/mg protein)		Respiratory control ratio	ADP-phosphorylation rate (nmol/min/mg protein)
				+ADP	−ADP		
Succinate	Young Adult	Untreated (12)	2.07 ± 0.06	25.17 ± 1.42	14.18 ± 1.05	1.87 ± 0.16	103.60 ± 5.96
		0.2 mg DHEA (12)	2.17 ± 0.10	33.90 ± 0.95^e	18.96 ± 0.88^d	1.83 ± 0.09	140.00 ± 7.13^e
		1.0 mg DHEA (12)	2.10 ± 0.07	28.60 ± 0.99	17.10 ± 0.67^a	1.67 ± 0.05	118.90 ± 3.44^a
	Old	Untreated (16)	2.10 ± 0.15	15.65 ± 0.75***	10.78 ± 0.77**	1.51 ± 0.07*	64.44 ± 4.63***
		0.2 mg DHEA (11)	2.09 ± 0.16	22.07 ± 1.50^e	14.96 ± 1.23^c	1.53 ± 0.12	91.62 ± 8.39^b
		1.0 mg DHEA (8)	2.07 ± 0.13	27.95 ± 1.20^e	21.60 ± 1.65^c	1.32 ± 0.07	113.20 ± 9.80^e
Ascorbate + TMPD	Young Adult	Untreated (12)	0.70 ± 0.03	22.99 ± 1.81	13.92 ± 1.12	1.66 ± 0.04	31.82 ± 2.35
		0.2 mg DHEA (12)	0.72 ± 0.03	28.98 ± 1.76^a	19.90 ± 1.23^a	1.46 ± 0.02	41.75 ± 3.26^a
		1.0 mg DHEA (12)	0.70 ± 0.03	31.44 ± 0.81^c	19.73 ± 0.90^e	1.61 ± 0.05	44.32 ± 2.38^d
	Old	Untreated (20)	0.72 ± 0.04	17.43 ± 1.04*	12.32 ± 0.64	1.41 ± 0.03**	24.03 ± 1.56**
		0.2 mg DHEA (16)	0.75 ± 0.05	22.18 ± 1.25^b	16.83 ± 0.85^c	1.32 ± 0.04	33.21 ± 2.61^c
		1.0 mg DHEA (12)	0.73 ± 0.04	22.03 ± 1.11^c	17.57 ± 0.69^e	1.25 ± 0.04	32.85 ± 1.38^e

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses.
[a] $p < 0.05$ compared with the corresponding untreated group.
[b] $p < 0.02$ compared with the corresponding untreated group.
[c] $p < 0.01$ compared with the corresponding untreated group.
[d] $p < 0.002$ compared with the corresponding untreated group.
[e] $p < 0.001$ compared with the corresponding untreated group.
[*] $p < 0.05$compared with the untreated young adult group.
[**] $p < 0.02$compared with the untreated young adult group.
[***] $p < 0.001$compared with the untreated young adult group.

Table 3
Effect of DHEA treatment on the cytochrome content of rat brain mitochondria

Age group	Treatment	Cytochrome content (pmol/mg protein)		
		aa_3	b	$c+c_1$
Young Adult	Untreated (8)	154.6 ± 8.46	176.2 ± 10.21	224.3 ± 14.80
	0.2 mg DHEA (8)	186.3 ± 3.87[b]	193.0 ± 7.64	203.6 ± 9.49
	1.0 mg DHEA (8)	267.4 ± 7.16[c]	353.9 ± 9.77[c]	198.8 ± 12.91
Old	Untreated (12)	96.5 ± 5.39*	185.3 ± 8.51	320.8 ± 16.88*
	0.2 mg DHEA (12)	143.8 ± 6.15[c]	216.5 ± 7.72[a]	304.9 ± 13.02
	1.0 mg DHEA (12)	213.3 ± 6.56[c]	268.0 ± 6.10[c]	247.3 ± 18.05[c]

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses.

[a] $p < 0.02$ compared with the corresponding untreated group.

[b] $p < 0.01$ compared with the corresponding untreated group.

[c] $p < 0.001$ compared with the corresponding untreated group.

* $p < 0.001$ compared with the untreated young adult group.

Table 4
Effect of DHEA treatment on ATPase activity in rat brain mitochondria

Age group	Treatment	Activity (μmol Pi liberated/h/mg protein)			
		Basal	+Mg^{2+}	+DNP	+Mg^{2+} +DNP
Young Adult	Untreated (8)	0.36 ± 0.02	6.03 ± 0.21	0.49 ± 0.03	6.86 ± 0.14
	0.2 mg DHEA (8)	0.81 ± 0.05[c]	8.34 ± 0.26[c]	0.89 ± 0.05[c]	8.66 ± 0.21[c]
	1.0 mg DHEA (8)	0.73 ± 0.05[c]	7.98 ± 0.31[c]	0.76 ± 0.05[c]	8.98 ± 0.19[c]
Old	Untreated (12)	0.35 ± 0.02	3.45 ± 0.25*	0.53 ± 0.03	4.08 ± 0.24*
	0.2 mg DHEA (12)	0.35 ± 0.02	4.48 ± 0.19[b]	0.46 ± 0.03	4.74 ± 0.12[a]
	1.0 mg DHEA (12)	0.44 ± 0.02[b]	3.33 ± 0.14	0.47 ± 0.02	3.88 ± 0.16

Experimental details are as given in the text. Results are given as mean ± S.E.M. of the number of observations indicated in the parentheses.

[a] $p < 0.05$ compared with the corresponding untreated group.

[b] $p < 0.01$ compared with the corresponding untreated group.

[c] $p < 0.001$ compared with the corresponding untreated group.

* $p < 0.001$ compared with the untreated young adult group.

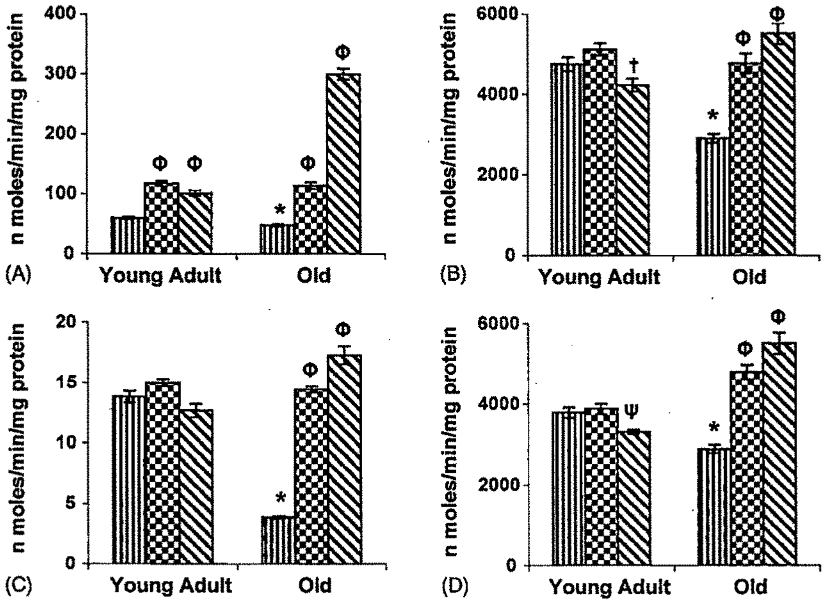

Fig. 1. Effect of DHEA treatment on mitochondrial and cytosolic dehydrogenases activities in rat brain. The results are given as mean ± S.E.M. of 12 and 20 independent observations for young adult and old animals, respectively. (A) Glutamate dehydrogenase; (B) malate dehydrogenase (Mitochondrial); (C) succinate DCIP reductase and (D) malate dehydrogenase (cytosolic); ▥, Untreated; ▦, 0.2 mg DHEA and ▧, 1.0 mg DHEA. $^{\dagger}p < 0.05$; $^{\Psi}p < 0.002$ and $^{\Phi}p < 0.001$ compared with the corresponding untreated group. $^{*}p < 0.001$ compared with the untreated young adult group.

activity in the cerebral mitochondria from the old rats are consistent with the latter observation. The results of the present study point out that DHEA treatment was effective in enhancing the respiratory potential of cerebral mitochondrial in old as well as young adult rats. The results also show that DHEA treatment was able to restore the respiratory parameters in old animals to values comparable to the untreated young adults. Interestingly, the increase in the respiratory parameters was of higher magnitude in young adults.

In this connection it may be mentioned that in the elderly population the plasma levels of DHEA decrease to about 10% of adult values at the age of around 70 years [13,22]. Considering the life-span of the rat of 3 years, the old animals (18–24 month old) in our studies may be equated with old humans of around 62–65 year age. If a similar situation prevails in the rats, then presumably the plasma DHEA levels in the old rats may be expected to be very low compared to the young adults. Age-dependent decrease in the content of DHEA in the human and rat brain has also been reported [17,35]. Therefore, it is possible that even after treating with exogenous DHEA it is unlikely that the adult plasma and/or brain levels of DHEA will be reached in the old animals. This could be one of the possible reasons for lower magnitude of stimulation in old animals. It is also important to note that higher dose of DHEA had no extra beneficial effects in young adults and occasionally showed adverse effects. These observations therefore cautions that dose of exogenous DHEA should be adjusted judiciously.

It is clear from the data presented that the GDH and cytosolic as well as mitochondrial MDH activities increased after treatment with DHEA. Additionally, under these treatment conditions the contents of cytochromes aa_3 and b also increased. These changes were accompanied by increase in the ATPase activity (Fig. 1, Tables 3 and 4). The dehydrogenases are known to be nuclear gene products whereas crucial polypeptides of cytochrome oxidase, i.e. cytochromes aa_3 and of cytochrome b and ATPase are known to be coded by mitochondrial DNA [28]. It may hence be suggested that DHEA treatment enhanced the respiratory activity of the mitochondria by selective activation of specific nuclear and mitochondrial genes. The most interesting part was that the contents of cytochromes $c + c_1$ decreased in the old animals after treatment with 1 mg DHEA, bringing the value close to control young adult level. This may suggest that DHEA has a negative regulatory role in cytochromes $c + c_1$ synthesis. Higher concentrations of DHEA occasionally showed less beneficial effects. It may hence be suggested that intricate age-dependent mechanisms may regulate DHEA action.

The possibility of non-genomic effects seems unlikely for two reasons. Firstly the treatment with DHEA was for a longer duration, i.e. for 1 week. Secondly, non-genomic membrane effects of steroids are evident within minutes [9]. Besides, it has been reported that incubation of brain mitochondria with DHEA under in vitro conditions inhibited all respiratory parameters [24].

It is well recognized that the concentrations of DHEAS are several times higher than those of DHEA in all brain regions [30]. DHEAS is the precursor for cerebral DHEA which is metabolized to 7α hydroxy DHEA and δ 5 androstene 3 β, 17 β diol [31,36]; the former is considered to be active metabolite [31,36]. The low concentrations of DHEA in the brain may be attributed to its rapid conversion to 7α hydroxy DHEA and δ 5 androstene 3 β, 17 β diol. It is possible that following treatment with exogenous DHEA there could be increased conversion to the active metabolites, which is responsible for the observed effects in terms of enhanced respiratory activity in the cerebral mitochondria. However, this possibility needs to be verified by more direct experiments using 7α hydroxy DHEA.

In conclusion our results show that treatment with DHEA can improve the respiratory parameters of cerebral mitochondria in old rats bringing them close to young adult levels. Since the electrophysiological function of the brain is known to be energy dependent [1,2] the enhanced respiratory function can help in improving memory and behavioral pattern in elderly.

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Dehydroepiandrosterone Treatment Alters Lipid/Phospholipid Profiles of Rat Brain and Liver Mitochondria

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Abstract: Stimulation of mitochondrial function following treatment with dehydroepiandrosterone (DHEA) has been demonstrated. Since the activity of several electron transport chain components is dependent on specific lipid/phospholipid components, we examined the effects of DHEA treatment (0.1-2.0 mg/kg body weight for 7 consecutive days) on lipid/phospholipids profiles of rat brain and liver mitochondria. In the brain mitochondria, contents of both total phospholipids (TPL) and cholesterol (CHL) increased. The major effect on phospholipids profile was increase in the contents of lysophospholipids (Lyso) and sphingomyelin (SPM) component followed by phosphatidylinositol (PI) and phosphatidylserine (PS). The contents of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) were not affected. At the higher dose (2.0 mg) the observed effects declined. The TPL and CHL contents of liver mitochondria were generally unchanged by DHEA treatment. Under this condition the content of PI and PS increased. The contents of other phospholipid components were not changed. Our results suggest that the observed changes may complement the function of electron transport chain components.

Key Words: Brain mitochondrial lipids; brain mitochondrial cholesterol brain mitochondrial phospholipid profile; liver mitochondrial lipids, liver mitochondrial cholesterol liver mitochondrial phospholipid profile; brain mitochondrial membrane fluidity, liver mitochondrial membrane fluidity.

INTRODUCTION

The steroids dehydroepiandrosterone (DHEA) and its sulfated conjugate DHEA-S are synthesized in highest concentrations by the adrenals and show a characteristic age-related pattern of secretion. The concentration of DHEA in the serum is low in the juveniles but starts increasing two years before the onset of puberty, peaks at adult stage and declines after the age of 35 to 40 years (Hinson and Raven 1999; Parker, 1999). In the elderly population the concentration of DHEA decreases to about 10% of the peak adult values (Hinson and Raven, 1999; Celec *et al.*, 2003). Based on this characteristic age-related pattern DHEA is considered to be the youth hormone (Hinson and Raven 1999; Celec *et al.* 2003). Interestingly, it has been reported that significant quantities of DHEA and DHEA-S are present in brain (Weill-Engerer *et al.*, 2002; Racchi *et al.*, 2003; Kazihnitkova *et al.*, 2004). DHEA, DHEA-S, and pregnenolone are known to be synthesized by the brain and are considered to be the neurosteroids (Racchi *et al.*, 2003). An age-dependent decline in the content of DHEAS in the brain and decreased contents of DHEA and DHEA-S in the brain in Alzheimer's disease and dementia have also been reported (Weill-Engerer *et al.*, 2002; Kazihnitkova *et al.*, 2004).

There are reports to indicate that the exogenous supplementation with DHEA helps to improve memory and behavior in elderly population (Buvat, 2003). A number of studies have been carried out to find usefulness of exogenous DHEA supplementation in health, disease and human well being especially in the elderly population (Celec *et al.*, 2003). While DHEA is freely available in the U.S. and needs no prescription (Hinson and Raven, 1999), the claims for its beneficial effects in health, disease and improvement of memory and behavior in elderly persons have not been clearly established and seem to be equivocal (Hinson and Raven, 1999; Oliver and Clemens, 1999).

Treatment of experimental animals with DHEA resulted in hypertrophy of the hepatocytes due to increased proliferation of mitochondria and peroxisomes (Bellei *et al.*, 1992). DHEA treatment also stimulated the mitochondrial electron transport functions (Song *et al.*, 1989; Swiercynski *et al.*, 2001). Earlier studies from our laboratory have shown that treatment of the old rats with DHEA resulted in significant stimulation of respiratory activity in the brain mitochondria (Patel and Katyare, 2006 a,b,c). Also, treatment with DHEA enhanced the maturation and development of mitochondrial functions in the brain as well as liver of the growing animals (Patel and Katyare, 2006 a,b,c).Dependence of mitochondrial membrane-bound enzymes on specific phospholipid classes is well recognized (Daum, G.,1985; Fry and Green, 1981; Mc Millin and Dowhan, 2002).

Since treatment with DHEA significantly influenced respiratory parameters of brain as well as liver mitochondria (Song *et al.*, 1989; Mohan and Cleary, 1991; Bellei *et al.* 1992; Swiercynski *et al.* 2001; Patel and Katyare 2006 a,b,c). We therefore examined the effect of DHEA treatment on lipid/phospholipid profiles of rat brain and liver mitochondria. The results of these investigations are described here.

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MATERIALS AND METHODS

Chemicals

3β-Hydroxy-5-androsten-17-one (+)-dehydroisoandrosterone (DHEA) was purchased from Sigma–Aldrich, USA. Bovine serum albumin fraction V (BSA), 4-morpholinopropanesulfonic acid (MOPS), disodium salt of ethylenediaminetetraacetic acid (EDTA), 1-6 diphenyl-1,3,5-hexatriene (DPH), were purchased from Sigma Chemical Co. USA. Silica gel G was from E. Merck, Darmstadt, Germany. All other chemicals were of analytical-reagent grade and were purchased locally.

Animals and Treatment with DHEA

Adult male albino rats (8 – 10 week old) of Charles–Foster strain weighing between 200–250 g were used. The animals received daily injections of 0.1, 0.2, 1.0 or 2.0 mg DHEA /Kg body weight subcutaneously (s.c.) for 7 consecutive days. Daily records of body weight were maintained. Suspension of DHEA was prepared fresh in saline prior to injection. The controls received equivalent volume of saline. The animals were killed on the 8th day. The experimental protocol was approved by the Departmental Animal Ethics Committee

Isolation of Mitochondria

Isolation of brain and liver mitochondria was essentially according to the procedures described previously with some modifications (Swegert *et al.*, 1999; Katyare *et al.* 2003.; Katewa and Katyare 2004, Pandya *et al.* 2004a; Katyare and Rajan 2005).

Lipid Analysis

Extraction of Lipids

Extraction of lipids, separation of phospholipids classes by thin layer chromatography (TLC) and determination of membrane fluidity were carried out essentially according to the procedures described previously (Folch *et al.*, 1957; Pandya *et al.*, 2004b). Aliquots of mitochondrial suspension (4-8 mg protein) were extracted with 4 ml of freshly prepared chloroform: methanol mixture (2:1 v/v). The samples were vortexed vigorously and centrifuged at 3000 rpm for 10 min and the organic phase was carefully removed with the help of a broad gauge needle. The samples were re-extracted with 3 ml of chloroform: methanol mixture as above. The pooled organic phase was treated with 0.1 volume of 0.017 % $MgCl_2$ by vortexing vigorously. The tubes were than centrifuged as described above to achieve phase separation. The organic phase was carefully removed with care being taken to avoid the protein / proteolipid layer present between organic and aqueous phases (Folch *et al.*, 1957; Pandya *et al.* 2004b). The solvent was completely evaporated under the stream of nitrogen after which the extracted lipids were redissolved in known volume of chloroform: methanol mixture. Suitable aliquots were taken for the estimation of phospholipids phosphorus (Bartlett, 1954) and cholesterol (Zlatkis *et al.*, 1953), and for TLC.

Separation of Phospholipids by TLC

Separation of phospholipids classes was carried out by one dimensional TLC using silica gel G. The conditions for chamber saturation were according to Stahl (1969). Aliquots of the reconstituted samples containing 8-10 µg of phospholipids phosphorous were spotted on TLC plate in a way such that the diameter of the spot was minimum. The solvent system used for separation of phospholipid classes was as described by Skipski (1967) and consisted of chloroform: methanol: acetic acid: water (25:15:4:2 v/v). After the run was completed, the solvent was allowed to evaporate completely and the plates were briefly exposed to iodine vapor to visualize the individual phospholipid spots and the spots were marked. The areas corresponding to marked spots were carefully scraped and the silica gel was transferred to clean test tubes. To each tube 0.5 ml of 10 N H_2SO_4 was added and the samples were heated on a sand bath for 8-10 hour. The tubes were allowed to cool after which a drop of 70 % perchloric acid was added. The tubes were again heated for 3-4 hours till the solution in the tubes was clear and smell of chlorine was undetectable (Pandya *et al.*, 2004b). The analysis of phosphorus content was according to the procedure of Bartlett (1954).

Contents of individual phospholipids were computed by multiplying total phospholipid by the % fraction of the said phospholipid (Pandya *et al.*, 2004b).

Determination of Membrane Fluidity

Membrane fluidity measurements were carried out spectroflourophotometrically at 25 °C using DPH as the probe in a Shimadzu spectroflourophotometer model RF 5000 (Pandya *et al.*, 2004b).

Protein estimation was according to the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Statistics

Results are given as mean ± SEM. Statistical evaluation of the data was by Students' t-test.

RESULTS

Effects on Brain Mitochondria

Data in Table **1** show that treatment with DHEA (0.1 mg and 0.2 mg) resulted in about 13 % increase in the total phospholipids content and 62 % to 66 % increase in the cholesterol (CHL) content of the brain mitochondria; the effect declined at higher doses of DHEA. The TPL: CHL ratio (mole: mole) decreased only in the animals treated with 0.2 mg DHEA. The membrane fluidity was, in general, unchanged (Table **1**).

Examination of phospholipid profile revealed that treatment with 1.0 mg DHEA resulted in about 70% increase in the lysophospholipid (Lyso) component. The sphingomyelin (SPM) component increased progressively up to 1.0 mg DHEA (1.30 to 2.01 fold increase) after which the effect diminished and became comparable to that noted for animals treated with 0.2 mg dose. The phosphatidylcholine (PC) component increased marginally only in those animals treated with 0.1 mg dose. The composition of acidic phospholipid phosphatidylinositol (PI) and phosphatidylserine (PS) was unaltered except for lowering of PI at the highest dose (2.0 mg) of DHEA. DHEA treatment also had a PE lowering effect which was evident up to the dose of 1.0 mg;

Table1. Effect of DHEA Treatment on Total Phospholipids (TPL) and Cholesterol (CHL) Content of Rat Brain and Liver Mitochondria

Tissue	Treatment	TPL (µg/mg protein)	CHL (µg/mg protein)	TPL/CHL (mole:mole)	Fluorescence polarization, p
Brain	Untreated (12)	410.0 ± 33.96	383.2 ± 10.23	0.54 ± 0.05	0.279 ± 0.006
	0.1 mg DHEA (12)	532.2 ± 46.23^{a}	621.0 ± 43.92^{d}	0.43 ± 0.03	0.263 ± 0.008
	0.2 mg DHEA (12)	534.4 ± 44.51^{a}	634.8 ± 48.30^{d}	0.42 ± 0.02^{a}	0.261 ± 0.008
	1.0 mg DHEA (12)	507.0 ± 9.91^{b}	532.2 ± 33.31^{d}	0.50 ± 0.04	0.273 ± 0.007
	2.0 mg DHEA (8)	416.9 ± 37.57	456.6 ± 27.67^{d}	0.44 ± 0.02	0.284 ± 0.003
Liver	Untreated (12)	178.0 ± 13.87	51.59 ± 2.16	1.79 ± 0.20	0.203 ± 0.009
	0.1 mg DHEA (12)	172.9 ± 7.80	56.22 ± 3.95	1.61 ± 0.12	0.209 ± 0.005
	0.2 mg DHEA (12)	185.2 ± 15.35	59.05 ± 5.08	1.62 ± 0.12	0.208 ± 0.005
	1.0 mg DHEA (12)	187.1 ± 8.88	66.98 ± 4.51^{c}	1.43 ± 0.07^{a}	0.228 ± 0.007^{a}
	2.0 mg DHEA (8)	166.8 ± 12.29	53.24 ± 2.36	1.57 ± 0.20	0.231 ± 0.004^{b}

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.
a, $p < 0.05$; b, $p < 0.02$; c, $p < 0.01$ and d, $p < 0.001$ compared with the corresponding untreated group.

the effect declined at the higher dose. DHEA treatment also resulted in decrease in the diphosphatidylglycerol (DPG) component at 1.0 mg dose (Table **2**).

Since the TPL contents and phospholipid composition changed significantly (Tables **1** and **2**), it was important to find out the extent to which the content of individual phospholipid class was altered by DHEA treatment. Data in Table **3** detail the contents of individual phospholipid classes. Thus, the content of Lyso increased progressively (42 to 104% increase) up to the dose of 1.0 mg; the effect was abolished at higher dose. The content of PC also increased following DHEA treatment with maximum increase (52 %) being seen at the lowest dose (0.1 mg) of DHEA after which the effect declined and at the highest dose the value became comparable to untreated controls. A 38% decrease in PI content at 2.0 mg dose and 55% increase in PS content at 0.2 mg dose were other interesting features. A similar 31% increase in DPG content was noted in animals treated with 0.2 mg DHEA. The content of PE was not affected under these conditions (Table **3**).

Effects on Liver Mitochondria

As can be noted (Table **1**) DHEA treatment did not influence the TPL content of liver mitochondria. However, the CHL content increased by 30% in animals treated with 1.0 mg DHEA regimen. This was also reflected in the lowering of the molar ratio of TPL: CHL. Consequently the membrane fluidity decreased in groups treated with 1.0 and 2.0 mg

Table 2. Effect of DHEA Treatment on Phospholipids Composition of Rat Brain Mitochondria

Phospholipid Class	Composition, (% of Total)				
	Untreated (12)	**0.1 mg DHEA (14)**	**0.2 mg DHEA (10)**	**1.0 mg DHEA (12)**	**2.0 mg DHEA (10)**
Lyso	3.43 ± 0.33	3.76 ± 0.34	3.81 ± 0.61	5.87 ± 0.27^{c}	3.42 ± 0.28
SPM	5.80 ± 0.36	7.54 ± 0.47^{a}	8.68 ± 0.23^{c}	11.71 ± 0.83^{c}	8.16 ± 0.33^{c}
PC	40.99 ± 0.79	44.85 ± 0.71^{b}	42.35 ± 0.70	42.35 ± 0.82	39.14 ± 0.81
PI	2.49± 0.11	2.62 ± 0.08	2.93 ± 0.29	2.29 ± 0.11	1.88 ± 0.17^{a}
PS	2.50 ± 0.08	2.76 ± 0.12	2.84 ± 0.18	2.32 ± 0.12	2.46 ± 0.21
PE	40.27 ± 0.59	34.60 ± 0.68^{c}	34.97 ± 0.99^{c}	32.64 ± 1.33^{c}	40.52 ± 0.60
DPG	4.53 ± 0.09	3.87 ± 0.20	4.42 ± 0.32	2.83 ± 0.47^{b}	4.43 ± 0.40

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.
a, $p < 0.01$; b, $p < 0.002$; and c, $p < 0.001$ compared with untreated group.

Table 3. Effect of DHEA Treatment on Phospholipids Content of Rat Brain Mitochondria

Phospholipid Class	Content, (μg/ mg protein)				
	Untreated (12)	0.1 mg DHEA (14)	0.2 mg DHEA (14)	1.0 mg DHEA (12)	2.0 mg DHEA (10)
Lyso	14.54 ± 1.30	20.60 ± 2.08^b	21.92 ± 1.69^d	29.68 ± 1.33^e	13.09 ± 1.11
SPM	24.03 ± 1.69	41.27 ± 3.56^e	47.16 ± 2.81^e	59.21 ± 3.99^e	31.78 ± 2.77^a
PC	166.55 ±12.69	253.81 ±22.39^b	233.89 ±17.70^c	214.43 ± 4.88^d	152.43 ±12.57
PI	10.30± 1.01	14.42 ± 0.99^c	16.71 ± 1.60^e	11.56 ± 0.56	6.34 ± 0.54^d
PS	10.35 ± 1.04	15.29 ± 1.18^c	16.11 ± 1.16^d	11.74 ± 0.68	8.50 ± 0.67
PE	165.81±14.52	194.77 ±15.92	192.27 ±13.75	165.99 ± 8.08	154.09 ±10.17
DPG	18.37 ± 1.38	21.30 ± 1.67	24.11 ± 2.17^a	14.42 ± 2.45	15.91 ± 1.29

Results are given as mean ± SEM of the number of observations indicated in the parentheses.
a, $p < 0.05$; b, $p < 0.02$; c, $p<0.01$; d, $p < 0.002$ and e, $p < 0.001$ compared with the untreated group.

DHEA. Analysis of phospholipid composition revealed that DHEA treatment in general had tendency to increase acidic phospholipids namely PI and PS. The effect was evident in animals receiving DHEA dose of up to 1.0 mg; at higher dose of 2.0 mg the effect was abolished. DHEA treatment up to 1.0 mg dose also had a PE lowering effect. Once again even in this case, at higher dose of 2.0 mg the effect was abolished (Table **4**).

The observed changes were also reflected in the contents of the two acidic phospholipids PI and PS which increased by 1.44 to 1.94 fold. Interestingly, the content of Lyso increased 1.71 fold in animals receiving 0.1 mg DHEA. There was a generalized tendency of increase in the content of SPM up to the dose of 1.0 mg. However, the content of SPM decreased by 11% in animals treated with 2.0 mg dose of DHEA. The contents of other phospholipid classes were unchanged (Table **5**).

DISCUSSION

The present studies were undertaken to examine the possible effect(s) of DHEA treatment on lipid/phospholipid profile on rat brain and liver mitochondria. From the data presented (Table **1-5**) it is clear that DHEA treatment influenced differently the lipid/phospholipid profiles of the mitochondria from the two tissues. Also, in general, in both the tissues the effects were evident at doses of DHEA of up to 1.0 mg and the effects diminished at the highest dose (2.0 mg) of DHEA.

The differential effects of DHEA treatment became manifest with respect to contents of TPL and CHL, and the composition and contents of the individual phospholipid components.

Thus, DHEA treatment significantly increased the TPL and CHL contents of brain mitochondria while it was with-

Table 4. Effect of DHEA Treatment on Phospholipids Composition of Rat Liver Mitochondria

Phospholipid Class	Composition, (% of Total)				
	Untreated (12)	0.1 mg DHEA (14)	0.2 mg DHEA (10)	1.0 mg DHEA (12)	2.0 mg DHEA (10)
Lyso	1.54 ± 0.08	1.76 ± 0.19	1.74 ± 0.12	1.69 ± 0.17	1.54 ± 0.18
SPM	3.00 ± 0.10	3.85 ± 0.30^b	3.32 ± 0.26	3.44 ± 0.33	3.33 ± 0.56
PC	46.91 ± 0.56	46.50 ± 0.82	46.71 ± 1.65	46.31 ± 1.18	45.97 ± 1.49
PI	1.76 ± 0.07	3.18 ± 0.28^d	3.26 ± 0.15^d	3.19 ± 0.52^b	2.19 ± 0.12
PS	1.87 ± 0.08	2.80 ± 0.19^d	2.99 ± 0.22^d	3.43 ± 0.44^c	2.31 ± 0.35
PE	33.20 ± 0.57	30.40 ± 0.39^d	29.24 ± 1.54^a	29.57 ± 1.65^b	31.27 ± 0.86
DPG	11.76 ± 0.44	10.51 ± 0.51	12.73 ± 0.59	12.38 ± 0.75	13.61 ± 0.63^a

Experimental details are as given in the text. Results are given as mean ± SEM of the number of observations indicated in the parentheses.
a, $p < 0.05$; b, $p < 0.02$; c, $p < 0.002$ and d, $p < 0.001$ compared with the untreated group.

Table 5. Effect of DHEA Treatment on Phospholipids Content of Rat Liver Mitochondria

Phospholipid Class	Content, (μg/ mg protein)				
	Untreated (12)	0.1 mg DHEA (14)	0.2 mg DHEA (10)	1.0 mg DHEA (12)	2.0 mg DHEA (10)
Lyso	2.79 ± 0.29	4.78 ± 0.52^b	3.31 ± 0.25	3.16 ± 0.23	2.28 ± 0.16
SPM	5.44 ± 0.55	6.59 ± 0.53	6.26 ± 0.46	6.47 ± 0.56	4.84 ± 0.30^a
PC	83.17 ± 6.29	79.84 ± 3.02	90.32 ± 7.69	86.33 ± 4.02	73.12 ± 6.24
PI	3.15 ± 0.29	5.54 ± 0.56^c	6.10 ± 0.57^c	6.07 ± 0.50^c	3.30 ± 0.24
PS	3.36 ± 0.35	4.84 ± 0.41^a	5.79 ± 0.47^c	6.52 ± 0.34^c	3.19 ± 0.27
PE	59.32 ± 4.98	52.35 ± 2.23	55.29 ± 5.43	55.21 ± 4.03	49.13 ± 3.25
DPG	20.72 ± 1.64	18.18 ± 1.24	24.59 ± 2.01	23.36 ± 2.10	20.77 ± 1.07

Results are given as mean ± SEM of the number of observations indicated in the parentheses.
a, $p < 0.02$; b, $p < 0.01$ and c, $p < 0.001$ compared with the untreated group.

out any effect on TPL content in the liver mitochondria; in the liver mitochondria only the CHL content increased marginally (Table **1**). The contents of Lyso and SPM increased substantially in the brain mitochondria in a dose-dependent manner with marginal effects being seen in the liver mitochondria. Under these conditions the content PI and PS increased in mitochondria from both the tissues with maximum effect being seen for the liver mitochondria. The content of PC, PE and DPG were generally not affected in either of the tissues except for a small but reproducible increase in DPG content of brain mitochondria from animals receiving 0.2 mg dose of DHEA (Tables **3** and **5**). These differential effects resemble the known tissue-specific effects of hormones. Interestingly, however, there is no known receptor of DHEA (Natawa *et al.*, 2002).That the effects diminished at the highest dose (2.0 mg) is not really surprising since adverse catabolic effects of pharmacologic doses of hormones such as thyroid hormones have been well documented (Satav and Katyare, 1981; Satav and Katyare, 1982).

Increased content of CHL in the brain mitochondria following DHEA treatment deserves some comment. It has been reported that the brain synthesizes its own cholesterol (Bjorkhem and Meaney, 2004). The increased content of CHL which we observe here resembles the similar increase in the brain mitochondria from developing animals (Pandya *et al.* 2004b). It has been reported that the content of CHL in the brains of Alzheimer's patients increases significantly which may have a role in β amyloid plaque formation (Kalman and Janka, 2005). Interestingly, however, the DHEA content in the brains of Alzheimer's patients is known to be significantly low compared to the age-matched controls (Weill-Engerer *et al.*, 2002). Apparently, this increase occurs due to decrease in brain specific CYP46A1 enzyme responsible for the degradation of cholesterol to a water soluble metabolite 24S- hydroxycholesterol rather than decreased synthesis (Kalman and Janka, 2005). The increased content of Lyso which we note in the present studies is suggestive of increased turnover. It may hence be suggested that DHEA treatment may selectively stimulate the biosynthesis of CHL in the brain. Important to no note in this context is the fact that the brain itself synthesizes DHEA, DHEA-S and pregnenolone (Racchi *et al.*, 2003). These three steroids are considered to be the neurosteroids and may possibly have a role in cerebral metabolism (Baulieu and Paul, 1998).

The increased content of PS is another interesting feature. It has been shown that in experimental animals and in human trials nutritional supplementation of PS improved memory and cognitive functions while PC was ineffective in this respect (McDaniel *et al.*, 2003). This observation assumes importance in the context of the fact that DHEA has been reported to have beneficial effects in improving memory and cognitive functions in elderly population (Buvat, 2003). Our results also show that while DHEA can influence the synthesis of PS and PI, apparently it has no or only marginal effect on synthesis of PC, PE and DPG. As pointed out above, the increased levels of Lyso which are suggestive of increased turnover rule out the possibility that the content of these phospholipids could have increased due to selective synthesis rather than due to decreased catabolism.

Changes in the fatty acid composition of serum, hepatic and adipose lipids in lean and obese Zucker rats, following treatment with DHEA have been reported by Abadie *et al.* (2000). Mc Intosh *et al.* (1999) reported decreased serum and liver lipid contents following DHEA treatment. Mohan and Cleary (1991) observed that the liver mitochondria in rats maintained on diet supplemented with DHEA had decreased levels of DPG and PE and increase in the content of PC. Our results on decrease in PE are consistent with the observations of Mohan and Cleary (1991). However, it may be pointed out that Mohan and Cleary (1991) fed the experimental animals diet containing 0.6 % DHEA. In our studies the animals received DHEA by s.c. route. It is possible that the differences in the dose regimen and route of administration may be responsible for the observed differences in our results.

Treatment with DHEA has been reported to lower the carbohydrate metabolism with simultaneous increase in metabolism of lipids (Miller *et al.* 1988; Song *et al.* 1989; Su and Lardy 1991). Abadie *et al.* 2000 reported that treatment with DHEA for 30 days resulted in significant alteration of fatty acid profiles of serum, hepatic and adipose lipid components in lean and obese Zucker rats. DHEA feeding also resulted in increased proportion of arachidonic acid in total lipids and decreased oleic acid in soleus as well as cardiac muscle (Abadie *et al.*, 2001; Imai *et al.*, 1999) reported that the increase in hepatic content of oleic acid induced by DHEA in the rat was attributable to the induction of stearoyl-CoA desaturase. McIntosh *et al.* observed that DHEA treatment reduced serum and hepatic lipids (1999). Mohan and Cleary observed that the mitochondria from rats treated with DHEA for 7 days had lower levels of cardiolipin and phosphatidylethanolamine and an increase in phosphatidylcholine (1991). Also, changes in fatty acid composition of these phospholipids occurred after 7 days and 24 h of DHEA treatment (1991). However, as far as we are aware, the detailed tissue-specific effects of DHEA treatment on compositional changes in the lipid/phospholipid profiles have not been reported thus far.

The compositional changes in the phospholipids which we observe here may lead to altered charge distribution across the mitochondrial inner membrane, which in turn could modulate the function of the components of the electron transport chain. Dependence of electron transport chain components of specific lipid/phospholipid components is well documented (Daum, 1985) Results of our other studies have shown that the increased respiratory activity in mitochondria was accompanied by the accretion of crucial components of the electron transport chain namely cytochromes, ATPase and dehydrogenases (Patel and Katyare, 2006a,b,c). The changes in lipid/phospholipids profiles which we report here would complement the function of these components. Abnormalities in lipid/phospholipid profiles are known to affect cerebral and mitochondria functions in pathological conditions such as Parkinson's disease, neural trauma, neurodegenerative disease and Alzheimer disease (Farooqui *et al.*, 2004).

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