Chapter 6

Dexamethasone Treatment Induced Changes in Lipid / Phospholipid Profiles of Rat Brain Mitochondria During Postnatal Development.

Introduction

Dexamethasone induced changes in oxidative phosphorylation in brain mitochondria have been detailed in Chapter 4 of the thesis Since the process of energy transduction requires the presence of lipid milieu (1), experiments were carried out to check the lipid/ phospholipid profiles of rat brain mitochondria following dexamethasone treatment in the rat belonging to different age groups The results of these experiments are described in this Chapter

Materials and Methods

Chemicals

Silica gel G was purchased from E Merck, Germany and 1,6 diphenyl-1,3,5 hexatriene (DPH) was purchased from Sigma, USA All other chemical were of analytical reagent grade and were purchased locally

Details of animal management, treatment with dexamethasone are as described in the earlier Chapter 2 of the thesis and isolation of mitochondria are as described in Chapter 4 of the thesis

Lipid analysis

Extraction of lipids

Aliquots of mitochondrial suspension containing 4 – 8 mg protein were extracted with 4 ml of freshly prepared chloroform methanol mixture (2 1 v/v) as described by Folch et al (2) The tubes were vortexed vigorously allowed to stand at room temperature and the organic phase was carefully removed with the help of a broad gauge syringe The samples were re-extracted with 3 ml of chloroform methanol mixture as above and the resulting

organic phases were pooled The pooled chloroform methanol extracts were treated with 0 1 volume of 0.017% $MgCl_2$, vortexed vigorously, allowed to stand at room temperature and organic phase was carefully removed with care being taken to avoid the proteolipid layer appearing between the organic and aqueous phases The solvent was completely evaporated under the stream of nitrogen, after which the lipids were dissolved in known volume of chloroform methanol mixture. Suitable aliquots were taken for the estimation of cholesterol (3) and phospholipid phospohorus in the sample (4) and thin layer chromatography (T L C)

Separation of phospholipids by thin layer chromatography (TLC)

Separation of phospholipid classes was carried out by one dimentional thin layer chromatography (5) using silica gel G A slurry of silica gel G (6 g/13ml distilled water per plate) was prepared by gentle mixing and spread on glass plates with the help of applicator with thickness of layer maintained to 0 25mm The layer was allowed to dry by leaving plates overnight at room temperature Prior to use the plates were activated in an oven at 100°C for 20-25 min

Aliquots of the reconstituted samples containing 8-10 µg of phospholipid phosphorus were spotted on TLC plate in a way such that the diameter of the spot was minimum which ensured better resolution The conditions for preparation of TLC plates, chamber saturation etc were according to Stahl (5) The solvent used for the chamber saturation was chloroform methanol acetic acid water (25 15 4 2 v/v) Before run, the plates were reactivated for 2min at 110 ^{0}C After the run was completed the plates were taken out and kept at room temperature for 3 to 4 hours to remove the solvents

After brief exposure of iodine vapor spots of individual phospholipid were marked and iodine was allowed to sublime off After this the spots were carefully scraped and transferred to marked test tubes To each tube 0 5 ml of 10 N H_2SO_4 were added and the samples were heated on a sand bath for 8-10 hours The tubes were allowed to cool afterwhich 0 1ml of 70% perchloric acid was added The tubes were then heated on the sand bath for 3-4 hours till the solution in the tubes were clear and smell of chlorine was undetectable The analysis of phosphorus content was according to the procedure of Bartlett(4)

Determination of membrane fluidity

Membrane fluidity determination was carried out at 25 °C spectrophotoflourimetrically using DPH as the probe Stock DPH solution (2mM) was prepared in tetrahydrofuran and stored at 0-4 °C in an amber coloured bottle For measurement of fluorescence polarization, samples were taken in 3ml of buffered sucrose solution (0 25M sucrose containing 10mM Tris-HCl, pH 7 4) at a final protein concentration of 0.2 mg/ml, and an aliquot of stock DPH solution was added so that the molar ratio of probe to lipid was between 1 200 to 1 300 (6,7) The mixture was vortexed vigorously and left in dark for 30 min to permit equilibration of probe into membranes Fluorescence polarization was measured in a Shimadzu RF 5000 spectrophotofluorimeter with a polarizer attachment Excitation and emission wavelengths were 360nm and 430nm, bandwidths were 5nm and 10nm respectively Data were accumulated for 5 sec for each polarization setting vertical (parallel) and horizontal (perpendicular) (7) The instrument has program for calculation of fluorescence polarization (P) from which the value of r, rα and S can be calculated The details of the methods have been described previously (8)

Protein estimation was according to method of Lowry et al (9)

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

Results

The age dependent changes in the data on total phospholipid (TPL) and cholesterol (CHL) content in rat brain mitochondira are shown in Fig 1(A-C) As can be noted, in the control animals the TPL content increased by about 25% in 4 week animals and remained at this level through the 5th week. in the adults a further 65% increase was seen CHL content showed progressive increase with development and compared to the 2 week group the CHL content in the adults was 2 to 3 fold higher The increase in CHL content from 3 week onwards parallels the overall process of brain development (10) Consequently, compared to the 2 week animals, TPL/CHL molar ratio was significantly low in all the age groups

Dexamethasone treatment had a negative effect on the TPL content which was evident especially from the 4th week onwards. the decrease in TPL content was progressive By contrast, dexamethasone treatment significantly elevated CHL content in 3 and 4 week animals Opposite effect i e decreased CHL content was seen for the 5 week and adult groups On a relative scale the TPL/CHL molar ratio were significantly low in 3 week, 4 week and adult animals By contrast this ratio was very high in the 5 week groups

The analysis of phospholipid (PL) composition (Fig 2,A-G) revealed that lysophospholipid component was relatively low in the control group throughout the developmental period and dexamethasone treatment generally suppressed it further Sphingomyelin is increased in 5 week group which is consistent with cerebeller development(11) Dexamethasone treatment effect was age specific, 2 week and 4 week

Fig. 1 Effect of dexamethasone treatment on rat brain mitochondrial (A) total phospholipid (TPL), (B) cholesterol and (C) TPL/CHL (mole mole) The TPL and CHL content is given as expressed as μg/mg protein Each value represent the mean ± SEM of 22 independent observations

[a] p < 0 05, [b] p< 0 01 and [c] p<0 001 as compared with corresponding control group

Fig. 1

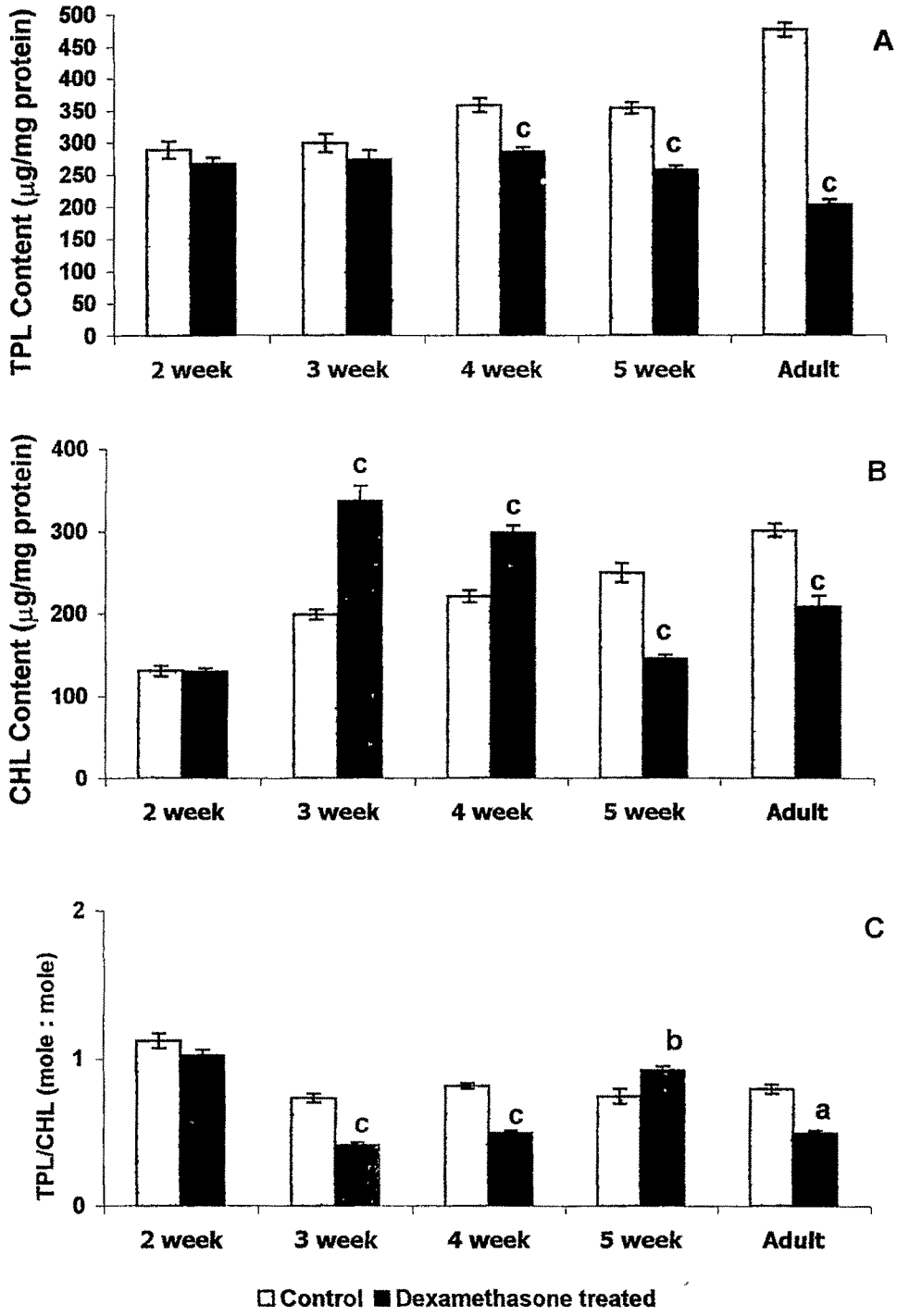
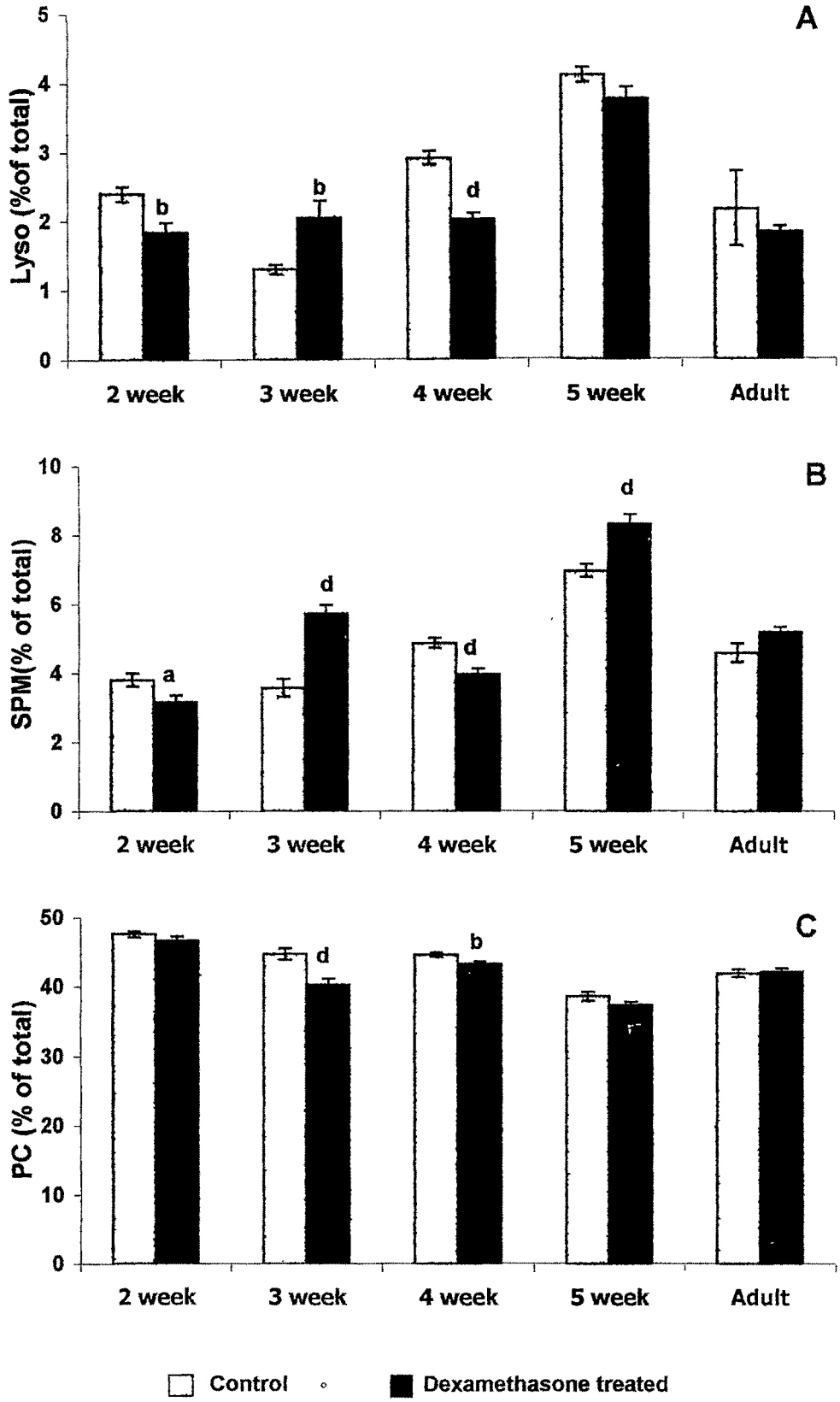
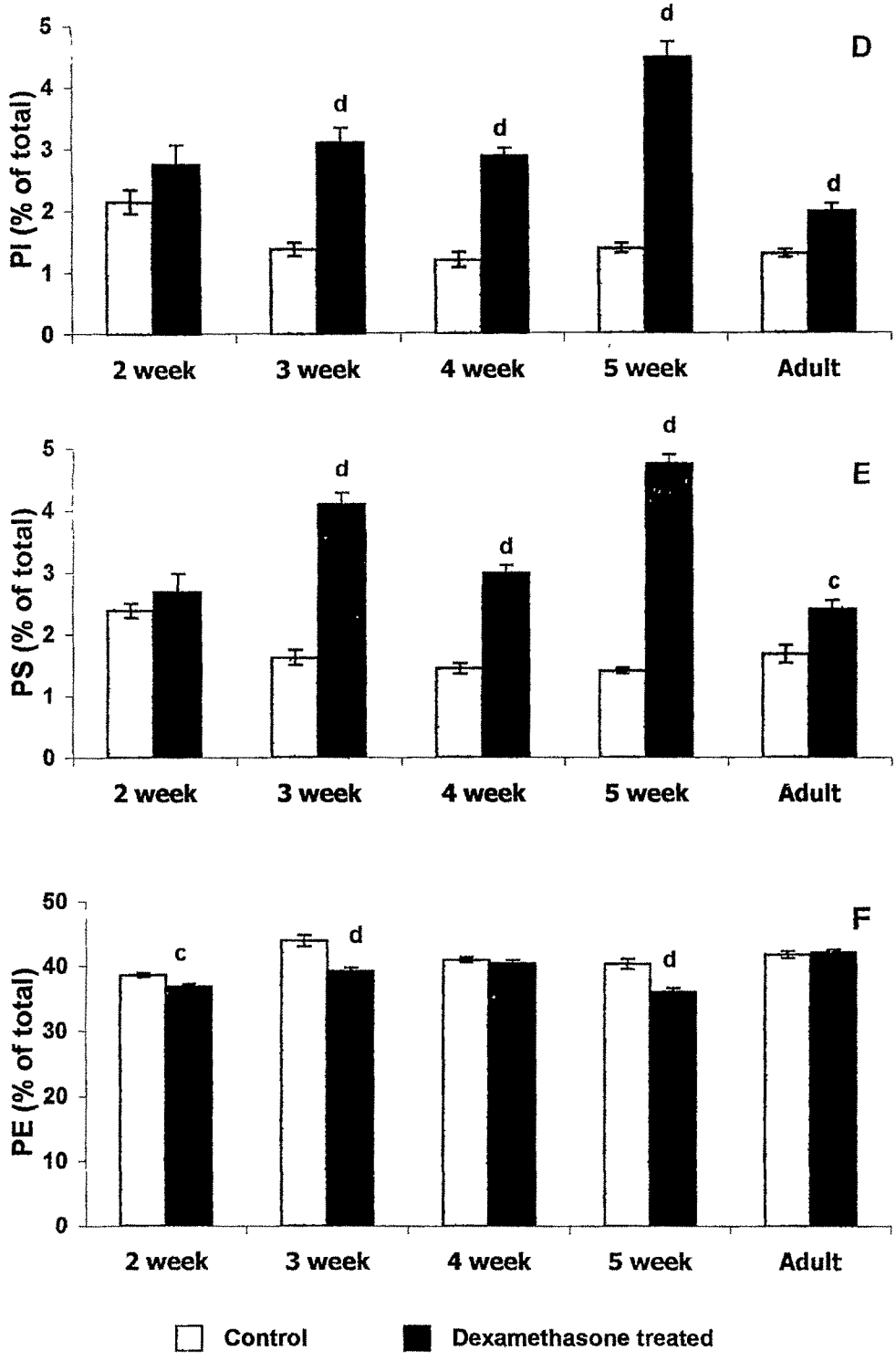

A
TPL Content (μg/mg protein)
500
450
400
350
300
250
200
150
100
50
0
c
c
c
2 week
3 week
4 week
5 week
Adult
B
CHL Content (μg/mg protein)
400
300
200
100
0
c
c
c
c
2 week
3 week
4 week
5 week
Adult
C
TPL/CHL (mole : mole)
2
1
0
c
c
b
a
2 week
3 week
4 week
5 week
Adult
Control
Dexamethasone treated

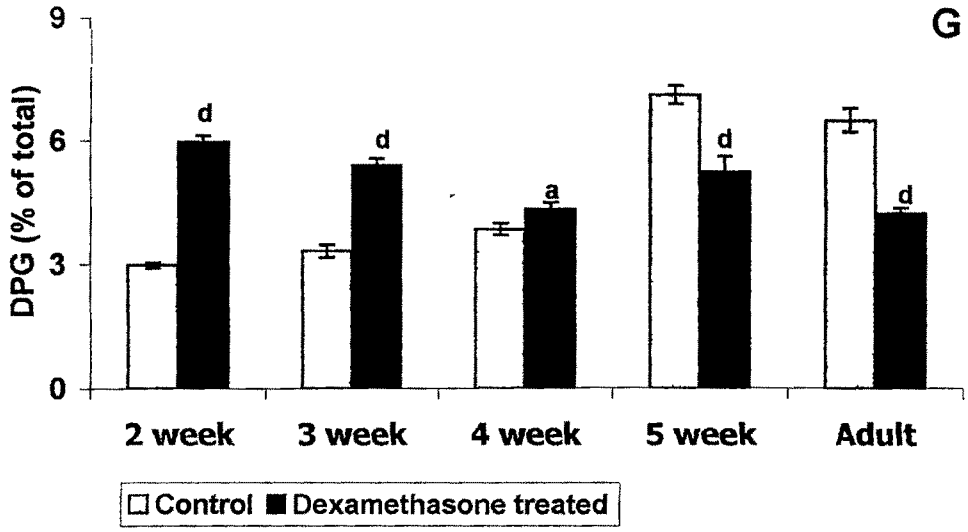
Fig. 2 Effect of dexamethasone treatment on rat brain mitochondrial phospholipid composition during development A) Lyso, B) SPM, C) PC, D) PI, E) PS, F) PE, G) DPG

[a] $p < 0\ 05$, [b] $p < 0\ 01$, [c] $p < 0\ 002$ and [d] $p < 0\ 001$ as compared with corresponding control group

Fig. 2


A
Lyso (%of total)
5
4
3
2
1
0
b
b
d
d
2 week
3 week
4 week
5 week
Adult
B
SPM(% of total)
10
8
6
4
2
0
a
d
d
d
2 week
3 week
4 week
5 week
Adult
C
PC (% of total)
50
40
30
20
10
0
d
b
2 week
3 week
4 week
5 week
Adult
Control
Dexamethasone treated


D
PI (% of total)
5
4
3
2
1
0
d
d
d
d
2 week
3 week
4 week
5 week
Adult
E
PS (% of total)
5
4
3
2
1
0
d
d
d
c
2 week
3 week
4 week
5 week
Adult
F
PE (% of total)
50
40
30
20
10
0
c
d
d
2 week
3 week
4 week
5 week
Adult
Control
Dexamethasone treated


G
9
6
3
0
DPG (% of total)
d
d
a
d
d
2 week
3 week
4 week
5 week
Adult
Control
Dexamethasone treated

animals registered decrease while 3 and 5 week animals showed elevation in SPM The PC decreased somewhat during development Dexamethasone treatment had only marginal lowering effect on PC in 3 and 4 week animals The PI and PS contents were around 2% in 2 week animals but decreased with development Dexamethasone treatment had tendency to increase both PS and PI in all the age groups Maximum increase was seen in the 4 week animals The PE increased somewhat with development Dexamethasone treatment induced small decrease (5 to 11%) in 2,3 and 5 week animals The DPG content was maximum bv the 5th week and remained so in the adults Dexamethasone treatment resulted in elevation of DPG up to 4th week Maximum effect (100% increase) was seen in 2 week animals and the minimum effect (i e 7% increase) was seen in 4 week animals. By contrast in 5 week and adult animals dexamethasone treatment caused a progressive 26 and 35% decrease

The changes were also reflected the content of the individual phospholipids derived from the TPL content and phospholipid composition of individual samples These results are shown in Fig. 3(A-G)

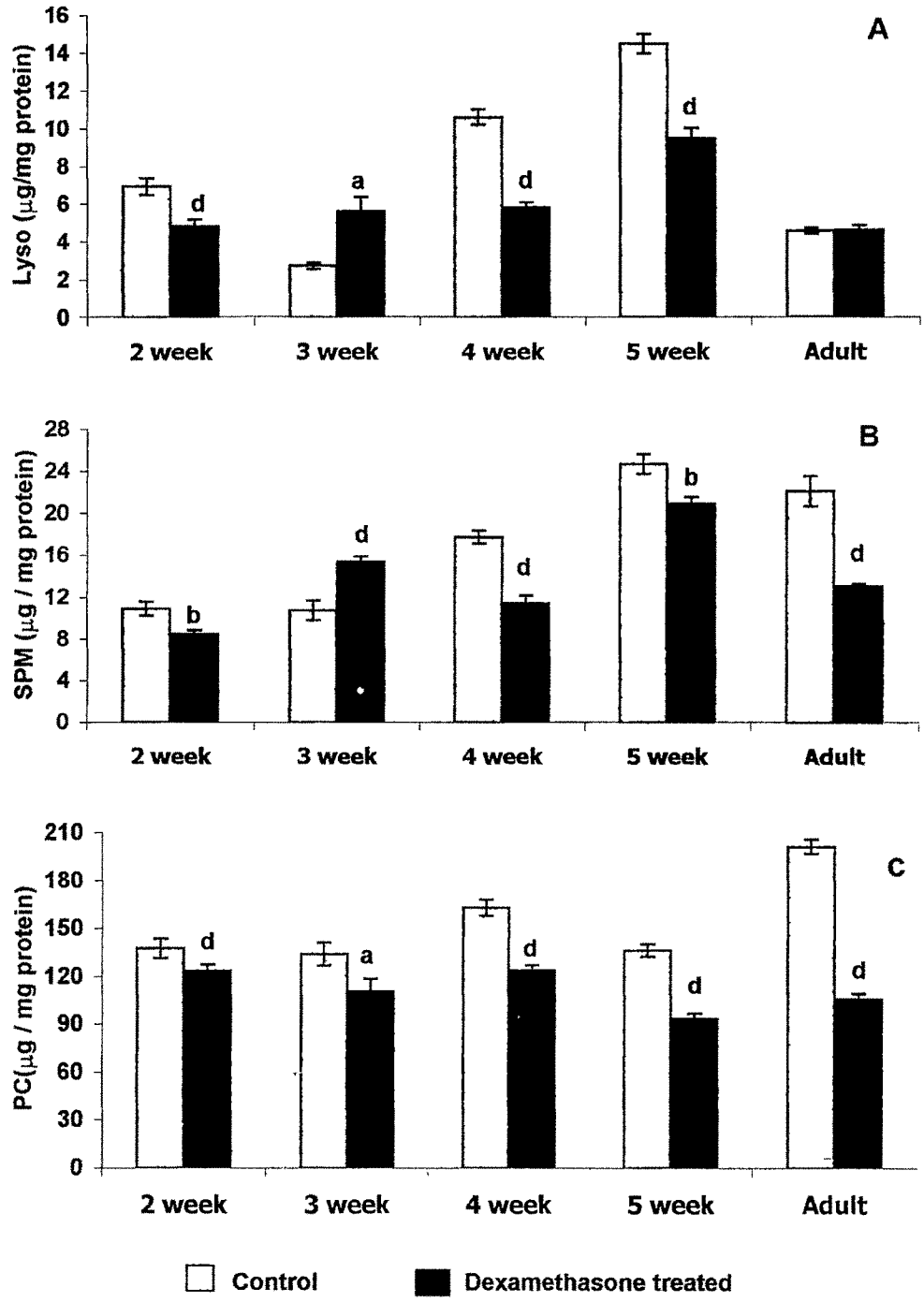
In the controls the fluidity of the membrane was unaltered up to the 4th week but decreased significantly in 5 week animals and in the adults Dexamethasone treatment resulted in membrane fluidization in 5 week and adult groups (Table 1)

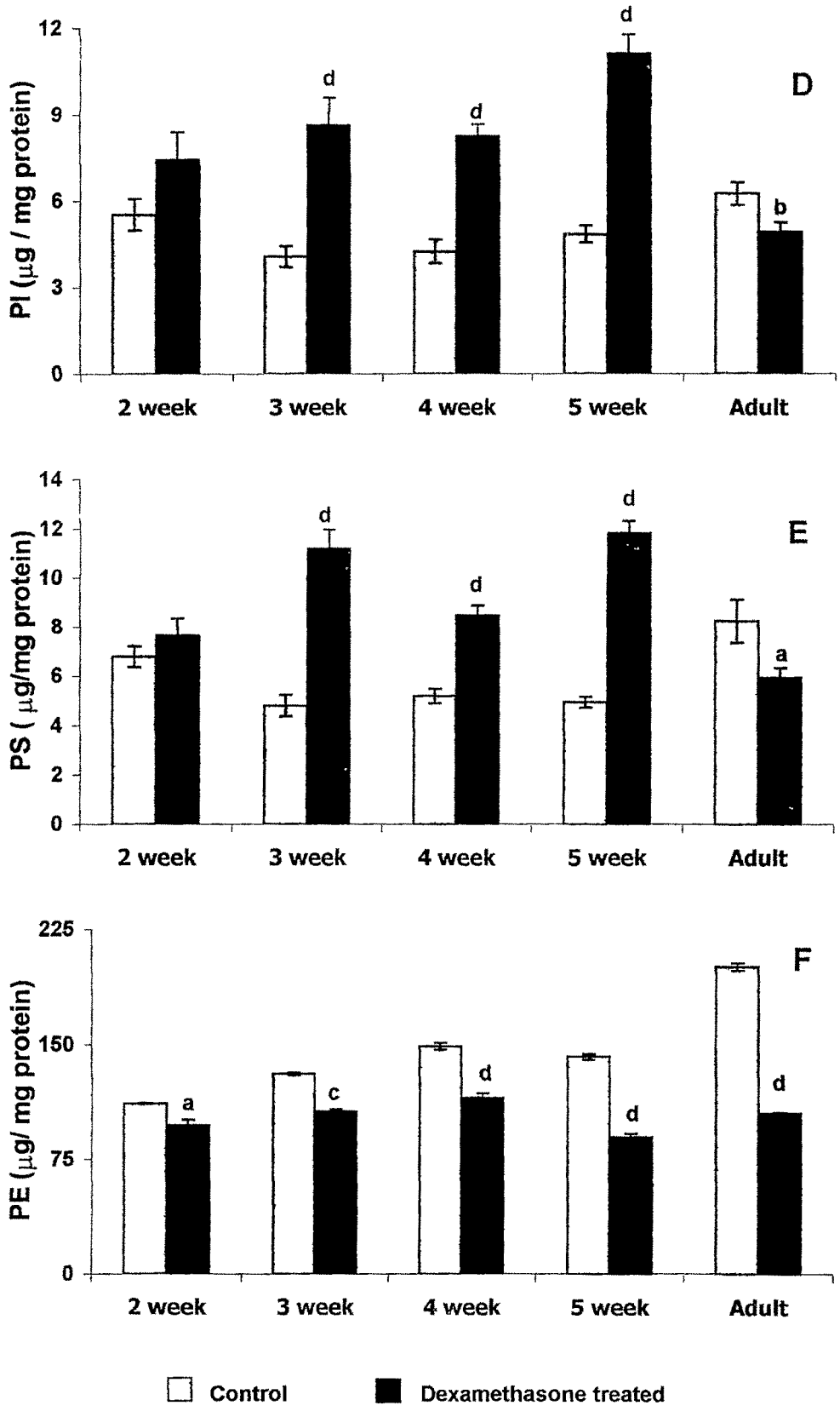
In the control group fluidity was negatively correlated with TPL and CHL content but was independent of the molar ratio Following dexamethasone treatment the fluidity correlated positively with CHL content but with TPL content there was no correlation The TPL/CHL ratio showed negative correlation The fluidity correlated positively with

Fig. 3 Effect of dexamethasone treatment on rat brain mitochondrial phospholipid content (μg/mg protein) during development A) Lyso, B) SPM, C) PC, D) PI, E) PS, F) PE, G) DPG

[a] $p < 0\ 05$, [b] $p< 0\ 02$, [c] $p< 0\ 01$ and [d] $p<0\ 001$ as compared with corresponding control group

Fig. 3


A
Lyso (μg/mg protein)
16
14
12
10
8
6
4
2
0
d
a
d
d
2 week
3 week
4 week
5 week
Adult
B
SPM (μg / mg protein)
28
24
20
16
12
8
4
0
b
d
d
b
d
2 week
3 week
4 week
5 week
Adult
C
PC(μg / mg protein)
210
180
150
120
90
60
30
0
d
a
d
d
d
2 week
3 week
4 week
5 week
Adult
Control
Dexamethasone treated


D
PI (µg / mg protein)
12
9
6
3
0
d
d
d
b
2 week
3 week
4 week
5 week
Adult
E
PS (µg/mg protein)
14
12
10
8
6
4
2
0
d
d
d
a
2 week
3 week
4 week
5 week
Adult
F
PE (µg/ mg protein)
225
150
75
0
a
c
d
d
d
2 week
3 week
4 week
5 week
Adult
Control
Dexamethasone treated

G

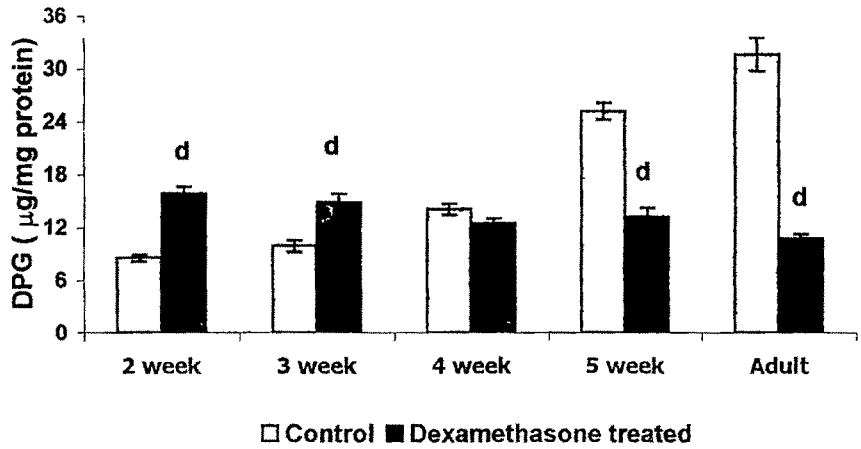

36
30
24
18
12
6
0
DPG (μg/mg protein)
d
d
d
d
2 week
3 week
4 week
5 week
Adult
□ Control ■ Dexamethasone treated

Table 1 The effect of dexamethasone treatment on fluidity parameters of rat brain mitochondria

Treatment Group	Fluidity parameter			
	Fluorescence polarization, p	Fluorescence anisotropy, r	Limited hindered anisotropy, rα	Order parameter, S
2 week Control (22)	0 213 ± 0 007	0 153 ± 0 006	0 104 ± 0 007	0 794 ± 0 015
Dex (16)	0 238 ± 0 025	0 183 ± 0.025	0 144 ± 0 034	0 783 ± 0 019
3 week Control (18)	0 215 ± 0 004	0 154 ± 0 003	0 106 ± 0 006	0 819 ± 0 019
Dex (22)	0 210 ± 0 008	0 151 ± 0 006	0 102 ± 0 008	0 774 ± 0 025
4 week Control (16)	0 211 ± 0 005	0 154 ± 0 003	0 106 ± 0 006	0 817 ± 0 013
Dex (22)	0 212 ± 0 004	0 152 ± 0 003	0 103 ± 0 004	0 810 ± 0 010
5 week Control (22)	0 235 ± 0 004	0 170 ± 0 004	0 127 ± 0 005	0 855 ± 0 006
Dex (28)	0 211 ± 0 005 [e]	0 152 ± 0 004 [c]	0 102 ± 0.005 [d]	0 788 ± 0 015 [e]
Adult Control (24)	0 231 ± 0 006	0 167 ± 0 005	0 123 ± 0 006	0 843 ± 0.011
Dex (16)	0 211 ± 0 004 [c]	0 151 ± 0 003 [e]	0 102 ± 0 003 [a]	0 811 ± 0 013

The experimental conditions are as described in the text The results are as mean ± SEM of the number of observations indicated in the parenthesis

[a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$, [d] $p < 0.002$, and [e] $p < 0.001$ as compared with the corresponding control

PC/PE ratio and basic phospholipid (BPL) content The reverse effect was seen for SPM/PE and SPM/PC ratios After dexamethasone treatment the correlation with PC/PE or SPM/PE ratio was reversed and no correlation with SPM/PE was seen The fluidity showed a strong negative correlation with DPG content with both control and dexamethasone treated group

Discussion

Influence of glucocorticoids on lipid metabolism is very well recognized (12) Glucocorticoids are known to alter the synthesis of triacylglycerol, cholesterol and phospholipid in different tissues including brain Glucocorticoids have been shown to induce marked decrease in cholesterol synthesis in various target tissues which is related with control of cell proliferation (13) Ramchandran et al (14) shown that dexamethasone action was linked to HMG CoA synthase synthesis

The glucocorticoid have been shown to inhibit fatty acid synthesis in HeLa cells (14,15) and lymphocytes whereas in the lungs they are known to enhance synthesis of lecithine which are involved in the constitution of lung surfactants (16) Brenner et al (17) showed that the dexamethasone treatment depressed D 5 and D 6 desaturase activities while increased D 9 desaturase and synthase activity in liver This author also reported that the phospholipid/ CHL molar ratio in the liver microsomes was somewhat lower, additionally this authors found that there was a tendency to decrease PC while PE, PI, PS tended to be higher Interestingly the arachidonic acid synthesis decrease while oleic and palmatic acid synthesis increase significantly there by lowering 18 0/ 20 4n-6 ratio in PC (17)

However, membrane fluidity was not altered Glucocorticoids are known to regulate the expression of glycerol phosphate dehydrogenase (α G3PDH), myelin basic protein, proteolipid protein and glial fibrillary acidic protein in developing rat brain (18) Importance of α G3PDH in providing precursor for biosynthesis of phospholipid is well recognized (19) However the effect of dexamethasone treatment on mitochondrial lipid/ phospholipid metabolism has not been reported thus form the data presented it is clear that dexamethasone treatment in general adversely affected the total lipid content of the brain mitochondria but resulted in increased CHL content in 3 and 4 week group Thus, the effects seem to be age specific This was also evident from the TPL/CHL molar ratios which were differentially affected in different groups The differential effects were also noted for the composition of Lyso, SPM and DPG Interestingly, dexamethasone treatment resulted in significant increases in the PI and PS components in all the age groups, thus suggesting the regulatory role of corticosteroids in acidic phospholipid biosynthesis (20) Dexamethasone treatment also resulted in substantially increased in the mitochondrially synthesized acidic phospholipid DPG up to 4th week of age: opposite effect was noted in the 5 week and adult animals The overall effect therefore seems to be that dexamethasone treatment interferes with the normal developmental pattern of the brain The earlier studies have shown that dexamethasone treatment brought about early significant stimulation of α G3PDH activity Despite that the phospholipid content of the mitochondria was generally low

Other studies referred to above (21) have shown that the changes in liver microsomal phospholipid composition were at marginal nature If the similar situation exist for brain microsomal phospholipid biosynthesis it is possible that possibly the transfer of

phospholipid from microsomes to mitochondria may be impaired by dexamethasone treatment

In spite of the significant changes in the phospholipid content and composition, the membrane fluidity increased only in the 5 week and adult groups Which seems somewhat puzzling However DPH is known to monitor bulk membrane fluidity Properties Therefore if micro heterogeneity in membrane fluidity existed, this was not detected by the DPH probe

Summary

Dexamethasone treatment resulted in decreased mitochondrial TPL content in rats from 4th week onwards till the adult stage. The CHL content decreased during the 4th and 5th week but was about 35 to 70 % increase was observed in 3 and 4 week groups Dexamethasone treatment altered the phospholipid composition which showed that in general the acidic phospholipids i e PI and PS increased in all age groups Similar effect was noted for DPG up to 4th week However, in later stages DPG decreased Effects on other phospholipid classes was either variable or marginal Dexamethasone treatment caused membrane fluidization in 5 week and adult groups

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