Synopsis

BIOCHEMICAL INVESTIGATIONS ON CARDIOVASCULAR FUNCTIONS IN DIABETES

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Diabetes mellitus, the clinical expression of absolute or relative insulin deficiency, afflicts more than 20 to 30 million people throughout the world. Current treatment of diabetes can lower the blood glucose level but can not prevent or rectify secondary complications. Long-term diabetes affects several tissues which include nerves, lens, large and small blood vessels, kidneys and retina. Amongst these secondary complications none is more detrimentally affected than the cardiovascular system. Overall, 66 % of all morbidity and mortality in diabetes is due to cardiovascular disease. The incidences of choronary heart disease (CHD) and congestive heart failure (CHF) are higher in diabetics compared with their age, weight and sex matched controls. Amongst the diabetics, the incidences of CHD and CHF are higher in females compared to the males. The epidemiological data suggest that classic cardiovascular risk factors can not account for all excess risk of CHD mortality and morbidity associated with diabetes. Several studies show biochemical, histological and functional alterations in diabetic hearts. The underlying biochemical defects, however, are not clearly delineated, although basement membrane alteration is recognized as the primary defect.

Heart has adrenergic as well as cholinergic innervations and cholinesterase is responsible for termination of nerve signal i.e. hydrolysis of the neurotransmitter acetylcholine. The heart tissue contains both acetylcholinesterase (AChE) as well as butyrylcholinesterase (BChE) activity. The BChE

activity is about 85 % of the total cholinesterase activity, while AChE activity is about 15 %. However it is believed that AChE is responsible for physiologic hydrolysis of acetylcholine. Function of BChE in the heart is unclear, and needs attention. Earlier workers have shown that kinetic properties of the membrane-bound form but not of the soluble form of AChE in the brain are altered significantly in the alloxan-diabetic rats. Other workers have shown that plasma BChE activity increases in diabetic rats and that especially in the female rat serum BChE activity is significantly higher than in the male rats. It has also been reported that induction of diabetes with streptozotocin in rats caused a significant rise in serum BChE activity.

Several studies have shown diabetes-induced alterations in membrane structure-function in different systems i.e. those from erythrocytes, platelets, sciatic nerve, kidneys, liver etc.

It is interesting to note that erythrocyte membranes contain AChE activity while the serum contains BChE activity. In view of the above, studying AChE activity in erythrocyte membrane can serve as a non-invasive parameter to monitor the progress and / or control of the disease. However, since the physiologic significance of AChE on erythrocyte membrane is not clear, in parallel studies we have also monitored the properties of Na^+,K^+ ATPase as an additional marker for

membrane function. Simultaneously we have also determined the BChE activity in the serum. These studies were carried out in humans and experimental animals i.e. rats.

Since it was not possible to carry out studies on the role of BChE in heart of diabetic patients in relation to increased incidences of CHD and CHF, we have employed alloxan-diabetic rat as a model system.

BChE in several respect resembles AChE except for its substrate specificity. However it is not known whether this enzyme in the heart exists in the membrane-bound form or in the soluble form. With a view to illustrating this point we have isolated the soluble and membrane-bound forms of BChE from the heart and checked the kinetic properties of the two forms of BChE. Studies were carried out in alloxan-diabetic male and female rats in comparison with the corresponding sex and age matched controls. Effect of insulin treatment was also examined. It is expected that these comparative studies on male and female rats may provide same insights regarding the mechanisms underlying the increased incidences of CHD and CHF in the females.

The present study has been divided in two parts : A) human studies, and B) rat studies.

A) Human studies :

For human studies, the volunteers were grouped in four groups : a) non-diabetic control (control) b) untreated diabetic (UTD) c) insulin-treated diabetic (ITD) and d) tablet treated diabetic (TTD). The diabetics in the group (d) received sulfonylurea type of drugs for varying durations. Every effort was made to get age and sex matched individuals in all the groups. Mean age of the volunteers in the all groups was about 52 years. The diabetic status was ascertained in terms of serum glucose level. Thus the values of serum glucose levels were about double in the UTD compared with the non-diabetic controls; in both the treated groups these values were somewhat lower than in the UTD group but were still higher than in the controls. Serum protein glycosylation was somewhat high in UTD, ITD and TTD groups but the increase was not statistically significant. However, for erythrocyte membrane proteins the glycosylation value in the UTD group showed a significant increase (more than three fold). Insulin treatment had no effect and in fact the extent of glycosylation further increased and the value was found to be five fold higher as compared to the controls. In the TTD group the extent of glycosylation was somewhat lower than in the UTD group but the value was still 2.5 fold higher than in the controls.

Activities of two erythrocyte membrane enzymes, Na^+,K^+ ATPase and AChE, and one serum enzyme BChE were checked. Na^+,K^+

ATPase activity decreased in UTD group. Insulin treatment brought about an increase in the enzyme activity but still it was not comparable with the controls. In the TTD group, the extent of decrease in the enzyme activity was even greater. Thus the enzyme activity was about one fourth of that seen in the control group. AChE activity did not change significantly in UTD and ITD groups but in the TTD group it became about half. BChE activity decreased in UTD group and no improvement was seen after insulin treatment. However in the TTD group the activity increased somewhat but was still lower than that in the control.

In view of the differential effects on the enzyme activities as outlined above, we have also checked effect of <u>in vitro</u> insulin addition on the enzymes activities. Pre-incubation with insulin resulted in decreased Na^+,K^+ ATPase activity in the ITD and TTD groups with the extent of decrease in activity being lower in the TTD than in the ITD group. Only in ITD group erythrocyte membrane AChE activity decreased with 10^{-7} M insulin concentration. For the rest of the groups only marginal changes were observed. No effect of insulin was seen on serum BChE activity in all the groups.

Experiments were also performed to check the substrate kinetic properties of the three enzymes. Thus in the erythrocyte membrane Na^+,K^+ ATPase from the control group two components were observed : one with high affinity and the

other with low affinity. In UTD group the Vmax of both the components and Km of component II decreased drastically. In ITD group the Km of both the components increased and Vmax of component I decreased, while in TTD group Km of component I increased and Km of component II and Vmax of both the components decreased significantly.

In erythrocyte membranes from the control group two components of AChE differing in their Km and Vmax were noted. No significant difference was observed in UTD and ITD. In the TTD group Vmax of both the components decreased.

In serum three components of BChE were found to be present. In UTD group Km of component I and Vmax of the first two components decreased. In ITD group Km and Vmax of component I and III decreased while component II was absent. In TTD group Km and Vmax of all three components decreased. These results are covered in Chapter II of the thesis.

In continuation of the above studies, possible erythrocyte membrane-structure function alterations were ascertained in terms of different parameters. These included : total phospholipid (TPL) and cholesterol (CHL) content of erythrocyte membranes, and phospholipid composition and membrane fluidity. TPL values were unchanged in the UTD group but decreased significantly in both the treatment groups. The CHL value was also unchanged in UTD but was significantly elevated in insulin treated group. In TTD group, the values

were comparable to the controls. The TPL / CHL ratio did not change in UTD but decreased significantly in both the treatment groups.

Erythrocyte membrane fluidity decreased in diabetic condition, but after insulin treatment, the membrane fluidity increased. In the TTD group, membrane fluidity was comparable with the control.

In erythrocyte membranes from the control group sphingomyelin (SPM), phosphatidylcholine (PC) and phosphatidylethenolamine (PE) were the major phospholipid components, while lysophosphatidic acid (Lyso), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA) were present as minor components. In UTD, only PI and PS increased significantly with marginal decrease in the PC component; all other phospholipid components were more or less the same as the control. In the ITD group the LYSO and PC values decreased significantly, while the PS content was still elevated. In ITD group Lyso, PI and PA decreased as compared to the UTD group. In TTD group SPM, PI and PS value increased, while PC value decreased as compared with the control group. If we compare TTD group with UTD group, there only increase in SPM was observed, while no changes were found in the rest of the phospholipid components.

Temperature kinetics analysis of erythrocyte membrane AChE

showed that the phase transition temperature (Tt) was near physiological temperature. In higher temperature range the energy of activation (E1) was higher and in lower temperature range the energy of activation (E2) was lower. No major changes were observed in the UTD group. In ITD group two trends were observed. Half of the population showed phase transition and E1, E2 and Tt values comparable with the control and the UTD groups, while in the remaining half of the population, phase transition was abolished. Similar two trends were observed even in the TTD group. Also in the sub-group of TTD having phase transition E1 value decreased as compared to the control and the UTD, while in sub-group of TTD having no phase transition E2 value was higher as compared to the control and the UTD groups.

Temperature kinetics analysis of BChE from control serum showed no phase transition; in the UTD group the phase transition was observed and the E1 value decreased as compared to the control. In the ITD group two trends were noted; half the population showed phase transition. Here the values of E1, E2 and Tt were comparable to those of the UTD. The remaining half did not show phase transition. In TTD group, phase transition was evident and values of E1, E2 and Tt were comparable with the UTD. These results are contained in Chapter III of the thesis.

B) Rat studies:

For the rat studies, mainly three groups : control, diabetic and insulin-treated diabetic were used. Experiments were carried out on the rats belonging to both the sexes i.e. males and females; diabetes was induced by injecting alloxan subcutaneously. In diabetic group of both the sexes decrease in the body weight was observed; after insulin treatment the body weight was restored almost to the control value. Diabetic state was ascertained in terms of hyperglycemia, glycosurea and polyurea. After insulin treatment serum glucose and urine volume were comparable with the control and sugar was absent in the urine.

Experiments were carried out to measure the TPL and CHL contents in the erythrocyte membranes. Thus in the control male rats the values of TPL and CHL were 494 and 200 µg / mg protein respectively. TPL value increased by about 3.2 fold in the diabetic group, with significant decrease in the CHL content. In ITD it decreased somewhat but was still about 2 fold higher than the control. The CHL value increased significantly after insulin treatment and became 2.3 fold higher compared to the control. These changes were also reflected in TPL / CHL molar ratio values.

In the erythrocyte membranes from the control female rats the values of TPL and CHL content were comparable to those in the

males. TPL value increased by 80 % in the diabetic group and decreased somewhat after insulin treatment. CHL value also increased in the diabetic group by 40 % but after insulin treatment it decreased although it was still higher than in the controls.

In erythrocyte membranes from control male and female rats major phospholipids were PC and PE, while the other component i.e. Lyso, SPM, PI, PS and PA. ranged from 2.0 to 10 %. The values for the males and females were comparable. In the male diabetic rats PC value increased while SPM and PI values decreased; after insulin treatment the SPM value increased beyond control. The PI value also increased but was still low compared with the controls. PC was restored to control level.

In female diabetic rats also the PC value increased significantly with drastic decrease in the PI and PS values. After insulin treatment SPM value increased beyond normal but PI value became 2.0 fold higher than in the control; PS value was comparable with the controls.

Erythrocyte membrane fluidity was checked in all the groups. In membranes from male rats the fluidity decreased in the diabetic group; insulin treatment hardly had any effect. In the female rats the membrane fluidity increased in the diabetic group and could be restored after insulin treatment. These results are included in Chapter IV of the thesis.

The erythrocyte membrane Na^+,K^+ ATPase activity increased in diabetic male rats and remained high after insulin treatment, whereas in the females the activity decreased in diabetic condition and after insulin treatment it increased and was higher than in the control. In the erythrocyte membranes from both males and females the AChE activity was almost the same. No AChE activity could be detected in the diabetic and insulin-treated diabetic rats.

In erythrocyte membranes of male rats the Na^+,K^+ ATPase substrate kinetics data showed presence of only one component in all the group. In diabetic condition Vmax value increased; after insulin treatment both Km and Vmax value increased further. In the female rat erythrocyte membrane Na^+,K^+ ATPase substrate kinetic data showed presence of two components : one having high affinity and the second one having low affinity in all the groups. In diabetic condition Vmax of both the components decreased and remained lower than in the controls; insulin treatment caused further greater increase.

Erythrocyte membrane AChE substrate kinetics data showed presence of three components in the male rats, one having high, second having intermediate and the third having low affinity. The same trend was observed in the females, although the Vmax of component I was somewhat lower. Surprisingly, as pointed out above it was found that the AChE activity was not detectable in the erythrocyte membranes

from the diabetic and insulin-treated diabetic rats of both the sexes.

Temperature kinetics data on erythrocyte membrane AChE from male rats showed presence of phase transition. For higher temperature range energy of activation (E1) was higher and for lower temperature range energy of activation E2 was lower and phase transition temperature (Tt) was near physiological temperature. In the females for higher temperature range energy of activation E1 was lower and for lower temperature range energy of activation E2 was higher. Phase transition temperature was decreased by about half in the females. These data are included in Chapter V of the thesis.

Serum BChE activity data show that in the control group the activity was about 4.4 fold higher in the females than in the males. In diabetic males the activity increased more than two fold and decreased somewhat after insulin treatment. In the female diabetic rats the activity was almost the same as in the controls; insulin treatment had no effect.

Substrate kinetics data showed presence of two components of BChE in the male serum, one having high affinity and the second having low affinity. Km of component II and Vmax of both the components increased in diabetic condition. After insulin treatment Km of component II decreased, while Vmax of both the components remained elevated.

In the female serum also two components of BChE were noted. Comparison of the control females and the control males revealed that the Km of both the components was lower in the females, while the Vmax of both the components was 4-5 fold higher. In diabetic females values of Km and Vmax were unchanged for both the components and insulin treatment had only marginal effect.

Temperature kinetics analysis of BChE in the control male serum showed that for higher temperature range the energy of activation (E1) was low and for lower temperature range the energy of activation (E2) was high. Diabetic condition had no effect on E2 but E1 was somewhat higher. After insulin treatment E1 value decreased and became comparable to the control, but the E2 value decreased significantly. Tt was unaffected under all the conditions.

The trend was about similar for the control AChE activity in the males and the female AChE except that E2 value was lower in the females than in the control males. In diabetic and insulin treated females only E2 value increased. Tt was unaffected. These results are included in Chapter VI of the thesis.

In the female rats soluble fraction of heart BChE activity was more than three fold higher than the males. In the diabetic male rats the activity increased by about three fold

and after insulin treatment it further increased and became five fold higher, while in the female rats in diabetic condition the activity decreased and after insulin treatment it increased and remained higher than in the controls.

In the next set of experiments, substrate kinetic analysis of the soluble and membrane-bound BChE in the heart was examined. In male rat heart two components of BChE were present : one having high affinity and the second having low affinity. In diabetic condition Km of component I decreased while the Vmax of component I as well as component II increased by about 3 fold. Insulin treatment caused further greater increase (7 to 10 fold increase) in the Vmax of both the components with decrease in the Km values.

In the female rat heart, for the soluble BChE in the control group Vmax of both component I and II was higher than in the male rats. Diabetic state had no effect. However, as in the case of the males, after insulin treatment, the Vmax of both component I and II increased and became comparable to the corresponding males, although the extent of increase was of much lesser magnitude.

Membrane-bound form of BChE activity was higher in females than in the males. In diabetic male rats the activity increased and became more than two fold higher. After insulin treatment also activity further increased. The same trend was observed in the females.

In membrane-bound form of BChE in the male rat heart also two components were observed, one having low affinity and the second one having high affinity. In diabetic condition Km of component I decreased while Vmax of both the components increased and became almost double. After insulin treatment Km of component I increased but still remained lower than the control. Km of component II further decreased. Besides, the Vmax of both the components increased further. In membrane-bound form of female rat heart BChE also two components were observed. Km of component I and Vmax of both the components were lower in females as compared to the males. The Km of component I increased in diabetic condition and decreased after the insulin treatment but still remained higher than the controls, while Vmax of both the components increased in diabetic condition and it further increased after insulin treatment. These data are included in Chapter VII of the thesis.

Temperature kinetics data on soluble form of BChE from control male rat heart showed presence of phase transition. For higher temperature range E1 value was lower and for lower temperature range E2 value was higher. In diabetic condition Tt decreased while E1 and E2 increased marginally. After insulin treatment E1 value came back to control level but E2 value still remained higher; phase transition temperature decreased by $4^{o}C$.

The results on the temperature kinetics of soluble form of control female rat heart BChE were almost the same as the control males and not much difference was observed in the rest of the groups.

Temperature kinetics of membrane-bound fraction of BChE from male rat heart showed phase transition. For higher temperature range E1 was lower and for lower temperature range E2 was higher. Only marginal changes were observed in the Arrhenius kinetics in the diabetic and insulin-treated diabetic groups. In the control female rats E2 value was lower than in the control males. E2 value increased in diabetic state and remained high after insulin treatment. No other changes were discernible. These data are included in Chapter VIII of the thesis.

In Conclusion, the present studies have tried to bring into focus the basic biochemical defects in the cardiovascular functions in diabetes in humans as well as in the rats. In particular, it was found that all the functional defects in the diabetic state could not be corrected by insulin treatment. This is consistent with the observations of earlier workers that insulin treatment can control the blood sugar level but can not rectify all the maladies of diabetes.