Chapter 12

Diabetic Cardiomyopathy and Reactive Oxygen Species (ROS) Related Parameters in Male and Female Rats. A Comparative Study

Introduction

Diabetes mellitus affects nearly every organ/system in the body (1-3). Kidneys, heart, pancreas and eyes etc. are the most severely affected systems and the effects are seen at an early stage of the disease (3). The sequels of chronic hyperglycemia in diabetes are manifested as micro- and macro-vascular complications. The macro-vascular changes result in higher risk for myocardial infarction, congestive heart failure (CHF), coronary heart disease (CHD) and stroke, collectively grouped as CVD (4-8).

The incidence of myocardial infarction, CHF and CHD are reported to be significantly high in diabetic patients (5,6,8). Thus, the diabetic population had 4-5 fold increased risk of CHF (5,9,10). The risk of increase in the development of CHF in insulin-dependant diabetes mellitus appears to be greater in all cases even after the age, blood glucose, blood pressure, weight and cholesterol values are well corrected or taken care of (6,7,10,11). It has also been reported that insulin treatment for one year did not reverse the myocardial abnormalities (8,12,13). Interestingly, the incidence of CVDs is higher in diabetic women than in diabetic men (14). Thus, in the diabetic population, the risk of CHF was 2.4 times higher in men and 5.3 times higher in diabetic women than the non-diabetic male and female populations (5-7,15). CVD is the primary cause of death in diabetics and especially so in women (5-7).

Involvement of reactive oxygen species (ROS) in etiologies of several disease conditions is now being increasingly recognized (16-21). It has been suggested that hyperglycemia in diabetes leads to excess production of: ROS, lipid peroxidation (LPO) and protein

glycation (22). Increased oxidative stress is believed to play an important role in the etiology and pathogeneses of chronic complications like atherosclerosis, myocardial infarction, hypertension etc. (23-25).

It has been shown earlier that in mitochondrial electron transport system, oxygen slippage to the extent of 3-5%, can give rise to ROS (16,26,27). It is also well documented that the rates of substrate oxidation are significantly high in cardiac mitochondria (28-31). This is consistent with the higher energy demand of the cardiac tissue (27,32). Therefore, even under normal physiological conditions, significantly higher amount of ROS may be generated in the cardiac tissue.

Our earlier reports suggested that streptozotocin-diabetes differentially affected oxidative energy metabolism in heart mitochondria from male and female rats (28). Also, insulin treatment resulted in differential hyper-stimulation of respiratory activity in cardiac mitochondria from the male and female animals (28). Since the effects of diabetes and insulin treatment were differential in the male and female rats (31), and the incidence of CVDs is reported to be higher in the diabetic females (5,6,11) it was of interest to find out if the cardiac ROS metabolism is differentially affected in a sex-dependant manner. It is expected that such studies can provide insights into the underlying role of ROS in etiology of CVDs in diabetes. Therefore we carried out experiments to determine and compare the ROS parameter in heart using alloxan-diabetic male and female rats as a model system. Effects of treatment with insulin were also examined. The results of these experiments are described in the present communication.

Materials and Methods

The experimental detailed as per given in Chapter 11 of the Thesis.

Isolation of mitochondrial and post-mitochondrial fractions was by the procedure given in Chapter 11 of the Thesis using the isolation medium: 0.3 M sucrose, 5 mM MOPS, pH 7.4, 1 mM EDTA and 250 μg BSA/ml.

Assay procedures are described in Chapter 11 of the Thesis.

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

Results

In the male rats the heart weight decreased by 11 and 25 % respectively at the end of one week and one month diabetic period and insulin treatment had restorative effect. In the female rats, in the control group the heart weights were somewhat higher than in the males. In the diabetic females, the decrease in heart weights was of greater magnitude initially (18 % decrease) and persisted at the end of one month (20 % decrease). Insulin treatment had no restorative effect. Thus in the females rats, in the diabetic as well as insulin treated diabetic groups, the heart weights were always low (Table 1).

The results of diabetes parameters are as detailed in the Chapter 11 of the Thesis.

Table 1. Effect of alloxan-diabetes and insulin treatment on body weight, heart weight and serum glucose of male and female rats

Animals	Treatment Period	Group	Final body weight, g	Heart weight	
				g	%
Male	One week	Control (8)	261.4 ± 5.9	0.744 ± 0.023	0.28 ± 0.01
		Diabetic (18)	208.3 ± 7.5[d]	0.663 ± 0.018[b]	0.32 ± 0.02[d]
		Diabetic + insulin (12)	228.1 ± 8.6[c]	0.767 ± 0.049	0.34 ± 0.02[d,§]
	One month	Control (8)	285.5 ± 5.3	0.783 ± 0.019	0.27 ± 0.01
		Diabetic (15)	172.6 ± 6.2[d]	0.584 ± 0.017[d]	0.34 ± 0.02[d]
		Diabetic + insulin (12)	275.5 ± 6.7[§]	0.780 ± 0.023[§]	0.28 ± 0.02[d,§]
Female	One week	Control (8)	235.4 ± 5.9	0.819 ± 0.039	0.35 ± 0.02
		Diabetic (16)	181.0 ± 4.0[d]	0.675 ± 0.025[b]	0.37 ± 0.03[d]
		Diabetic + insulin (15)	189.8 ± 8.4[d]	0.698 ± 0.031[a]	0.37 ± 0.02[d,§]
	One month	Control (8)	258.7 ± 6.7	0.825 ± 0.035	0.32 ± 0.01
		Diabetic (16)	165.8 ± 5.5[d]	0.663 ± 0.041[c]	0.40 ± 0.02[d]
		Diabetic + insulin (20)	185.0 ± 15.5[d]	0.684 ± 0.043[a]	0.37 ± 0.03[c,§]

The results are given as mean ± S. E. M. 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 control values.

§, $p<0.001$ compared with corresponding diabetic values.

The results on ROS related parameters in one week and one month control were comparable. Hence, these values were pooled and control is given only as one group in Tables 2 and 3.

The effects of diabetes and insulin treatment on ROS related parameter in heart from the male rats are detailed in the Tables 2. It may be seen that the extent of lipid peroxidation in mitochondria was not influenced by the diabetic state. Peculiarly, however, the LPO value increased by 34 % in the one week diabetic group treated with insulin. In the post-mitochondrial fraction, extent of LPO increased in the early diabetic condition by 32 %, while at the later stage a 16 % decrease was noted. Insulin treatments resulted in 44% increase in the LPO level (Table 2). The diabetic state resulted in hyper-induction of XO activity (5-6.6 fold increase). Paradoxically, insulin treatments caused further (8-9 fold) increase in the XO activity (Table 2). The SOD activity in the mitochondria increased by 45 % in the one week diabetic group and was 2.8 fold high in the one month diabetic animals; insulin treatment resulted in a further 1.7 and 3.2 fold increase (Table 2). In the post-mitochondrial fraction also the SOD activity increased by 47 and 85 % respectively in the two diabetic groups. However insulin treatments had no corrective effect. The catalase activity in the diabetic groups was marginally lower and insulin treatment was of no avail (Table 2). The GPox activity in the mitochondrial and the post-mitochondrial fractions was unchanged in the two diabetic groups. Insulin treatment stimulated the GPox activities by 56-75 % (Table 2). In the diabetic animals the G6PDH activity decreased by 36-50 %. Early insulin treatment had partial restorative effect whereas insulin treatment in one month diabetic group completely restored the G6PDH activity

Table 2.Effect of alloxan diabetes on reactive oxygen species related parameters in heart from male rats

Parameter	Control (16)	One week		One month	
		Diabetic (18)	Diabetic + Insulin (12)	Diabetic (15)	Diabetic + Insulin (12)
LPO (Mitochondrial)	6.31 ± 0.27	6.74 ± 0.17	8.43 ± 0.47^{d,§}	6.66 ± 0.35	6.73 ± 0.29
LPO (Post-mitochondrial)	7.68 ± 0.36	10.15 ± 0.30^{e}	9.51 ± 0.22^{e}	6.45 ± 0.23^{a}	11.08 ± 0.13$^{e,\Omega}$
Xanthine oxidase	1.00 ± 0.08	6.60 ± 0.50^{e}	9.71 ± 0.44$^{e,\Omega}$	4.99 ± 0.15^{e}	8.69 ± 0.35$^{e,\Omega}$
SOD (Mitochondrial)	2.61 ± 0.06	3.79 ± 0.30^{d}	4.38 ± 0.29^{d}	7.31 ± 0.21^{e}	8.35 ± 0.40$^{e,\Psi}$
SOD (Post-mitochondrial)	3.39 ± 0.50	4.99 ± 0.31^{c}	5.00 ± 0.36^{b}	6.29 ± 0.10^{e}	5.74 ± 0.40^{d}
Catalase	9.09 ± 0.16	8.56 ± 0.13^{e}	7.26 ± 0.01$^{e,\Omega}$	8.15 ± 0.30^{e}	7.38 ± 0.11^{e}
GPox (Mitochondrial)	371.7 ± 9.10	337.1 ± 20.0	652.3 ± 54.5$^{e,\Omega}$	406.4 ± 18.1	650.0 ± 44.7$^{e,\Omega}$
GPox (Post-mitochondrial)	1202.2 ± 45.4	1209.7 ± 74.8	1875.9 ± 102.9$^{e,\Omega}$	1420.2 ± 93.6^{e}	2071.5 ± 163.3^{e,§}
G6P dehydrogenase	8.42 ± 0.42	4.12 ± 0.10^{e}	6.23 ± 0.23$^{e,\Omega}$	5.35 ± 0.29^{e}	8.21 ± 0.37$^{\Omega}$
GSH (Mitochondrial)	4.48 ± 0.44	2.84 ± 0.20^{c}	6.23 ± 0.23$^{c,\Omega}$	N.D.	N.D.
GSH (Post-mitochondrial)	17.20 ± 1.04	6.05 ± 0.25^{e}	13.20 ± 0.86$^{c,\Omega}$	21.30 ± 1.66^{a}	16.70 ± 1.03$^{\Psi}$

The data are presented as mean ± S. E. M. 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 control values.

Ψ, $p<0.05$; §, $p<0.01$; and Ω, $p<0.001$ compared with corresponding diabetic values.

Table 3.Effect of alloxan diabetes on reactive oxygen species related parameters in heart from female rats

Parameter	Control (16)	One week		One month	
		Diabetic (16)	Diabetic + Insulin (15)	Diabetic (16)	Diabetic + Insulin (20)
LPO (Mitochondrial)	5.83 ± 0.16	7.84 ± 0.26^{d}	7.63 ± 0.37^{d}	6.54 ± 0.30^{a}	7.77 ± 0.35$^{d,\Psi}$
LPO (Post-mitochondrial)	7.77 ± 0.53	9.21 ± 0.64	9.91 ± 0.43^{b}	6.99 ± 0.46	12.43 ± 0.49$^{d,\Omega}$
Xanthine oxidase	0.91 ± 0.09	7.57 ± 0.33^{d}	11.08 ± 0.33$^{d,\Omega}$	5.89 ± 0.36^{d}	9.93 ± 0.56$^{d,\Omega}$
SOD (Mitochondrial)	2.81 ± 0.09	4.66 ± 0.28^{d}	4.99 ± 0.20^{d}	7.76 ± 0.13^{d}	8.38 ± 0.32^{d}
SOD (Post-mitochondrial)	4.26 ± 0.08	5.30 ± 0.20^{d}	5.10 ± 0.21	6.20 ± 0.26^{d}	6.04 ± 0.35^{d}
Catalase	11.30 ± 0.19	10.34 ± 0.98	8.35 ± 0.35^{d}	9.22 ± 0.40^{d}	7.68 ± 0.30^{d,§}
GPox (Mitochondrial)	340.0 ± 10.4	367.6 ± 17.4	546.3 ± 29.8$^{d,\Omega}$	363.9 ± 14.2	834.7 ± 19.2$^{d,\Omega}$
GPox (Post-mitochondrial)	1253.3 ± 47.9	1372.9 ± 29.8	2003.8 ± 116.3$^{d,\Omega}$	1565.6 ± 123.4^{a}	2212.2 ± 63.03$^{d,\Omega}$
G6P dehydrogenase	9.24 ± 0.33	6.54 ± 0.11^{d}	9.90 ± 0.40$^{\Omega}$	4.44 ± 0.11^{d}	8.36 ± 0.53$^{\Omega}$
GSH (Mitochondrial)	5.76 ± 0.33	2.25 ± 0.13^{d}	4.09 ± 0.28$^{c,\Omega}$	N.D.	N.D.
GSH (Post-mitochondrial)	22.40 ± 0.89	9.31 ± 0.61^{d}	23.10 ± 0.38$^{\Omega}$	13.80 ± 0.38^{d}	15.50 ± 2.58^{d}

The data are presented as mean ± S. E. M. 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 control values.

Ψ, $p<0.02$; §, $p<0.01$; and Ω, $p<0.001$ compared with corresponding diabetic values.

N.D., not detectable

(Table 2). As early as one week, the mitochondrial GSH levels decreased by 37 % and were undetectable at end of one month; insulin treatment had no corrective effects. In the post-mitochondrial fraction also the GSH levels decreased (65 and 23 % decrease respectively in two diabetic groups). Insulin treatment had restorative effect (Table 2).

The effect of alloxan diabetes on ROS related parameters in the heart from the female rats are summarized in Table 3. Thus compared to the male rats, the extent of increase in LPO level in mitochondria was higher in the females. In the post-mitochondrial fraction the pattern was comparable to that with male rats (Tables 2 and 3). In the diabetic condition, the XO activity increased to a greater extent than in the male rats (6.5-8.3 fold increase). The trend for mitochondrial SOD activity was comparable to that in the males (Tables 2 and 3). Increase in the SOD activity in the post-mitochondrial fraction was of lesser magnitude. The catalase activity decreased by 18 % in the one month diabetic animals. Pattern for the GPox activity -both mitochondrial and post-mitochondrial- was comparable to males. The G6PDH activity decreased by 29 and 52 % in one week and one month diabetic groups respectively in the female rats. In early diabetic condition the GSH level in the mitochondria decreased to a greater extent in the females. The GSH levels in the post-mitochondrial fraction decreased in the diabetic condition with one month diabetic females registering greater decrease than in the males (Tables 2 and 3). Insulin treatment had no restorative effects on SOD activity. The catalase activity decreased while XO, GPox and G6PDH activities increased following insulin treatment. Paradoxically, the LPO levels also increased beyond the diabetic values.

Discussion

The most significant observation of the present study was several fold increase in the XO activity in the diabetic animals with the extent of increase being higher in the female rats. The increase in XO activity is a matter of concern since XO is recognized as one of the extra-mitochondrial sites of superoxide (O_2^-) generation (26, 33). O_2^- generated extra-mitochondrially should be eliminated in the first instance by SOD of the post-mitochondrial fraction. However, as can be noted, compared to several fold increase in the in the XO, the SOD activity in the post-mitochondrial fraction was only miniscule (Tables 2 and 3). The problem gets further aggravated because the catalase activity diminished to a very significant extent in the cardiac tissue, the effect being more pronounced in the females. Because of this, the major route of elimination of hydrogen peroxide (H_2O_2) generated by SOD action, would seem to be via GPox. As can be noted from the data (Tables 2 and 3) at the late stage of the diabetes the mitochondrial GSH is totally depleted in the heart and early depletion is evident in the female diabetic group. GSH is the substrate for the GPox activity. Non-availability of GSH in the mitochondria is further compounded by low G6PDH activity in the heart and greater impairment of G6PDH activity in diabetes in the females (Tables 2 and 3). G6PDH produces NADPH which is the substrate for regenerating reduced glutathione (GSH) from its oxidized form GSSG. Although NADPH can be generated by systems other than G6PDH (34), it is not clear at this stage, whether these alternate systems are also affected in diabetes. Especially, noteworthy is the fact that the GSH level in the post-mitochondrial fraction in the female heart is significantly low (Tables 2 and 3); cytosolically synthesized GSH is the source of GSH pool in the mitochondria.

Earlier we have reported that the cardiac tissue has low ROS defense mechanism than the liver (35). Taking this into consideration, the results would suggest that low ROS

defense mechanism and several fold increase in XO activity may initiate the ROS mediated damage in the cardiac tissue, which is reflected in terms of relatively high LPO levels (Table 2 and 3) as compared to liver (36). The results also suggest that the lesion may be at the level of mitochondria which are faced with the problem of GSH depletion, and then spreads to extra-mitochondrial regions. Besides, the GSH content of the post-mitochondrial fraction itself is significantly low in the females than in males and insulin treatment is generally ineffective in the females.

Earlier we have shown that, streptozotocin-diabetes differentially affected the respiratory function in mitochondria from male and female rats and insulin treatment resulted in hyper-stimulation of respiration in the mitochondria from female heart (28). Hyper-stimulation of respiration would lead to enhanced ROS generation due to oxygen slippage (16,27,37) and this would, ultimately be reflected in terms of increased LPO levels and possible depletion of GSH. Results of our present studies (Table 2 and 3) are consistent with these presumptions.

It has been reported that in the humans the incidence of CVDs is higher in diabetic females and insulin treatment has no beneficial effects (5-7,15). Results of our present studies also suggest that the cardiac tissue in the diabetic female rats is more susceptible to ROS damage due to weak ROS defense system, which is further compromised in diabetes and the fact that insulin treatment is generally ineffective. However, at this stage it is not clear if similar situation prevails in humans.

Summary

The diabetic state severely compromised the ROS defense mechanism in the cardiac tissue and the effects were more pronounced in the female than in the male rats.

There was several fold increase in the xanthine oxidase (XO) activity in general and the magnitude of increase was higher in the females; insulin treatment resulted in further increase in the XO activity.

The glucose-6-phosphate dehydrogenase (G6PDH) and catalase activities decreased and the reduced glutathione (GSH) content in mitochondria was completely depleted in diabetic state with significant decrease in the GSH levels in the post-mitochondrial fraction; the effect was more pronounced in the females.

The superoxide dismutase (SOD) and glutathione peroxidase (GPox) activities increased in the diabetic state to a greater extent in male rats. Insulin treatment had restorative action only on some parameters.

In conclusion, our results suggest that diabetic state may further compromise the week ROS defense systems in the heart thus initiating a lesion at the level of mitochondria which ultimately leads to cardiomyopathy and the effects are especially more pronounced in the females. Our results also pointed out that insulin treatment was ineffective in restoring ROS related parameters.

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