DIABETIC CARDIOMYOPATHY AND REACTIVE OXYGEN SPECIES (ROS) RELATED PARAMETERS IN MALE AND FEMALE RATS : A COMPARATIVE STUDY

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ABSTRACT

Studies were carried out to examine and compare the effects of alloxan-diabetes on reactive oxygen species (ROS) related parameters in the heart from male and female rats. Effects of insulin treatment were also evaluated. 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 weak 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.

KEY WORDS

Alloxan-diabetes; ROS parameters in Diabetes; Diabetic cardiomyopathy

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 macrovascular complications. The macrovascular changes result in higher risk for myocardial infarction, congestive heart failure (CHF), coronary heart disease (CHD) and stroke, collectively grouped as cardiovascular diseases (CVDs) (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 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

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oxidative stress is believed to play an important role in the etiology and pathogenesis 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.

Earlier, we have shown 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 rats (28). Since the effects of diabetes and insulin treatment were differential in the male and female rats, and the incidence of CVDs is reported to be higher in the diabetic females (5,6,11, 31) 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

Chemicals : Alloxan was purchased from National Chemicals, Vadodara, India. Bovine serum albumin (BSA) fraction V and 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) were from SRL, India. Sodium salts of NADP⁺, glucose-6-phosphate (G6P), dichlorophenolindophenol (DCIP), ter-butyl hydroperoxide (t-BOOH) and 4-morpholinopropanesulfonic acid (MOPS) were obtained from Sigma Chemical Co., St. Louis, MO, U. S. A. Xanthine was purchased from Hofmann La Roche, Switzerland. Reduced glutathione (GSH) was obtained from Hi Media, India and NPH insulin (40 U/ml) was obtained from Lilli, France S.A.S. All other chemicals were of analytical-reagent grade and were purchased locally.

Animals : Adult male and female albino rats (8-10 week old) of Charles-Foster strain were used. At the starting of the experiments, the initial body weights in the male groups ranged from 230-250 g while these values for the female groups were

in the range of 210-220 g. The initial body weights of male and female rats belonging to various experimental groups (details given below) were matched within 5 g. The animals were fasted overnight and diabetic state was induced by injecting once 12 mg of alloxan/100 g body weight subcutaneously (s. c.) (33-35). Alloxan solution was prepared fresh in saline (120 mg/ml) prior to use. The controls received equivalent volume of the vehicle. The diabetic state was confirmed by hyperglycemia, polyuria and glucosuria (33,36). Glucose content in the serum and the urine was estimated according to the methods described previously (30,33,36).

Experiments were carried out at the end of one week or one month of induction of diabetic state to ascertain the early-onset and long-term effects (30,37). For one week study diabetic animals received insulin from the fifth day of induction of diabetes for three consecutive days and for one month studies diabetic animals received insulin starting from the forth week of induction of diabetes for seven consecutive days at a dose of 0.8 unit of NPH insulin/100 g body weight twice daily (around 8 AM and 6 PM) (s. c.). Animals were killed 18 h after receiving the last insulin injection. The number of animals used in various experimental groups ranged from 8-20 as indicated in Tables 1-3.

Isolation of mitochondrial and post-mitochondrial fractions :

At the end of the experimental period the animals were sacrificed by decapitation and the heart was quickly removed and placed in beaker containing chilled (0-4 °C) isolation medium: 0.3 M sucrose, 5 mM MOPS, pH 7.4, 1 mM EDTA and 250 µg BSA/ml. The tissue was minced and washed repeatedly with the isolation medium to remove adhering blood and 10% (w/v) homogenates were prepared using a Potter-Elvehjem type glass-Teflon homogenizer. The nuclei and cell debris were sedimented by centrifugation at 650 x g for 10 min and discarded. The supernatant was subjected to a further centrifugation at 7,500 x g for 10 min. The resulting mitochondrial pellet was washed by suspending gently in the isolation medium and by re-sedimenting at 7,500 x g for 10 min. Finally, the mitochondria were suspended in the isolation medium to give about 12-15 mg mitochondrial protein/ml (29,32,38). The post-mitochondrial supernatant was then centrifuged at 12,000 x g for 10 min to remove light mitochondria (39). The pellet was discarded and the resulting supernatant was used as the source of cytosolic enzymes.

ROS parameters : Determination of lipid peroxidation (LPO, n mole malondialdehyde/mg protein) was by the method of Uchiyama and Mihara (40). Xanthine oxidase activity (XO), (P mole DCIP reduced/min/mg protein) was estimated by the

methods described (34,41). Superoxide dismutase (SOD) activity (units/mg protein; 1 unit represents the amount of enzyme required for 50 % inhibition of pyrogallol autoxidation under standard assay conditions) was determined by the procedures described previously (42, 43). Estimation of catalase activity (Sigma units/mg protein), was according to the method of Satav et al. (34). Measurement of glutathione peroxidase (GPox) activity (units/min/mg protein) was carried out by the procedure of Hafeman et al. (44). Determination of glucose-6-phosphate dehydrogenase (G6PDH) activity (n mole NADPH formed/min/mg protein) was according to the procedure described by Cohen and Rosemeyer (45). Content of glutathione (GSH) (p mole/mg protein) was quantified by the method of Kaushal et al. (39).

Estimation of protein was according to the method of Lowry et al. with bovine serum albumin fraction V used as the standard (46). Statistical evaluation of the data was done by Students' 't'-test.

RESULTS

At the end of one week and one month diabetic state resulted in 20 and 40% reduction respectively in the body weight of the male rats. In one week group insulin treatment had marginal restorative effect on body weight; in the one month group insulin treatment almost completely restored the body

weight (59 % increase in body weight compared to the diabetic group). The trend was comparable for diabetic female rats, except that insulin treatments had only marginal restorative effect on body weight (Table 1). These observations are consistent with our previously reported results (31).

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, decrease in heart weight 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 serum glucose levels increased by 3.3 and 4.1 folds in one week and one month diabetic male rats compared to the corresponding controls. Similarly, in the female diabetic groups, the serum glucose levels were 3.7 and 4.2 fold higher. Insulin treatments had only partial restorative effect and the serum glucose levels were always higher compared to the corresponding controls. This was especially true for the females (Table 1). Diabetic state resulted in 16 and 20 fold increase in the urine volume respectively in the male diabetic groups. Insulin treatment restored the urine volume to normality

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)	Serum glucose level (mM)
Male	One week	Control (8)	261.4 ± 5.9	0.744 ± 0.023	4.61 ± 0.28
		Diabetic (18)	208.3 ± 7.5 ^d	0.663 ± 0.018 ^b	15.01 ± 0.54 ^d
		Diabetic + insulin (12)	228.1 ± 8.6 ^c	0.767 ± 0.049	6.79 ± 0.25 ^{d,§}
	One month	Control (8)	285.5 ± 5.3	0.783 ± 0.019	4.55 ± 0.21
		Diabetic (15)	172.6 ± 6.2 ^d	0.584 ± 0.017 ^d	18.88 ± 0.62 ^d
		Diabetic + insulin (12)	275.5 ± 6.7 [§]	0.780 ± 0.023 [§]	5.69 ± 0.09 ^{d,§}
Female	One week	Control (8)	235.4 ± 5.9	0.819 ± 0.039	4.45 ± 0.33
		Diabetic (16)	181.0 ± 4.0 ^d	0.675 ± 0.025 ^b	16.48 ± 0.30 ^d
		Diabetic + insulin (15)	189.8 ± 8.4 ^d	0.698 ± 0.031 ^a	7.18 ± 0.21 ^{d,§}
	One month	Control (8)	258.7 ± 6.7	0.825 ± 0.035	4.56 ± 0.41
		Diabetic (16)	165.8 ± 5.5 ^d	0.663 ± 0.041 ^c	19.30 ± 0.22 ^d
		Diabetic + insulin (20)	185.0 ± 15.5 ^d	0.684 ± 0.043 ^a	6.35 ± 0.26 ^{c,§}

The 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 control values.

§, p<0.001 compared with corresponding diabetic values.

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,Ω}
Xanthine oxidase	1.00 ± 0.08	6.60 ± 0.50 ^e	9.71 ± 0.44 ^{e,Ω}	4.99 ± 0.15 ^e	8.69 ± 0.35 ^{e,Ω}
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,Ψ}
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 ^c	7.26 ± 0.01 ^{e,Ω}	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,Ω}	406.4 ± 18.1	650.0 ± 44.7 ^{e,Ω}
GPox (Post-mitochondrial)	1202.2 ± 45.4	1209.7 ± 74.8	1875.9 ± 102.9 ^{e,Ω}	1420.2 ± 93.6 ^c	2071.5 ± 163.3 ^{e,§}
G6P dehydrogenase	8.42 ± 0.42	4.12 ± 0.10 ^e	6.23 ± 0.23 ^{e,Ω}	5.35 ± 0.29 ^e	8.21 ± 0.37 ^Ω
GSH (Mitochondrial)	4.48 ± 0.44	2.84 ± 0.20 ^c	6.23 ± 0.23 ^{c,Ω}	N.D.	N.D.
GSH (Post-mitochondrial)	17.20 ± 1.04	6.05 ± 0.25 ^e	13.20 ± 0.86 ^{c,Ω}	21.30 ± 1.66 ^a	16.70 ± 1.03 ^Ψ

The data 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 control values.

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

N.D., not detectable

(2.0-2.5 ml/24 h). More or less similar results were obtained even for the female rats (data not shown). The urinary sugar excretion amounted to about 1.0-1.25 g / 24 h in the two male and female diabetic groups; insulin treatment completely abolished the urinary sugar excretion (data not shown).

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 alloxan-diabetes and subsequent treatment with insulin on ROS related parameter in heart from the male rats are detailed in Tables 2. It may be seen that the extent of lipid peroxidation in mitochondria was not influenced by diabetic state. Peculiarly, however, LPO value increased by 34 % in one week diabetic group treated with insulin. In post-mitochondrial fraction, extent of LPO increased in early diabetic condition by 32 %, while at the later stage a 16 % decrease was noted. Insulin treatments resulted in 44% increase in LPO level (Table 2). Diabetic state resulted in hyper-induction of XO activity (5-6.6 fold increase). Paradoxically, insulin treatments caused further (8-9 fold) increase in XO activity (Table 2). SOD activity in the mitochondria increased by 45 % in one week diabetic group and was 2.8 fold high in one month diabetic animals; insulin treatment resulted in a further 1.7

and 3.2 fold increase (Table 2). In post-mitochondrial fraction also SOD activity increased by 47 and 85 % respectively in the two diabetic groups. However insulin treatments had no corrective effect. The catalase activity in diabetic groups was marginally lower and insulin treatment was of no avail (Table 2). The GPox activity in mitochondrial and post-mitochondrial fractions was unchanged in two diabetic groups. Insulin treatment stimulated GPox activities by 56-75 % (Table 2). In diabetic animals 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). As early as one week, mitochondrial GSH levels decreased by 37 % and were undetectable at the end of one month; insulin treatment had no corrective effects. In post-mitochondrial fraction also GSH levels decreased (65 and 23 % decrease respectively in two diabetic groups). Insulin treatment had restorative effect (Table 2).

The effect of alloxan-diabetes and subsequent treatment with insulin on ROS related parameters in heart from the female rats are summarized in Table 3. Thus compared to the male rats, extent of increase in LPO level in mitochondria was higher in the females. In post-mitochondrial fraction the pattern was comparable to that with male rats (Tables 2 and 3). In the diabetic condition, XO activity increased to a greater extent

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 (18)	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,Ψ}
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,Ω}
Xanthine oxidase	0.91 ± 0.09	7.57 ± 0.33 ^d	11.08 ± 0.33 ^{d,Ω}	5.89 ± 0.36 ^d	9.93 ± 0.56 ^{d,Ω}
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,Ω}	363.9 ± 14.2	834.7 ± 19.2 ^{d,Ω}
GPox (Post-mitochondrial)	1253.3 ± 47.9	1372.9 ± 29.8	2003.8 ± 116.3 ^{d,Ω}	1565.6 ± 123.4 ^a	2212.2 ± 63.03 ^{d,Ω}
G6P dehydrogenase	9.24 ± 0.33	6.54 ± 0.11 ^d	9.90 ± 0.40 ^Ω	4.44 ± 0.11 ^d	8.36 ± 0.53 ^Ω
GSH (Mitochondrial)	5.76 ± 0.33	2.25 ± 0.13 ^d	4.09 ± 0.28 ^{c,Ω}	N.D.	N.D.
GSH (Post-mitochondrial)	22.40 ± 0.89	9.31 ± 0.61 ^d	23.10 ± 0.38 ^Ω	13.80 ± 0.38 ^d	15.50 ± 2.58 ^d

The data 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 control values.

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

N.D., not detectable

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 SOD activity in post-mitochondrial fraction was of lesser magnitude. In one month diabetic animals, catalase activity decreased by 18 %. 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 early diabetic condition GSH level in mitochondria decreased to a greater extent in the females. The GSH levels in post-mitochondrial fraction decreased in diabetic state 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. Following insulin treatment catalase activity decreased while XO, GPox and G6PDH activities increased. Paradoxically, the LPO levels also increased beyond the diabetic values.

DISCUSSION

The most significant observation of the present study was several fold increase in XO activity in 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 (26,34). 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 XO, SOD activity in 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 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 (Tables 2 and 3). GSH is the substrate for 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 (47), 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 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 cardiac tissue has low ROS defense mechanism than the liver (48). 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 (Tables 2 and 3) as compared to liver (49). 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 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 (16,27,50) and this would, ultimately be reflected in terms of increased LPO levels and possible depletion of GSH. Results of our present studies (Tables 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.

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