Chapter 11

Effect of Alloxan-Diabetes and Subsequent Insulin treatment on Reactive Oxygen Species (ROS) Related Parameters in Mitochondrial and Post-mitochondrial Fractions from Rat Liver

Introduction

Diabetes mellitus, the single most important metabolic disease affects more than 150 million people in the world (1). It affects nearly every organ/system in the body in a differential manner and to different extents (2, 3). Thus the kidneys, heart, pancreas and eyes etc. are the most severely affected systems whereas the peripheral nervous system and the brain are affected last; the liver is the least affected tissue (4).

Involvement of reactive oxygen species (ROS) in etiologies of several disease conditions including diabetes is now being increasingly recognized (5-10). Interesting to note in this context is the suggestion that hyperglycemia in diabetes leads to excessive production of: ROS, lipid peroxidation (LPO) and protein glycation (11).

Although the liver seems to be the least affected tissue in diabetes (4), changes in the mitochondrial respiratory activities do occur which we have reported previously (12). It is therefore of interest to evaluate the extent to which diabetic state and insulin treatment affect the ROS parameter in the liver. In the present studies these aspects employing alloxan-diabetic rat as the model system was examined. The results are summarized in this Chapter.

Materials and Methods

Chemicals

Alloxan was purchased from National Chemicals, Vadodara, India. Bovine serum albumin fraction V (BSA) and 5, 5' -dithiobis (2-nitrobenzoic acid) (DTNB) were

obtained from SRL, India. Sodium salts of $NADP^+$, glucose-6-phosphate (G6P), dichlorophenol indophenol (DCIP) and ter-butyl hydroperoxide (t-BOOH) were obtained from Sigma, USA. Xanthine was obtained from Hofmann La Roche, Switzerland. Reduced glutathione (GSH) was a product of Hi Media, India. All other chemicals were of the highest purity grade and were purchased locally.

Animals

Adult male and female albino rats (8-10 week old) of Charles-Foster strain were used. Induction of diabetes, treatment with insulin and confirmation of diabetes were as detailed in Chapter 2 of the Thesis.

Isolation of mitochondrial and post-mitochondrial fractions

The tissues were minced in the isolation medium (detailed given in Chapter 2 of the Thesis) if necessary 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 resedimenting 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. The post-mitochondrial supernatant was then centrifuged at 12,000 x g for 10 min to remove light mitochondria (13). The pellet was discarded and

the resulting supernatant was used as the source of cytosolic enzymes, care being taken to note the volume of the supernatant.

Assay procedures

Determination of lipid peroxidation (LPO) in terms of thiobarbituric acid reactive materials (TBARS) was according to the method of Uchiyama and Mihara (23) in which TBARS are extracted in n butanol: pyridine (15:1) mixture and optical density was determined at 530 nm. Results are given as n moles of malondialdehyde formed.

Xanthine oxidase activity was determined as described previously (15, 16) by following the reduction of DCIP induced after addition of xanthine. The reaction was monitored at 600 nm. The activity is expressed as n moles DCIP reduced / min / mg protein.

Superoxide dismutase activity in mitochondria and post-mitochondrial fractions was determined by following the inhibition of pyrogallol autoxidation under standard assay condition as described (17, 18). The reaction was measured at 420 nm. One unit represents the amount of enzyme which inhibits pyrogallol autoxidation by 50 % under standard assay conditions.

Measurement of catalase activity was monitored by following the decrease in H_2O_2 concentration as determined by decrease in absorbance at 240 nm (15). Activity is expressed as Sigma units i.e. μ moles of H_2O_2 decomposed / min/ mg protein (15)

Glutathione peroxidase activity was measured according to the method of Hafeman et al. (19) in which oxidation of reduced glutathione (GSH) by ter-butyl hydroperoxide mediated by either mitochondrial or post-mitochondrial source of enzyme was monitored. One unit of enzyme activity represents Δ log [GSH] / min / mg protein (19). GSH measurements were according to the method described previously (13).

Determination of glucose 6 phosphate dehydrogenase (G6PDH) activity was by spectrophotometric method where formation of NADPH was monitored at 340 nm (20). The activity is expressed as n moles NADPH formed / min / mg protein.

Protein estimation was according to the method of Lowry et al. using bovine serum albumin as the standard (21).

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

Results

In the male rats alloxan-diabetes resulted in 19 and 39 % reduction in the body weight respectively at the end of one week and one month. Insulin treatment had marginal restorative effect in one week group, while in the one month group, insulin treatment almost completely restored the body weight (59 % increases in body weight compared to the diabetic group). In the female rats, diabetic state resulted in 18 and 33 % reduction in the body weight. Treatment with insulin was ineffective in restoring the body weight to normality (Table 1).

Table 1. Effect of alloxan-diabetes and insulin treatment on body weight and liver weight of the rats

Animals	Treatment Period	Group	Final body weight, g	Liver weight	
				g	%
Male	One week	Control	258.1 ± 5.6	8.25 ± 0.33	3.27 ± 0.19
		Diabetic	211.0 ± 6.9[c]	6.88 ± 0.34[a]	3.26 ± 0.24[c]
		Diabetic + insulin	233.4 ± 8.7[a]	12.73 ± 0.63[c,§]	5.45 ± 0.25[c,§]
	One month	Control	280.7 ± 4.7	8.96 ± 0.41	3.51 ± 0.26
		Diabetic	170.6 ± 5.2[c]	5.88 ± 0.49[b]	3.44 ± 0.23[c]
		Diabetic + insulin	270.5 ± 7.2[§]	13.41 ± 0 86[c,§]	4.95 ± 0.29[c,§]
Female	One week	Control	232.9 ± 7.3	7.45 ± 0.36	3.19 ± 0.22
		Diabetic	191.4 ± 6.9[c]	5.61 ± 0.41[a]	2.93 ± 0.24[c]
		Diabetic + insulin	205.3 ± 8.7[a]	10.26 ± 0.59[c,§]	4.99 ± 0.35[c,§]
	One month	Control	253.4 ± 5.1	7.77 ± 0.38	3.07 ± 0.19
		Diabetic	170.4 ± 8.2[c]	5.35 ± 0.39[b]	3.13 ± 0 15[c]
		Diabetic + insulin	193.5 ± 9.9[§]	10.11 ± 0.46[c,§]	5.22 ± 0.24[c,§]

The results are given as mean ± S. E. M. of 8-12 independent observations.

a, $p<0.05$; b, $p< 0.01$ and c, $p<0.001$; compared with control values.

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

ND, not detectable

At the end of one week and one month alloxan-diabetes the liver weight of the male rats decreased by 17 % and 34 % respectively. Treatment with insulin brought about significant increase in the liver weight not only compared to the diabetic groups (85 and 128 % increase respectively), but also beyond the corresponding values for control groups (54 and 50% increase respectively) (Table 1). The similar trend was seen for the female rats.

In the male rats, the levels of glucose in the serum increased by 3.2 and 4.3 folds in one week and one month diabetic rats. Insulin treatments had partial restorative effect and the serum glucose levels were always higher compared to those in the corresponding control groups (Table 2). Polyurea in the diabetic state was reflected in terms of 18 and 21 fold increase in the urine volume respectively in the two diabetic groups. Insulin treatments restored the urine volume to normality (2.0-2.5 ml/24 h). The urinary sugar excretion amounted to about 1.0-1.25 g / 24 h in the two diabetic groups; insulin treatment completely abolished the urinary sugar excretion (Table 2). Here also, in the female rats, almost identical results were found (Table 2)

The effects of short-term and long-term diabetic state and of insulin treatment on ROS related parameters in liver from the male rats are detailed in the Table 3. As can be noted, the LPO levels in the mitochondrial fraction increased marginally (19%) only in one month diabetic group where the LPO level in the post-mitochondrial fraction registered a 19% decrease. The changes were corrected by insulin treatment. The XO activity decreased significantly in both the diabetic group (55 and 60 % decrease respectively in

Table 2 Effect of alloxan-diabetes and insulin treatment on diabetes parameters.

Animals	Group	Treatment	Serum glucose level, mM	Urine volume, ml/24 h	Urine sugar, mg/ml	Sugar excretion, g/24 h
Male	One week	Control	4.21 ± 0.35	2 53 ± 0.17	ND	ND
		Diabetic	15 02 ± 0.65[b]	45.63 ± 1.08[b]	22.44 ± 0.32	1.02 ± 0 03
		Diabetic + insulin	6.01 ± 0 35[a,§]	3.37 ± 0.12[a,§]	ND	ND
	One month	Control	4.31 ± 0.33	2.71 ± 0.22	ND	ND
		Diabetic	19.69 ± 0 98[b]	54 61 ± 1.19[b]	22.87 ± 0.52	1.24 ± 0.03
		Diabetic + insulin	6.63 ± 0.39[a,§]	3 01 ± 0.15[§]	ND	ND
Female	One week	Control	4.45 ± 0.55	2.23 ± 0.27	ND	ND
		Diabetic	16.48 ± 0 30[b]	43.51 ± 3.00[b]	24.11 ± 1.15	1.05 ± 0 06
		Diabetic + insulin	7.18 ± 0.21[b,§]	3.37 ± 0.12[b,§]	ND	ND
	One month	Control	4.56 ± 0.33	2.43 ± 0.19	ND	ND
		Diabetic	19.30 ± 0 22[b]	51 24 ± 1.56[b]	23.99 ± 0.52	1.23 ± 0.05
		Diabetic + insulin	6.35 ± 0.26[a,§]	3.11 ± 0.20[§]	ND	ND

The results are given as mean ± S E. M. of 8-12 independent observations.

a, $p<0.01$ and b, $p<0.001$; compared with control values

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

Table 3.Effect of alloxan diabetes on reactive oxygen species related parameters in liver of male rat

Parameter	Control	One week		One month	
		Diabetic	Diabetic + Insulin	Diabetic	Diabetic + Insulin
LPO (Mitochondrial)	2.23 ± 0.10	2.12 ± 0.08	2.22 ± 0.11	2.66 ± 0.11[a]	2.39 ± 0.11
LPO (Post-mitochondrial)	3.90 ± 0.16	3.78 ± 0.09	3.87 ± 0.23	3.16 ± 0.22[a]	3.56 ± 0.13
Xanthine oxidase	7.29 ± 0.36	3.30 ± 0.27[a]	2.90 ± 0.10[e]	2.60 ± 0.10[e]	3.60 ± 0.10[e,Ψ]
SOD (Mitochondrial)	7.14 ± 0.16	8.10 ± 0.21[c]	7.99 ± 0.64	3.27 ± 0.13[e]	5.15 ± 0.12[e,Ψ]
SOD (Post-mitochondrial)	11.10 ± 0.21	12.30 ± 0.69	10.1 ± 0.40[a,§]	6.59 ± 0.48[e]	7.00 ± 0.30[e]
Catalase	108.2 ± 2.00	100.7 ± 5.80	62.0 ± 0.40[e,Ψ]	70.3 ± 3.20[e]	51.5 ± 1.0[e,Ψ]
GPox (Mitochondrial)	797.1 ± 41.5	644.3 ± 54.2[a]	767.0 ± 55.6	693.0 ± 46.3	961.8 ± 83.3[§]
GPox (Post-mitochondrial)	1312.2 ± 117.3	1802.5 ± 96.4[e]	1962.4 ± 67.8[e]	1891.0 ± 169.1[b]	2469.3 ± 189.3[e,§]
G6P dehydrogenase	41.5 ± 1.21	7.88 ± 0.21[e]	77.6 ± 4.40[e,Ψ]	12.5 ± 0.68[e]	50.3 ± 3.13[a,Ψ]
Reduced glutathione (Mitochondrial)	3.36 ± 0.23	4.32 ± 0.29[a]	4.09 ± 0.28	0.99 ± 0.08[e]	1.00 ± 0.02[e]
Reduced glutathione (Post-mitochondrial)	25.0 ± 1.45	12.5 ± 0.70[e]	29.2 ± 0.89[a,Ψ]	16.9 ± 0.78[e]	18.0 ± 1.00[d]

The data are presented as mean ± S. E. M. of 8-20 independent observations.

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.

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

the two diabetic groups) and was not corrected by insulin treatment. This is consistent with our earlier observation (15). The mitochondrial SOD activity increased initially by 13 % but declined subsequently by 64 %. Insulin treatment in one month group was able to restore the activity to about 64% of the control value (Table 3). The SOD activity in the post-mitochondrial fraction decreased by almost 40 % in one month diabetic group and treatment with insulin had no restorative effect (Table 3). The catalase activities were 93 and 65% respectively of the control in the early and the late stag of diabetes and insulin treatments had rather an adverse effect. Mitochondrial GPox activity decreased (13-19% decrease) in the two diabetic groups and insulin treatments either normalized or marginally stimulated the GPox activity. In the post-mitochondrial fraction the GPox activity increased in the two diabetic groups; effects of insulin treatment were comparable to those in mitochondria. The G6PDH activity decreased significantly (70-80 % decrease) in the two diabetic groups. Treatment with insulin at early stage had a greater stimulatory effect. The GSH level in mitochondria was high in the initial stage of the diabetic condition but decreased substantially (70 % decrease). Insulin treatments had no effect. The GSH level in the post-mitochondrial fraction was low in both the diabetic groups and could be restored by insulin treatment only at the early stage (Table 3).

The effects of short-term and long-term diabetic state and of insulin treatment on ROS related parameters in liver from the female rats are detailed in the Table 4.

Discussion

As can be noted, the major effects of diabetic state noted at the early stage i.e. one week were: diminished levels of XO, mitochondrial GPox, G6PDH and GSH in post-

mitochondrial fraction. By contrast, mitochondrial SOD and GPox activity in the post-mitochondrial fraction were elevated. At the late stage, i.e. at the end of one month, XO, mitochondrial and post-mitochondrial SOD, catalase, G6PDH and GSH content in mitochondrial as well as post-mitochondrial fractions decreased. Once again the GPox activity in the post-mitochondrial fraction was elevated significantly

Insulin treatment, in general was ineffective in restoring the XO, mitochondrial and post-mitochondrial SOD, and catalase activities. Also the GSH levels in mitochondrial as well as post-mitochondrial fractions were not restored in one month group.

The decreased XO activity may relate to lowering of the purine catabolic rate but would also imply that the cytosolic generation of O_2^- is also diminished under these conditions. Lowering of catalase and SOD activities in long run would weaken the ROS defense mechanism. Although the catalase and SOD activities decreased in long run in the diabetic state and were insulin insensitive, still the residual activities are sufficiently high especially when compared to their contents in tissues such as heart (18). This may perhaps explain as to why the liver is the least affected tissue in diabetes (4).

Additionally, one redeeming feature is that the GPox activities, both mitochondrial as well as cytosolic, were only marginally affected or were stimulated in diabetic state and stimulation beyond control level was noted following insulin treatment. In other words, the results would imply that the ROS elimination occurred mainly by the GPox route.

As is well recognized, GSH is an essential component for GPox action and gets oxidized to GSSG. GSH is regenerated by reduction of GSSG in the cytosol for which NADPH is used as the reducing equivalent. As can be noted (Table 2), the G6PDH activity is sensitive to insulin deficiency. Obviously this will result in diminished availability of NADPH in the diabetic state thus impairing GSH regeneration. It may, however be pointed out that NADPH can also be generated by alternate routes such as pentose phosphate pathway, isocitrate dehydrogenase or glutamate dehydrogenase (12). In our earlier studies we have shown that glutamate dehydrogenase activity is not sensitive to insulin status (12). The deficiency of GSH particularly in the mitochondria would suggest that either the GSH is extensively used to combat the ROS generated mitochondrially or that there may be impairment in import of GSH to mitochondria from the cytosol especially at the late stage of diabetes. Our previously reported impairments of mitochondrial oxidative metabolism may relate to the depletion of intra-mitochondrial GSH.

In other studies, we have noted that diabetic state differentially affected cardiac and brain mitochondrial metabolism in male and female rats (22, 23). Therefore, in parallel experiments we evaluated the ROS parameters in the female rats; the results were almost similar to those noted for males (Table 4). These results would therefore suggest that unlike the heart, insulin status does not affect the ROS parameters in the liver in a sex-dependent manner.

Table 4.Effect of alloxan diabetes on reactive oxygen species related parameters in liver of female rat

Parameter	Control	Diabetic		Diabetic+ Insulin	
		1 Week	1 Month	1 Week	1 Month
LPO (Mitochondrial)	1.89 ± 0.10(20)	2.17 ± 0.20(12)	1.98 ± 0.10(8)	2.18 ± 0.17(8)	2.08 ± 0.09(8)
LPO (Post-mitochondrial)	3.47 ± 0.26(20)	4.88 ± 0.37(11)	3.86 ± 0.24(8)	5.02 ± 0.57(14)	3.58 ± 0.12(8)
Xanthin oxidase	6.09 ± 0.20(16)	4.40 ± 0.14(8)	2.89 ± 0.11(14)	4.00 ± 0.12(8)	2.11 ± 0.20(12)
SOD (Mitochondrial)	8.23 ± 0.25(16)	10.90 ± 0.39(12)	4.66 ± 0.25(16)	9.28 ± 0.56(11)	6.62 ± 0.10(8)
SOD (Post-mitochondrial)	12 90 ± 0.39(16)	12.90 ± 0.45(8)	6.19 ± 0.53(9)	9.76 ± 0.65(12)	7.66 ± 0.26(8)
Catalase	113.2 ± 2.8(30)	87.6 ± 3.80(12)	62.8 ± 2.4(19)	46.1 ± 3.5(12)	37.5 ± 1.3(13)
GPox (Mitochondrial)	798.6 ± 71.6(16)	606.4 ± 50.3(12)	539.9 ± 31.6(16)	967.1 ± 86.4(8)	1060.1 ± 90.3(8)
GPox (Post-mitochondrial)	1173.5 ± 37.5(12)	1928.1 ± 106.4(12)	1555.6 ± 122.2(8)	2340.7 ± 189.3(8)	2768.2 ± 113.8(8)
G6P dehydrogenase	52.40 ± 1.87(8)	9.29 ± 0.26(8)	25.10 ± 0.30(8)	103.90 ± 3.46(7)	58.30 ± 3.10(9)
Reduced glutathione (Mitochondrial)	3.44 ± 0.27(20)	3.89 ± 0.28(8)	0.84 ± 0.04(8)	3.76 ± 0.26(8)	0.77 ± 0.06(8)
Reduced glutathione (Post-mitochondrial)	21.10 ± 1.00(18)	10.10 ± 0.88(8)	16.90 ± 0.78(8)	22.30 ± 1.11(8)	13.33 ± 0.70(8)

The data are presented as mean ± S. E. M. of 8-20 independent observations.

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.

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

Summary

In the early stage, XO, mitochondrial GPox, G6PDH, and GSH in post-mitochondrial fraction decreased while mitochondrial SOD and GPox in post-mitochondrial fraction increased.

At the late stage i.e. at the end of one month, XO, mitochondrial and post-mitochondrial SOD, catalase, G6PDH and GSH in mitochondrial as well as post-mitochondrial fractions decreased while GPox in post-mitochondrial fraction increased significantly.

Insulin treatment, in general was ineffective in restoring XO, mitochondrial and post-mitochondrial SOD and catalase activities or GSH content in mitochondrial as well as post-mitochondrial fractions in one month group.

Our results suggest that GPox activity may be a major route of eliminating ROS in the liver of diabetic and insulin-treated diabetic rats.

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