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Diabetes-induced Metabolic Abnormalities in Myocardium: Effect of Antioxidant Therapy

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Effects of hyperglycemia (both diabetes and experimental galactosemia) on cardiac metabolism have been determined. In addition, the effect of supplemental antioxidants on these hyperglycemia-induced abnormalities of cardiac metabolism has been investigated. Diabetes or experimental galactosemia of 2 months duration in rats significantly increased oxidative stress in myocardium, as demonstrated by elevation of thiobarbituric acid reactive substances (TBARS) and lipid fluorescent products in left ventricle. Activity of protein kinase C (PKC) was elevated in the myocardium, and the activities of (Na,K)-ATPase and calcium ATPases were subnormal. Adminisiration of supplemental antioxidants containing a mixture of ascorbic acid, Trolox; α-tocopherol acetate, N-acetyl cysteine, β-carotene, and selenium prevented both the diabetes-induced and galactosemia-induced elevation of oxidative stress and PKC activity, and inhibited the decreases of myocardial (Na,K)-ATPase and calcium ATPases. The results show that these metabolic abnormalities are not unique to diabetes *per se*, but are secondary to elevated blood hexose levels, and supplemental antioxidants inhibit these metabolic abnormalities. Our findings suggest that antioxidants inhibit abnormal metabolic processes that may contribute to the development of cardiac disease in diabetes, and offer a potential clinical means to inhibit cardiac abnormalities in diabetes.

Keywords: Antioxidants, diabetes, galactosemia, heart, oxidative stress

INTRODUCTION

Cardiovascular disease is the leading cause of mortality and morbidity in the diabetic population.[1] Despite the increased frequency of atherosclerosis in diabetics, congestive heart failure occurs more frequently in diabetics than the frequency of coronary artery disease would predict. Cardiac disease in diabetic subjects is reported to be due to a combination of microangiopathy, macroangiopathy, autonomic neuropathy, and various other factors which produce structural and functional alterations in heart.[2] Diabetes Control and Complications Trial has shown that intensive glucose control has beneficial effects on cardiac abnormalities,[3] but in many patients such glycemic control is difficult to achieve and maintain indefinitely.

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Multiple biochemical sequelae of hyperglycemia have been postulated to contribute to the heart disease initiated by diabetes. Diabetes has been shown to impair (Na,K)-ATPase and calcium ATPase activities of the myocardium,[4–6] and alterations in the activities of these enzymes, possibly by altering ion concentrations in myocytes, have been implicated in the development of cardiomyopathy.[4,5,7,8] Likewise, diabetes increases activity of protein kinase C (PKC) in cardiomyocytes,[9–11] a change which can result in ventricular hypertrophy and impaired contractility. In diabetes elevated levels of free radicals in heart muscle have been reported to be involved in the regulation of activities of both ATPases and PKC.[12–14]

Antioxidants have been found to have potential value in treatment of diabetes-induced heart disease, ascorbic acid supplementation improves myocardial performance in diabetic rats,[15] and α-tocopherol supplementation inhibits diabetes-induced myocytosis, and prevents autonomic neuropathy in hearts of diabetic rats.[16] Antioxidants normalize diabetes-induced abnormalities in ATPases, PKC and oxidative stress in other tissues such as retina.[17] Since cardiac tissue develops these similar biochemical abnormalities in diabetes, antioxidants might have beneficial actions also on cardiac tissue metabolism. Recent clinical and epidemiological studies suggest that antioxidant treatment might reduce the risk of diabetes-induced cardiac disease.[18]

Experimental galactosemia, induced by feeding of normal rats galactose-supplemented diet, provides an experimental model to differentiate sequelae of hyperglycemia from other sequelae of insulin deficiency. Galactose-fed animals have elevated levels of blood hexose, but do not develop other sequelae of insulin deficiency, such as alterations in lipids and protein metabolism. This experimental elevation of tissue aldohexose levels has been shown to reproduce complications of diabetes in several tissues, including retina[19] and peripheral nerve.[20] In contrast to retina, the effects of experimental galactosemia on cardiac structure and function is not well understood, only a couple of such studies have appeared which report subnormal resting beat rate of right atrium, and prolonged contraction and relaxation times in galactose-fed rats.[21,22]

In the present study, effects of experimental galactosemia on cardiac metabolism have been compared to experimental diabetes in rats. In addition, the effect of supplemental antioxidants on these hyperglycemia-induced abnormalities of cardiac metabolism has been determined.

MATERIALS AND METHODS

Sprague–Dawley rats (200–220 g) were randomly assigned to normal, diabetic or galactosemic groups. Diabetes was induced by an injection of alloxan monohydrate (45 mg/kg, i.v.) after a 24 h fast. Insulin was given as needed to diabetic rats to allow slow weight gain while still allowing polyuria, hyperphagia, and hyperglycemia (blood glucose levels of 20–50 mM). In a group of normal rats, experimental galactosemia was induced by feeding Purina 5001 rat diet enriched with 30% D-galactose (w/w). These experiments conformed to the ARVO Resolution on the Use of Animals in Research. Half of the diabetic rats and galactose-fed rats prospectively were assigned to receive diets supplemented with an antioxidant mixture containing ascorbic acid, 1 g/kg; Torolox 500 mg/kg; α-tocopherol acetate, 250 mg/kg; N-acetyle cysteine, 200 mg/kg; β-carotene 45 mg/kg; and selenium 0.1 mg/kg of diet. In order to achieve complete protection against oxidative stress, a mixture of antioxidants (including both water soluble and lipid soluble forms of tocopherol) having different mode of actions was used. Since diabetic rats consume more food than experimentally galactosemic rats, the concentration of each antioxidant in the diet for galactose-fed rats was increased by 40% in order to achieve similar antioxidant consumption (per unit body weight/day) by both diabetic and galactosemic animals. The antioxidants were mixed into

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powdered diets and the diets were replaced weekly, and food consumption was measured to calculate the amount of antioxidants consumed. Nonenzymatically glycated hemoglobin (GHb) was measured at 2 months of diabetes or galactosemia using affinity columns (Glyc-Affin; Pierce, Rockford, IL). All of these methods have previously been described by us.[23]

At the end of 2 months of diabetes or experimental galactosemia (with or without therapy), rats were fasted overnight, anesthetized, and the heart was collected and weighed. In order to be consistent, all biochemical parameters were measured in tissues obtained from left ventricle. A small portion of left ventricle (0.3–0.5 cm) was immediately rinsed with cold phosphate buffer saline and frozen at −80°C until further use.

To estimate lipid peroxide levels, TBARS were quantitated in left ventricle by measuring malonaldehyde–thiobarbituric acid adducts formed by acid hydrolysis at 100°C using 1,1,3,3-tetramethoxy propane as a standard, and the absorbance was measured at 535 nm.[24] This assay is sensitive up to 0.3 μmol/l.

Lipid fluorescent products, which represent cross-linking of membrane protein with malondialdehyde, were measured in heart by the method described by Jain.[24] Lipids were extracted from heart using isopropanol and chloroform, and fluorescence was measured at excitation of 360 nm and emission of 440 nm.

Activity of PKC was determined in left ventricle using an *in situ* assay.[25–27] Briefly, a small piece of left ventricle (50–75 mg wet weight) was incubated at 30°C for 15 min with a buffered salt solution containing 50 μg/ml digitonin to permeabilize the tissues, 100 μM [γ-^{32}P ATP] containing 1000–1400 cpm/pmole and 100 μM of PKC-specific peptide substrate ((RKRTLRRL) corresponding to the threonine phosphorylation site of the epidermal growth factor receptor (residue 691–698)). PKC activity was assessed based on the transfer of ^{32}P from [γ-^{32}P ATP] to the octapeptide, and represented the sum of PKC activity of various isoforms present in the tissue. PKC activity was validated by measuring enzyme activity in the presence and absence of Phorbol esters (100 nM tetradecanoylphorbol acetate) and staurosporine (50 nM). Tetradecanoylphorbol acetate stimulated PKC activity by 2–3 fold, and staurosporine inhibited by more than 90%.

(Na,K)-ATPase and calcium ATPase were measured as reported previously,[28] except that the enzymes were assayed using 5 mM of [γ^{32}P] ATP as substrate. A small portion of left ventricle (about 100 mg) was homogenized in 20 mM Tris-HCl pH 7.5 containing 0.1 mM phenylmethylsulfonyl fluoride and 0.2 M sucrose and the cell debris was separated by centrifuging the homogenate at 2000× *g* for 5 min. In order to reduce the amount of contaminating cytosolic proteins and hemoglobin, crude membrane fractions were prepared by centrifuging homogenate at 15,000× *g* for 10 min. This membrane preparation was suspended in 50 mM Tris-HCl pH 7.5 containing PMSF, and was used for enzyme assay after being frozen and thawed three times in order to permeabilize the membranes. Calcium ATPase was measured using the same reaction mixture containing ouabain and 90 μM $CaCl_2$. (Na,K)-ATPase activity was calculated by determining the difference between the activities obtained in the absence and presence of ouabain, and calcium ATPase activity was calculated as the difference between the activities obtained in the presence and absence $CaCl_2$.

Tissue protein was measured by the Bradford method[29] using bovine serum albumin as standard. Data are reported as mean ± SD and analyzed statistically using the nonparametric Kruskal–Wallis test followed by Mann–Whitney test for multiple group comparison. Similar conclusions were reached also by using ANOVA followed by Fisher or Tukey tests.

RESULTS

Blood aldohexose concentrations were significantly increased both in diabetic rats and in

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galactose-fed rats, as shown by GHb levels (Table I). The severity of hyperglycemia was not affected by the dietary supplementation of antioxidants; GHb, body weight and food consumption were similar in control and antioxidant-supplemented groups of diabetic animals and of galactose-fed animals. As intended, the consumption of antioxidants by the treated diabetic rats and galactosemic rats was comparable (Table II).

In diabetes, oxidative stress (as estimated by TBARS and lipid fluorescent products) was elevated in myocardium by 60–80% ($p < 0.05$) compared to normal (Figure 1) Similarly, levels of TBARS and lipid fluorescent products were elevated by 40–50% also in experimentally galactosemic rats. Two months of hyperglycemia (diabetes or galactose feeding) resulted in elevation of myocardial PKC activity by more than 80% (Figure 2). In the same hyperglycemic animals, activities of myocardial (Na,K)-ATPase and calcium ATPases were significantly (p value < 0.05) decreased (Figure 3).

Dietary supplementation with the antioxidant mixture had beneficial effect on all of these

TABLE I Severity of hyperglycemia is not affected by antioxidant supplementation

	GHb (%)	Body weight (g)	Food intake (g/day)
Normal	3.9 ± 0.8	404 ± 35	32 ± 3
Diabetes	12.6 ± 1.8	251 ± 28	45 ± 10
Diabetes + antiox	11.6 ± 1.2*	248 ± 15*	43 ± 7*
Galactose	6.0 ± 1.1	343 ± 18	36 ± 4
Galactose + antiox	6.6 ± 1.4**	349 ± 32**	36 ± 8**

Values are mean ± SD of 6–8 rats in each group. p value > 0.5 compared with *diabetes or **experimental galactosemia.

TABLE II Supplemental antioxidants consumption was similar both in diabetic rats and experimentally galactosemic rats in antioxidant supplementation groups

	Supplemental antioxidants (mg/kg body weight/day)					
	Ascorbic acid	Trolox	α-tocopherol acetate	N-acetyl cysteine	β-carotene	Selenium
Diab + antiox	175 ± 28	88 ± 14	44 ± 7	35 ± 6	7.9 ± 1.2	0.017 ± 0.003
Gal + antiox	162 ± 19	81 ± 10	41 ± 5	32 ± 5	7.3 ± 1.7	0.016 ± 0.002

Data are mean ± SD of 6–8 rats/group.

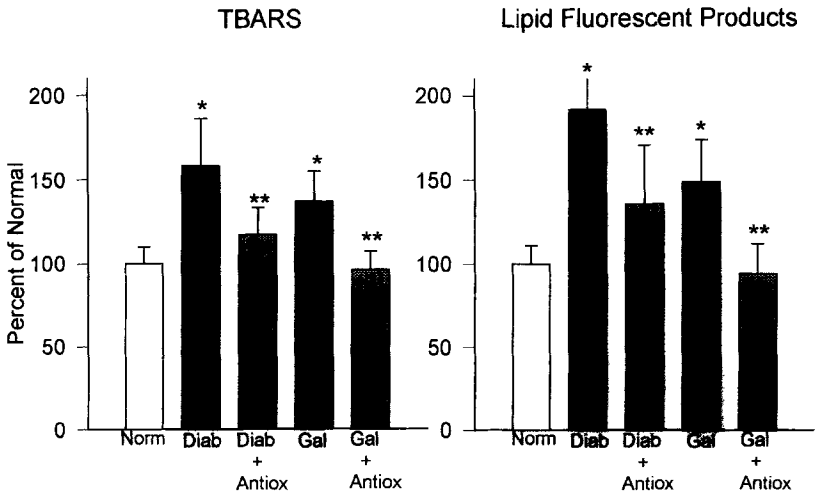

FIGURE 1 Effect of diabetes or experimental galactosemia on oxidative stress in myocardium. Oxidative stress was measured in left ventricle by estimating TBARS and lipid fluorescent products after 2 months of hyperglucemia. Total numbers of rats used in each group varied from 7 to 10. Results are mean ± SD. *p value < 0.05 compared with normal, and **$p < 0.05$ compared with diabetes or galactosemia.

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diabetes- and galactosemia-induced abnormalities of metabolism in the myocardium. Hyperglycemia-induced elevated levels of TBARS and lipid fluorescent products (Figure 1) and PKC activity (Figure 2) were normalized by antioxidant supplementation. Likewise, decreases in activities of (Na,K)-ATPase and calcium ATPase were prevented by antioxidant supplementation in diabetes and in experimental galactosemia (Figure 3).

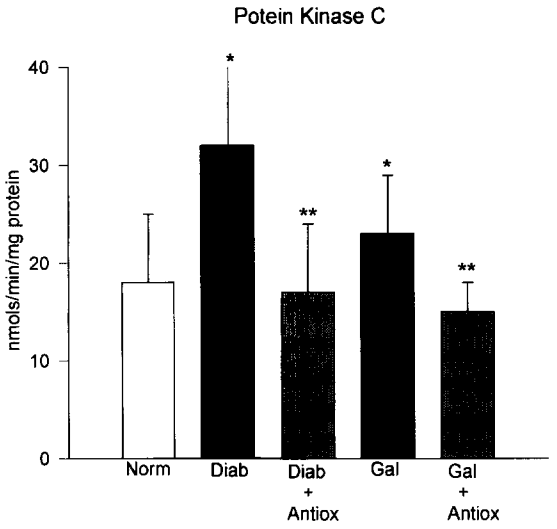

FIGURE 2 Antioxidant-supplementation prevents diabetes- or galactosemia-induced abnormalities of PKC. PKC was measured by *in situ* method in left ventricle using 50–75 mg wet weight of tissue. Each group had 6–8 rats and the values are mean ± SD. *p value < 0.05 compared with normal, and **p value < 0.05 compared with diabetes or galactosemia.

DISCUSSION

Diabetes results in increased oxidative stress and PKC activity, and subnormal key ATPases activities in cardiac tissue. Evidence that these defects are replicated also in cardiac tissue of experimentally galactosemic animals indicates that these metabolic abnormalities are not unique to diabetes *per se*, but are secondary to elevated blood hexose levels.

Each of these biochemical defects potentially contribute to cardiac dysfunction in diabetes. Oxygen-derived free radicals have been implicated in the pathogenesis of myocardial ischemia/reperfusion injury and atherosclerosis.[30] Increased production of free radicals by inhibiting

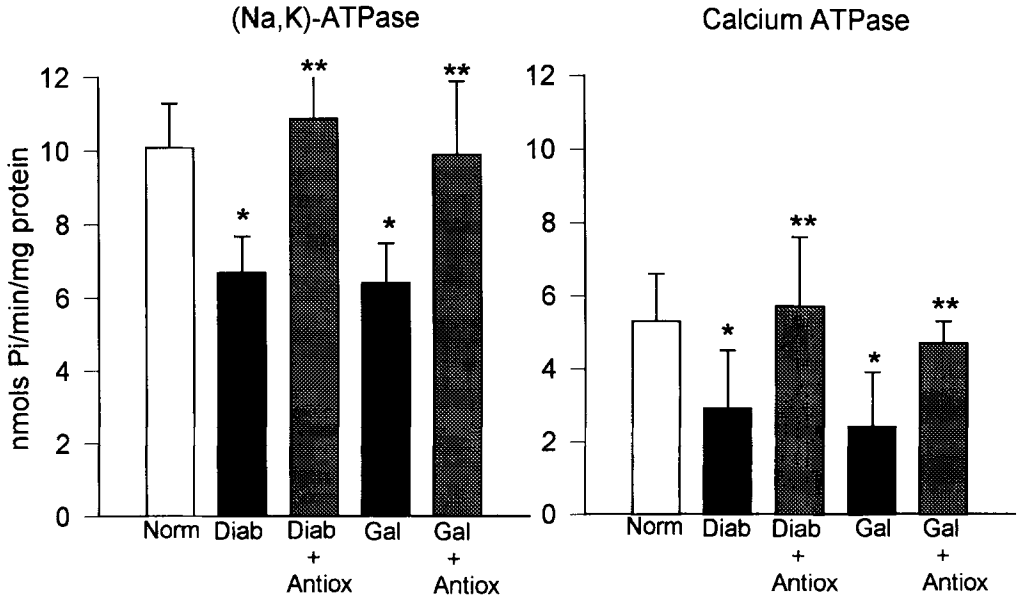

FIGURE 3 Diabetes- or galactosemia-induced abnormalities in (Na,K)-ATPase and calcium ATPase; (Na,K)-ATPase and calcium ATPase were measured in the crude membrane fraction of left ventricle, and the effect of antioxidant supplementation on ATPases was determined. Data represent mean ± SD of 6–8 rats in each group. *p value < 0.05 compared with normals, **p value < 0.05 compared with diabetes or galactosemia.

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prostacyclin synthesis may impair vascular contractile and relaxation mechanisms.[31] ATPases are reported to play a crucial role in the development of cardiomyopathy,[4,5,7,32] possibly by altering ion concentrations in myocytes. The ability of the sarcoplasmic reticulum to take up calcium is impaired in diabetic heart, and prolonged relaxation time of the diabetic heart is correlated with decreasd sarcoplasmic calcium uptake and decreased calcium ATPase.[33]

Elevation of PKC activity in myocardium also is associated with impaired regulation of cardiomyocyte contractility and vascular hemodynamics.[34,35] Over-expression of the β-isoform of PKC in cardiomyocytes of transgenic mice has been shown to result in cardiac hypertrophy and cardiomyopathy.[36] Antioxidants are able to prevent hyperglycemia-induced alterations of oxidative stress, PKC and ATPases in cardiac tissue. The beneficial effects of antioxidant supplementation on myocardial metabolism in our hyperglycemic animals seem not to be mediated by amelioration of hyperglycemia since GHb and body weights were not affected in antioxidant-treated groups. The severity of blood hexose elevation intentionally was kept comparable between control and antioxidant-treated animals in order to avoid confounding potential effects of antioxidants with the effects due to differences in glycemia.

Antioxidant-supplementation has been reported to reduce the risk of cardiac disease in diabetes.[16,18] The mechanism by which the antioxidants mediate their beneficial effect on cardiac metabolism is not clear. α-tocopherol has been shown to inhibit proliferation of vascular smooth muscle cells, and in this process inhibition of PKC translocation induced by phorbol esters has been implicated,[37] but other water and lipid soluble antioxidants are inactive.[38] It is recognized, however, that these dietary supplements can act also in ways other than as antioxidants. α-tocopherol directly influences levels of DAG (a regulator of PKC activity) in glomeruli via its effect on modulation of diacylglycerol kinase activity.[39,40] In addition, immunoblotting studies in glomeruli showed no difference in the protein levels of DAG kinase α and γ isoforms, thus suggesting that d-α-tocopherol is modulating the kinetics of DAG kinase.

Abnormalities of PKC, ATPases and oxidative stress seem closely inter-related in cardiac tissue. Pharmacologic inhibition of the hyperglycemia-induced elevation in PKC activity has been shown to inhibit increased free radical production,[41] and reactive oxygen species have been reported to be capable of increasing the activity of PKC.[14,42] In retina[27] normalization of the diabetes-induced increase in PKC activity by a selective inhibitor of β isoform of PKC corrects also the defects in the activities (Na,K)-ATPase and calcium ATPase, suggesting that increased PKC is playing a crucial role in the regulation of these ATPases. In addition, other mechanisms which are reported to regulate the ATPase, e.g, phosphorylation/dephosphorylation of its catalytic subunit,[43,44] and influence of free radicals on both (Na,K)-ATPase and calcium ATPase[12,13] cannot be ruled out in hyperglycemia.

Data from diabetes control and complications trial research group, however, suggest that hyperglycemia *per se* may not be the major determinant of diabetes-induced cardiac dysfunction.[3] In heart of diabetic rats, antioxidant-supplementation improves myocardial performance,[15] and inhibits diabetes-induced myocytosis, and prevents autonomic neuropathy.[16] Thus, our results suggest that antioxidants inhibit abnormal biochemical processes that may contribute to the development of cardiac disease in diabetes, and offer a potential clinical means to inhibit cardiac disease in diabetes.

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