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Myocardial Effects of Ethanol Consumption in the Rat With Streptozotocin-Induced Diabetes

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Background: Rats with streptozotocin (STZ)-induced diabetes exhibit alterations in cardiac function, ventricular remodeling, and changes in cell signaling, which includes protein kinase C (PKC) isoforms. Moderate consumption of ethanol has a beneficial effect on cardiovascular outcomes in the general population, an effect that has recently been found to extend to patients with diabetes mellitus. We studied the effect of low-dose ethanol consumption on cardiac function, geometry, and PKC isoforms in the rat with STZ-induced diabetes.

Methods: Four groups of rats were studied over 8 to 10 weeks: control, STZ-induced diabetes, 12% (v/v) ethanol consumption, and STZ-induced diabetes plus 4% (v/v) ethanol consumption. Invasive hemodynamic measurements were performed; myocardial tissue was obtained for analysis for total PKC and cytosolic and membrane protein content of PKC-α, PKC-δ, and PKC-ε, and two-dimensional and M-mode echocardiograms were obtained.

Results: Compared with rats with diabetes alone, consumption of 4% ethanol prevented the decrease in left ventricular dP/dt seen with diabetes alone, as well as the increase in left ventricular internal dimension. Up-regulation of PKC-α, -δ, and -ε occurring in the diabetic animals was also prevented by ethanol consumption, whereas ethanol alone had no effect on PKC isoform pattern.

Conclusions: These data suggest that STZ-induced cardiac remodeling and dysfunction are associated with increases in PKC activity, particularly PKC-α, -δ, and -ε, and that consumption of ethanol can prevent these changes.

Key Words: Diabetes Mellitus, Protein Kinase C, Ethanol, Myocardium.

THE CARDIOVASCULAR CONSEQUENCES of diabetes mellitus are the principal causes of death and disability (Deckert et al., 1978; Garcia et al., 1974; Ruderman and Haudenschild, 1984) and include macrovascular (atherosclerosis) and microvascular sequelae and cardiomyopathy. Cardiomyopathy, and the resultant heart failure, is common among patients with diabetes mellitus, even in the absence of coronary artery disease (CAD) (Kannel et al., 1977). Cardiomyopathy also may account for the increased mortality rate of diabetic patients with acute myocardial infarction (twice that of nondiabetic patients) that is not explained by the size of the infarct. The importance of a diabetic cardiomyopathy in cardiovascular mortality is demonstrated by the findings of the Honolulu Heart Study, in which a direct effect of diabetes on the myocardium was demonstrated (Burchfiel et al., 1993).

Individuals who consume moderate to large amounts of ethanol are susceptible to an increased risk of CAD and cardiomyopathy. High consumption of ethanol is associated with the development of cardiomyopathy, hypertension, and hemorrhagic stroke. As many as 21% of subjects with excessive ethanol intake had clinical evidence of heart failure (Schenk and Cohen, 1970), and between 60 and 80% of patients with idiopathic dilated cardiomyopathy have abused ethanol (Alexander, 1966; Massumi et al., 1965). It is recognized that consumption of moderate amounts of ethanol, compared with abstinence, is associated with a lower risk of death from CAD and recurrent myocardial infarction for the population as a whole (Klatsky et al., 1992; Steinberg et al., 1991). Thus, the risk/benefit of ethanol on the cardiovascular system may be a J- or U-shaped relationship. Although the specific pathophysiological process by which ethanol affects the heart is not yet defined, the ultimate severity of injury seems to be influenced by genetic, metabolic, and environmental factors (Mousmoush and Abi-Mansour, 1991; Regan, 1984).

Because ethanol may alter glycemic regulation and potentially aggravate the cardiovascular, neurological, and immunosuppressive changes in patients with diabetes mellitus, the previous lack of data regarding the influence of ethanol in diabetes resulted in the doctrine that ethanol

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consumption was universally detrimental in diabetic patients (Keilman, 1983). This doctrine persisted even though studies suggested that consumption of moderate amounts of ethanol may be beneficial in diabetes (Connor and Marks, 1965; Klatsky et al., 1992; McDonald, 1980). It had been reported recently that alcohol consumption exerts an overall beneficial effect in decreasing the risk of death due to coronary heart disease in patients with diabetes (Tanascescu et al., 2001; Valmadrid et al., 1999). That finding is of great importance because cardiovascular diseases are responsible for the greatest mortality in diabetes.

We studied the effect of low-dose ethanol on the development of myocardial dysfunction in rats with streptozotocin (STZ)-induced diabetes. Echocardiography was used to determine whether ethanol consumption was associated with myocardial protection or was detrimental, because the myocardial disease in diabetes may be responsible for increased morbidity, with and without coronary atherosclerosis. We studied alterations in myocardial protein kinase C (PKC) isoforms because we, and others, have shown that both Ca^{2+}-dependent and -independent myocardial PKC isoforms (PKC-α, -δ, and -ϵ) increase in activity and content in rats with both alcoholic and diabetic heart disease (Giles et al., 1998; Given et al., 1998; Lee et al., 1989; Okumura et al., 1988).

METHODS AND MATERIALS

Model of Diabetes and Ethanol Administration

Specific pathogen-free Sprague-Dawley male rats (~125 g) were used for all experiments. Experimental diabetes was induced by the administration of a single dose of STZ (55 mg/kg intravenously) as we described previously (Given et al., 1994). Control rats received an equal volume of 0.02 M sodium citrate buffer (vehicle, pH 4.5). The experimental groups were controls consuming water, rats consuming 12% ethanol, diabetic rats consuming water, and diabetic rats consuming 4% ethanol (n = 5 per group). The decrease in ethanol concentration in the diabetic rats was required because they ingested three times more water per day than did euglycemic rats; thus, the total ethanol ingested daily did not differ between the experimental groups (22 ml/day of 12% ethanol versus 63 ml/day of 4% ethanol). All groups were maintained on Purina (St. Louis, MO) Rat Chow and were provided fluid ad libitum.

Echocardiographic Analysis of Cardiac Performance

Rats were anesthetized with ketamine (50 mg/kg intramuscularly). Echocardiograms were recorded by using a Toshiba (Tokyo, Japan) Model 270 echocardiography instrument with a 7-MHz transducer that was calibrated with phantoms before use. M-mode recordings were obtained of the left ventricle (LV) at the level of the mitral papillary muscle by using two-dimensional echocardiographic guidance in the long-axis view. Data were recorded on a SuperVHS 0.5-in. tape for playback. Six consecutive cardiac cycles for each view per parameter were digitized from tape onto a Freehand (Macromedia, San Francisco, CA) digital acquisition system and the average value calculated. Echocardiographic measurements included M-mode end-diastolic interventricular septal thickness, LV internal dimension, and posterior LV wall thickness, with the leading edge method, and were made by personnel blinded to treatment. LV mass (LVM) was calculated from the echocardiographic data by using the formula described by Litwin et al. (1995). Because there were significant differences in body weight in the diabetic groups, the LVM was normalized to body weight and reported as LVM index.

Determination of Mean Arterial Blood Pressure (MAP) and LV Performance In Vivo

MAP and LV function were determined in each of the rats after completion of the echographic analysis. Anesthetized rats were placed on a temperature-controlled surgical table, and the right carotid artery, jugular vein, and left femoral artery were isolated. A polyethylene catheter was introduced into the jugular vein for drug administration and measurement of venous pressure. A similar catheter was introduced into the carotid artery and advanced into the LV for measurement of ventricular pressures by using a Digi-Med Heart Performance Analyzer (Micro-Med, Louisville, KY). A third catheter was introduced into the left femoral artery for determination of MAP by using a Digi-Med Blood Pressure Analyzer (Micro-Med). After a 15-min stabilization period, heart rate, LV pressure, LV ± dP/dt, and MAP were continuously recorded for 5 min by using a Digi-Med Heart Performance Analyzer.

Analysis of PKC

For total tissue extract, hearts were rapidly removed, briefly perfused with ice-cold phosphate-buffered saline in a retrograde manner to remove residual blood, and then frozen in liquid nitrogen. At time of assay, portions of the frozen ventricles were homogenized in 3 to 5 volumes of homogenization buffer [20 mM Tris-HCl, 250 mM sucrose, 2 mM ethylenediaminetetraacetic acid, 2 mM ethyleneglycoltetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 0.02% leupeptin, and 0.1% Triton-X 100 (Rohm & Haas Co., Philadelphia, PA); pH 7.5], incubated for 1 hr at 4°C, then centrifuged at 3000 rpm for 30 min at 4°C. The supernatants were stored at −20°C until used. Membrane and cytosolic fractions of myocardial tissue were prepared by homogenizing tissue (Polytron, Paterson, NJ; setting 7 for 30 sec) in homogenization buffer without Triton X-100. The homogenates were centrifuged at 1000 × g for 10 min at 4°C. The resulting supernatant was ultracentrifuged at 100,000 × g for 30 min at 4°C. The supernatant was retained as the cytosolic fraction. The remaining pellet was resuspended and incubated for 1 hr at 4°C in homogenization buffer containing 0.1–1.0% Triton X-100, then it was centrifuged at 1000 × g for 10 min. The supernatant resulting from this last centrifugation was retained as the membrane fraction. Protein content of the various fractions was measured with the bicinchoninic acid (BCA) method.

PKC activity was measured by using a kit for PKC activity (Amersham, Inc., Arlington Heights, IN) which measured the phosphorylation of the specific substrate octapeptide (RKRTLRRL) in the presence of Ca^{2+}, phosphatidylserine, and diacylglycerol (DAG) by using [^{32}P]adenosine triphosphate (Amersham Searle, Waltham, MA). The protein content of the fractions was measured by the BCA method. The enzymatic data are expressed as picomoles per milligram of protein per minute present in the total tissue, membrane, and cytosolic fractions after correction for the nonspecific kinase activities found in the absence of Ca^{2+}, phosphatidylserine, and DAG.

Tissue samples containing 50 to 100 mg of protein (BCA method) were separated on 10% SDS-PAGE gels and assayed by Western blot analysis for PKC isozyme proteins by using polyclonal antibodies specific for each isozyme. The bound antibody was detected by an enhanced chemiluminescence method conducted in accordance with the manufacturer's instructions (Amersham, Inc.). Exposure times of immunoblots to Hyperfilm (Amersham Bioscience, Piscataway, NJ) were 5 min. The gel image was scanned with DeskScan II (Hewlett-Packard, Palo Alto, CA). The band of specific PKC' protein was quantified with NIH Image (Scion Corp., Frederick, MD) software after corrections for film background and β-actin protein density. The specificity of the antibodies for each isozyme was demonstrated by incubating each antibody with authentic samples of each rat RKC isozyme.

Statistical Analyses

A one-way ANOVA was used to test for differences among the experimental groups. Dunnett's post hoc test was used to identify groups that were statistically different from the control group, and Duncan's multiple range test was used for intergroup comparisons. The Kruskal-Wallis one-way ANOVA for ranks was used when data failed the test for normality

(i.e., end-diastolic pressure data). Statistical significance was accepted at $p \leq 0.05$. All data are reported as mean ± SD.

RESULTS

The blood ethanol concentrations in the groups at time of death were (mean ± SD) 1.6 ± 0.8 mM for controls maintained on water, 13.6 ± 0.9 mM for rats receiving 12% ethanol, 1.8 ± 0.6 mM for diabetic rats on water, and 14.2 ± 1.6 mM for diabetic 4% ethanol–treated rats. The two ethanol-treated groups had significantly greater blood ethanol concentrations when compared with controls ($p < 0.05$) but were not different from each other ($p > 0.05$). Glycosylated hemoglobin of the diabetic rats was 13.8 ± 5.7 and was not statistically different from that of diabetic rats treated with ethanol (11.8 ± 4.1). Fasting blood glucose levels were 378 ± 34 mg/dl in diabetic rats and 365 ± 28 mg/dl in diabetic rats treated with ethanol and were not statistically different from each other. Blood glucose levels in the control and ethanol-treated rats were 94 ± 4 mg/dl and 84 ± 7 mg/dl, respectively ($p > 0.05$). Mean body weight at the initiation of the study was 215 ± 10 g (combined data; rats were weighed before randomization to groups). At the time of death, body weights per group were control, 593 ± 30 g; 12% ethanol, 581 ± 25 g; diabetic, 315 ± 24 g; and diabetic plus 4% ethanol, 310 ± 16 g.

Hemodynamic and Cardiac Function

The hemodynamic data and LV functional data are presented in Table 1. Systemic arterial blood pressure was higher in rats consuming ethanol compared with control and diabetic animals. The decrease in ventricular function (i.e., ±dP/dt) that occurred with diabetes was prevented by the consumption of 4% ethanol even though the systemic arterial pressure was higher than in diabetic rats not consuming ethanol.

Echocardiographic Analysis

Echocardiographic data from a cohort of rats examined at 10 weeks after STZ dosing are presented in Table 2. The increased interventricular septal and posterior wall thickness recorded from rats consuming 12% ethanol is consistent with LV concentric remodeling in response to the increased systemic arterial blood pressure. The increased LV dimension observed in diabetic rats was not found in diabetic rats chronically treated with 4% ethanol. LVM index was significantly increased in both the diabetic and diabetic plus ethanol groups when compared with controls, although the ethanol treatment showed some degree of attenuation (Fig. 1).

PKC

Both total PKC activity and membrane PKC activity were increased in the ventricles of diabetic rats. However, PKC activity (total and membrane) in diabetic rats maintained on 4% ethanol was similar to that of control rats (Fig. 2). In addition, the up-regulation of PKC isozymes (α, δ, and ϵ) in membrane fractions observed in both alcoholic and diabetic rats was normalized in diabetic rats treated with 4% ethanol (Figs. 3 and 4). There were no differences between groups in PKC isozymes in the cytosolic fractions (Fig. 5).

DISCUSSION

Data from this study indicate that consumption of 4% ethanol by diabetic rats has the effect of preventing some of the functional and biochemical alterations of the myocardium recorded from rats with STZ-induced diabetes or ethanol consumption alone. Specifically, ethanol consumption prevented the decrease in peak LV +dP/dt and −dP/dt and the up-regulation of PKC—in particular the α, δ, and ϵ isoforms—that occurred in the diabetic rat. The increase in MAP seen in the rats consuming 12% ethanol alone did not occur in the diabetic rats consuming 4% ethanol, although the pressures in the ethanol-consuming diabetic rats tended to be higher than in the diabetic rats not consuming ethanol.

Table 1. Hemodynamic Measurements at 10 Weeks of Diabetes

Parameter	Control	12% Ethanol	Diabetic	Diabetic + 4% ethanol
MAP (mm Hg)	93 ± 5	116 ± 8*	84 ± 6	100 ± 10
EDP (mm Hg)	5 ± 2	3 ± 2	3 ± 1	1 ± 1*
LV MaxP (mm Hg)	120 ± 5	134 ± 11	81 ± 30*	123 ± 17
+dP/dt (mm Hg/sec)	6657 ± 1433	7963 ± 1057	2940 ± 615*	7340 ± 1171
−dP/dt (mm Hg/sec)	4329 ± 962	5628 ± 1283	1602 ± 508*	5026 ± 403

MAP, mean arterial pressure; EDP, left ventricular end-diastolic pressure; LVP MaxP = peak left ventricular pressure; +dP/dt and −dP/dt, positive and negative rate of change in left ventricular pressure.
*$p < 0.05$ compared with the control group.

Table 2. Myocardial Echocardiographic M-Mode Measurements at 10 Weeks of Diabetes

Parameter	Control	Ethanol (12%)	Diabetic	Diabetic + 4% ethanol	ANOVA
IVSD (cm)	0.117 ± 0.008	0.151 ± 0.004*	0.111 ± 0.004	0.105 ± 0.005	$p \leq 0.002$
PWD (cm)	0.140 ± 0.006	0.159 ± 0.002*	0.128 ± 0.002	0.125 ± 0.004*	$p \leq 0.001$
LVD (cm)	0.614 ± 0.028	0.593 ± 0.018	0.713 ± 0.036*	0.618 ± 0.032	$p \leq 0.045$

IVSD, interventricular septal defect; LVD, left ventricular dimension.
*$p \leq 0.05$ versus control in the respective experiment.

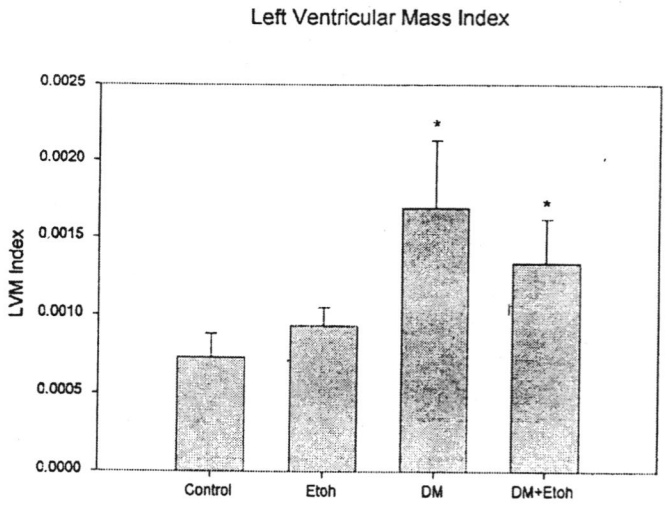

Fig. 1. Histogram showing left ventricular mass (LVM) index in control rats, 12% ethanol (Etoh) rats, diabetic (DM) rats, and diabetic rats maintained on 4% ethanol (DM+Etoh). Diabetic rats were treated with 4% ethanol because they consumed approximately 3 times the fluid volume of nondiabetic rats maintained on ethanol. *Significant statistical difference compared with the control group.

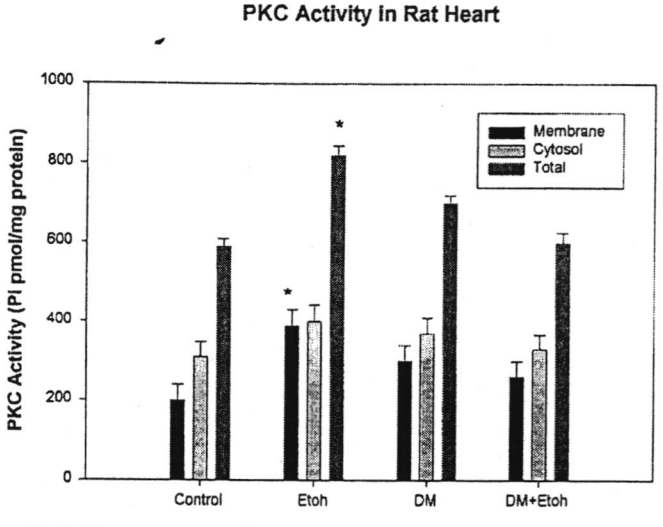

Fig. 2. Histogram showing the effect of 12% ethanol (Etoh), diabetes (DM), and chronic 4% ethanol treatment in diabetic rats (DM+Etoh) on total PKC activity in the rat heart. Diabetic rats were treated with 4% ethanol because they consumed approximately 3 times the fluid volume of nondiabetic rats maintained on ethanol. *Significant statistical difference compared with the control group.

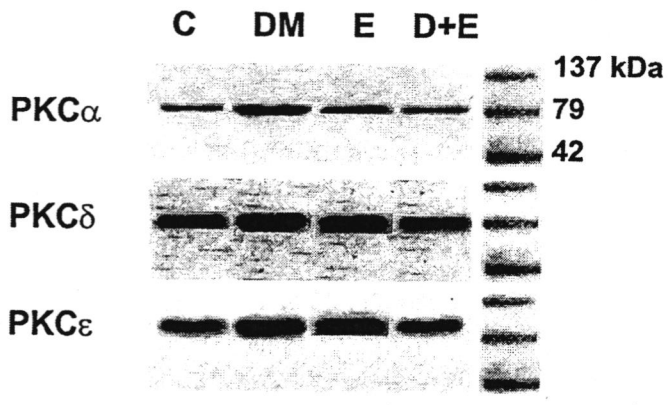

Fig. 3. Western blot of PKC-α, PKC-δ, and PKC-ε isoforms in normal rats (control; C), rats chronically maintained on 12% ethanol (E), diabetic rats (D), and diabetic rats chronically maintained on 4% ethanol (D+E). The bands of the marker proteins are indicated on the right.

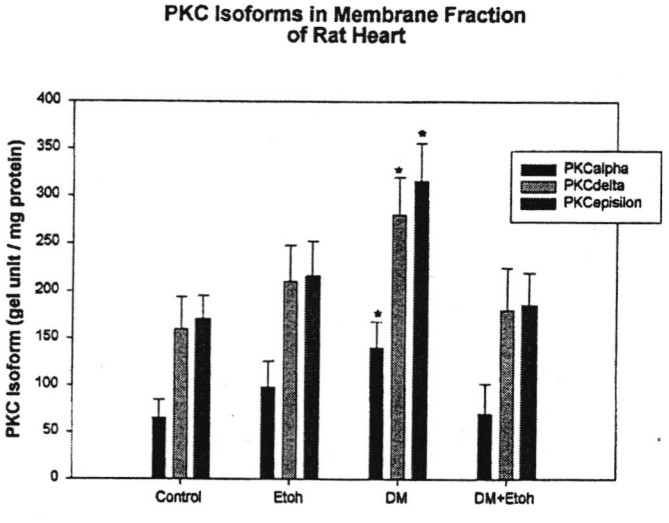

Fig. 4. Histogram showing changes in PKC-α, PKC-δ, and PKC-ε levels in myocardial tissue in control rats, rats maintained on 12% ethanol (Etoh), diabetic rats (DM), and diabetic rats chronically maintained on 4% ethanol (DM+Etoh). The increase in PKC-α, PKC-δ, and PKC-ε with diabetes was prevented by consumption of 4% ethanol. *Statistical difference compared with control.

To our knowledge, this is the first study investigating the effect of ethanol on the myocardium of the diabetic rat. Consequently, there are no reported similar in vivo data with which to compare our data. The in vitro effects of acute exposure to ethanol (80 to 240 mg/dl) on the contractile properties of LV papillary muscles obtained from healthy rats and those with STZ-induced diabetes have been studied (Brown et al., 1993). Ethanol decreased developed tension, time to peak tension, time to 90% relaxation, and the maximum rate of tension development in the muscles from both groups. The negative inotropic effects of ethanol were concentration dependent, and it seemed that the papillary muscles obtained from the diabetic rats were more sensitive to the negative inotropic effects of clinically relevant concentrations of ethanol.

An influence of ethanol on the changes in myocardial PKC that occur in diabetic cardiomyopathy provides support for the beneficial effects of ethanol on myocardial function. Hyperglycemic up-regulation of PKC by an increase in DAG has been proposed as a mechanism for the development of vascular complications in diabetes (Xia et al., 1994). Increased concentrations of DAG have been found in the myocardium of the rat with STZ-induced diabetes (Okumura et al., 1988) and in other tissues exposed to increased glucose concentrations. In hearts from rats with STZ-induced diabetes, up-regulation of PKC-ε occurred and was associated with a 5-fold increase in phosphorylation of troponin-I (Malhotra et al., 1997). Alterations in PKC may also interfere with the contractile proteins troponin-T, troponin-tropomyosin complex, and

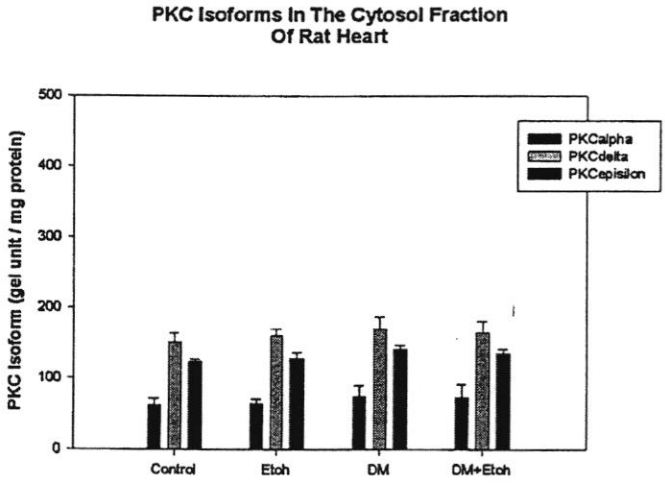

Fig. 5. Histogram showing no differences between groups in cytosolic PKC isoform (α, δ, and ϵ) levels in myocardial tissue in control rats, rats maintained on 12% ethanol (Etoh), diabetic rats (DM), and diabetic rats chronically maintained on 4% ethanol (DM+Etoh).

troponin-C, as well as with inhibition of Ca^{2+}-activated myofibrillar actomyosin Mg/adenosine triphosphatase activity and contractility (Malhotra et al., 1997). The PKC-δ isozyme can increase intracellular Ca^{2+} through L-type Ca^{2+} channels in *Xenopus* myocytes (Singer-Lahat et al., 1992). Increased PKC activity also influences nuclear gene transcription via the mitogen-activated protein kinase cascade to induce the immediate early gene program, with subsequent stimulation of the late genes that increase production of β-major histocompatibility complex, skeletal α-actin, inducible nitric oxide synthase, and angiotensin-converting enzyme (ACE) (Nishizuka, 1995). The influence of PKC on ACE expression and the demonstration that troponin-I phosphorylation by PKC-ϵ in the diabetic rat was prevented by an angiotensin II-AT_1-receptor antagonist receptor antagonist (Malhotra et al., 1997) indicates a relationship between PKC and the renin/angiotensin system. This is of interest because it agrees with our previous finding that chronic treatment with an ACE inhibitor attenuates the development of cardiomyopathy in the rat with STZ-induced diabetes (Given et al., 1994). Thus, persistent PKC activation resulting from chronic hyperglycemia may lead to alterations in cell growth and function via PKC-dependent phosphorylation of myocardial and skeletal muscle enzymes and proteins that may affect muscle contractility, gene expression, and growth.

The mechanism whereby 4% ethanol consumption prevents PKC up-regulation in the myocardium was not determined in this study. However, phospholipase D represents one possibility. PKC may up-regulate the activity of phospholipase D (Gustavsson, 1995), and this would result in increased formation of phosphatidic acid, which can degrade to form DAG and hence produce a further increased activation of PKC. Ethanol is known to have an inhibitory effect on phospholipase D and could therefore inhibit this amplification loop (Diamond and Gordon, 1997). This may account for the lack of an increase in PKC observed after chronic 4% ethanol ingestion in the diabetic myocardium.

It has been suggested that the benefits of alcohol consumption in diabetes may be related to improved insulin and glucose metabolism (Facchini et al., 1994; Lazarus et al., 1997). However, in this study the blood glucose concentrations and glycosylated hemoglobins were not different in the diabetic animals receiving alcohol compared with the diabetic animals not receiving ethanol. Also, although the model of STZ-induced diabetes in the rat in our laboratory was insulinopenic, the rats continued to gain weight, albeit at a slower rate, and did not demonstrate overt evidence of malnutrition.

We recognize that there is no perfectly suitable way to feed ethanol to experimental animals. We chose to have the rats consume ethanol in the drinking water to avoid the stress of gastric tube feeding. The blood ethanol concentrations were essentially the same for the diabetic and nondiabetic rats consuming ethanol because we allowed for the increased fluid intake manifested by the diabetic rats. Nevertheless, our model is not the pattern of humans consuming moderate amounts of ethanol, where the time of consumption may be very short.

CONCLUSION

These and other data suggest that STZ-induced cardiac remodeling and functional changes are associated with increases in PKC activity—particularly PKC-α, -δ, and -ϵ—and that ethanol consumption can prevent or attenuate these changes. Although this apparent relationship between changes in PKC and functional myocardial improvement needs to be confirmed by future cause/effect studies, these data may explain the observed benefit of moderate ethanol consumption on cardiovascular mortality and morbidity in patients with diabetes.

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