Cardiac Performance and Plasma Lipids of Omega-3 Fatty Acid–Treated Streptozocin-Induced Diabetic Rats

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We studied the effect of omega-3 fatty acid (ω3FA) treatment on plasma lipids and cardiomyopathy in the diabetic rat. The ω3FA preparation used was Promega. Male Wistar rats (250–275 g) were rendered diabetic by streptozocin (STZ; 55 mg · kg^{-1}). Nondiabetic control rats received the vehicle alone. Two weeks after STZ or vehicle injection, control and diabetic rats were randomly assigned to either a treated or untreated group. Promega was administered at a dose of 0.5 ml · kg^{-1} · day^{-1} by oral gavage for 4 wk, after which the rats were decapitated, plasma collected, and isolated working heart performance studied. Promega treatment did not affect plasma glucose, triglyceride, or cholesterol concentrations of either the control or diabetic rats. Cardiac performance was assessed by measuring the left ventricular response to changing left atrial filling pressures (7.5–20 cm H_2O). The treatment had no effect on peak left ventricular developed pressure (LVDP) or maximal rate of change of left ventricular pressure during systole ($+dP/dt_{max}$) or diastole ($-dP/dt_{max}$) in the nondiabetic control rats. LVDP and $+/-$ dP/dt were significantly improved ($P < .05$) in the treated diabetic rats compared with untreated diabetic rats, although cardiac performance did not improve to the nondiabetic level. Cardiac sarcoplasmic reticulum (SR) calcium transport activity was not affected by the treatment in the control rats but was significantly improved ($P < .05$) in the treated diabetic rats. These data suggest that ω3FA treatment partially blocks the development of experimental diabetic cardiomyopathy, possibly by affecting SR calcium transport activity. *Diabetes* 38:969–74, 1989

Cholesterol 1 mM = 38.7 mg/dl	Glucose 1 mM = 18 mg/dl

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Cardiac disease independent of coronary vascular disease is one of the secondary complications of chronic type I (insulin-dependent) diabetes mellitus (1). Experimentally, the cardiomyopathy is manifest as impaired left ventricular contractility and relaxation (2,3). Several biochemical and morphological alterations have been identified that may contribute to the diabetic cardiomyopathy. Clinical and experimental studies have demonstrated elevated tissue collagen levels in diabetic heart muscle in the absence of coronary vascular changes (4). In the diabetic rat, cardiac membrane calcium transport activities are attenuated: sarcoplasmic reticulum (SR) calcium transport and calcium ATPase activities are reduced (5,6) as is sarcolemmal calcium-pump activity (7). In addition, reduced oxygen delivery (8) and reduced levels of cellular ATP (9) may contribute to the impaired heart function. However, the etiology of the complex pathological alterations of diabetic cardiomyopathy remains incompletely defined.

Epidemiological data suggest an association between diets enriched in long-chain polyunsaturated omega-3 fatty acids (ω3FAs) and a reduced incidence of cardiovascular disease (10–12). Of possible relevance to these results are data indicating that ω3FAs reduce plasma lipid levels of both healthy and hypertriglyceridemic patients (13,14). In type I diabetes, ω3FA treatment has been shown to increase (15), decrease (16,17), or not affect (18) plasma triglyceride concentrations and to have similarly variable effects on plasma cholesterol. In type II (non-insulin-dependent) diabetes, the results are also variable because no effect or reduction in plasma triglyceride has been reported (19,20). Additionally, in an animal model of type II diabetes, insulin resistance was prevented by a diet enriched in ω3FAs (21), whereas in humans with type II diabetes, such treatment increased hepatic glucose output and impaired insulin secretion (20). Clearly the role of ω3FAs in diabetes-related lipid metabolic alterations, and the contribution these changes may make

toward reducing and/or exacerbating the risk of cardiovascular disease is equivocal.

Membrane lipid alterations can affect the activity of membrane-associated enzymes (22–24). Direct effects of ω3FAs on the heart are suggested by diet-induced modifications of cardiac membrane phospholipid fatty acyl composition (25–27). That ω3FAs may directly affect the heart is also suggested by the reduction in myocardial creatine kinase loss after coronary artery ligation in rats treated with a menhaden-oil diet (26), and the reduced incidence of spontaneous tachyarrhythmias in rat papillary muscle after an ω3FA-enriched diet (28). Other cardiovascular effects of ω3FAs in human type II diabetes include reductions in both systolic and diastolic blood pressures (19). However, in a swine model of myocardial ischemia, despite ω3FA-induced membrane phospholipid alterations, there was no effect on the incidence of postischemic arrhythmias or on the recovery of cardiac function (29). Cardiac effects of ω3FAs may therefore depend on the species and disease state investigated and on the ω3FA treatment protocol.

To date, effects of ω3FAs on diabetic cardiomyopathy have not been reported. Therefore, because cardiac phospholipid acyl-chain composition is altered by diets enriched in ω3FAs, and because these alterations may affect the heart, the effect of an ω3FA supplement on the development of streptozocin (STZ)-induced diabetic cardiomyopathy was investigated. This study also examined the effects of the ω3FA supplement on plasma lipid parameters and cardiac SR calcium transport activity. The results suggest that treatment with ω3FA may have an ameliorative effect on the development of diabetic cardiomyopathy.

RESEARCH DESIGN AND METHODS

Animal model and treatment protocol. Male Wistar rats, used in all experiments, were obtained from the University of British Columbia animal-care facilities. They weighed 250–275 g. Diabetes was induced with a single injection of 55 mg/kg i.v. STZ (Upjohn, Kalamazoo, MI). Three days after STZ administration, diabetes was confirmed by the presence of glycosuria (>2%). All animals given STZ became diabetic. Control and diabetic animals were randomly assigned to either untreated or treated groups 2 wk after STZ or vehicle administration. Animals in each experimental group were fed Rodent Laboratory Chow 5001 (Purina, St. Louis, MO). Food and water were supplied ad libitum to animals in each of the four experimental groups, and the animals were kept on a 12-h light-dark cycle.

The treatment protocol chosen to explore effects of ω3FA supplementation in the diabetic and control animals was conducted with Promega (Warner-Lambert, Morris Plains, NJ), a refined fish-oil product concentrated in long-chain polyunsaturated ω3FAs. The principal ω3FAs in the preparation are 5,8,11,14,17-eicosapentaenoic acid, C20:5,n-3 (EPA), and 4,7,10,13,16,19-docosahexaenoic acid, C22:6,n-3 (DHA). An initial dose-response study of the effect of Promega on isolated heart function in control and diabetic rats demonstrated an ameliorative effect of Promega at 0.5 ml · kg^{-1} · day^{-1} and 1.0 ml · kg^{-1} · day^{-1} (30). Based on those data, Promega was administered to control and diabetic treated rats at a dose of 0.5 ml · kg^{-1} · day^{-1}. Promega was obtained in bulk form; therefore, to prevent oxidation of the highly unsaturated lipids, aliquots of the stock were drawn and stored in glass vials under nitrogen at −80°C until they were used. Control and STZ-induced diabetic (STZ-D) animals were given Promega between 0830 and 0930 daily for the duration of the treatment. Promega was administered to the rats via oral gavage with a 75-mm stainless-steel tube. None of the animals treated by gavage demonstrated any apparent signs of discomfort or pain during the course of the study. Promega treatment was initiated 2 wk after STZ or vehicle injection and was continued for a period of 4 wk. Body weight and water consumption were recorded daily.

Experimental methods. Cardiac function was determined with an isolated working heart apparatus as described previously (2). After decapitation of the rat, the heart was rapidly excised, placed in cold perfusion buffer, and trimmed of pericardial fat and extraneous tissue. The Chenoweth-Koelle perfusion buffer (consisting of 10 mM glucose, 2.1 mM $MgCl_2$, 5.6 mM KCl, 120 mM NaCl, 2.2 mM $CaCl_2$, and 19 mM $NaHCO_3$) was aerated with 95% CO_2/5% O_2 at 37°C. The heart was initially perfused with Langendorff's method for 10 min. A 20-gauge stainless-steel cannula was then inserted into the apex of the left ventricle and fixed to a Stratham P23AA pressure transducer by means of an interconnecting 3-cm length of polyethylene 90 tubing. A 16-gauge stainless-steel cannula was fixed to the left atrial appendage. Intrinsic working heart function was determined by monitoring peak left ventricular developed pressure (LVDP) and the maximal rates of change of pressure during left ventricular systole and diastole while imposing various left atrial filling pressures (LAFP) on the heart. LAFP was regulated by an adjustable reservoir connected to the left atrial cannula. The height of the reservoir was calibrated to effect changes in LAFP from 7.5 to 20.0 cm H_2O. The hearts of both control and diabetic rats were paced at 300 beats/min at twice the threshold voltage by square-wave pulses of 5 ms duration. The ventricular pressure pulse and its first derivative were recorded by a Grass model 79D polygraph interfaced with an Apple II microcomputer curve-fitting program (31).

After this procedure, hearts were frozen in liquid N_2 and stored at −80°C until microsomes enriched in SR, were prepared. SR was prepared as previously described (32). Ventricular tissue was rapidly minced with scissors, followed by two 15-s homogenizations in 10 mM ice-cold $NaHCO_3$, pH 7.4. The homogenate was diluted to 25 ml with homogenization buffer and centrifuged at 500 × *g* for 15 min (all centrifugation at 4°C). The resulting pellet was discarded and the supernatant centrifuged at 7000 × *g* for 15 min. The pellet was discarded and the supernatant centrifuged at 31,000 × *g* for 30 min. The resulting pellet was washed in 12 ml of 0.6 M KCl, 30 mM histidine chloride, pH 7.0, and centrifuged at 31,000 × *g* for 30 min. The final pellet was resuspended in 0.75 ml of 0.25 M sucrose, 0.3 M KCl, and 0.1 M Tris-chloride, pH 7.2, frozen in liquid N_2, and stored at −80°C until it was used. The yield of SR protein was quantified by the method of Lowry et al. (33). Calcium transport activity was determined by a filtration technique. Free calcium concentrations ($[Ca^{2+}]_{free}$) were calculated with a FORTRAN program, TCATIONS.BC, that we modified slightly from previous studies (34). The SR (10–15 μg) was prein-

cubated for 3 min at a reaction temperature of 30°C in a medium consisting of (in mM): 40 histidine chloride, pH 6.8; 110 KCl, 5 $MgCl_2$, 5 NaN_3, 2.5 Tris-oxalate, and 5 Tris-ATP (Sigma, St. Louis, MO). The reaction was initiated by the addition of EGTA-buffered calcium (yielding $[Ca^{2+}]_{free}$ of 0.1 and 5.3 μM from 63 nM total added calcium, containing $^{45}CaCl_2$ [200,000 dpm/tube; Amersham, Arlington Heights, IL] to monitor calcium transport), proceeded for 5 min, and was terminated by filtration of an aliquot of the reaction mixture through a Millipore (South San Francisco, CA) HA 0.45-μm filter. The filter was washed once with 15 ml of 40 mM Tris-chloride, pH 7.2, to remove low-affinity, nonspecific-bound calcium. After drying, the filters were counted for radioactivity in 5 ml liquid-scintillation fluid.

Plasma glucose, cholesterol, and triglyceride were analyzed by an enzymatic colorimetric method with standard diagnostic assay kits (Boehringer Mannheim, Indianapolis, IN). Plasma immunoreactive insulin concentrations were determined by a double-antibody radioimmunoassay technique (Amersham).

Statistical methods. All data are presented as means ± SE. Data were analyzed by two-way analysis of variance (ANOVA). When group, treatment, or interactive differences were found, data were analyzed by one-way ANOVA, followed by Newman-Keuls test. Data were considered different at $P < .05$.

RESULTS

The data in Fig. 1 show that STZ-D rats did not grow as much as age-matched control animals during the experimental period. Figure 1 also shows that treatment with Promega did not affect body weight of either control or diabetic rats. The body weight of the control and control-treated animals at decapitation were 515 ± 15 g and 532 ± 9 g, respectively. The body weights of the diabetic and diabetic-treated rats at death were significantly lower than those of control rats at 385 ± 25 g and 403 ± 14 g, respectively. Characteristically, the untreated and treated diabetic rats exhibited signs typical of type I diabetes; polyphagia, polydipsia, and polyuria were apparent throughout the study period. The ω3FA

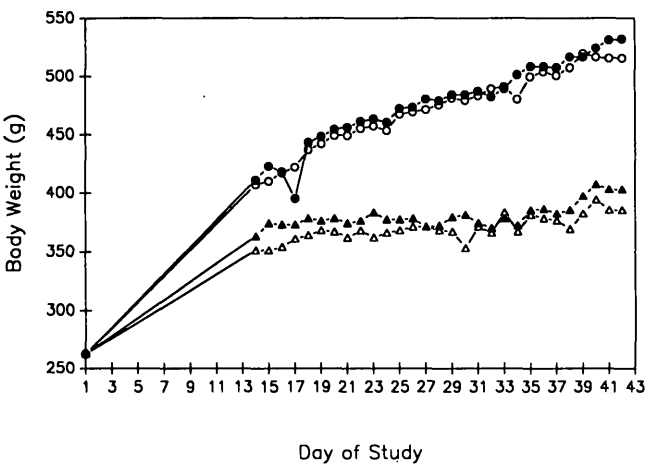

FIG. 1. Average daily body weight (g) of control (○), omega-3 fatty acid (ω3FA)–treated control (●), diabetic (△), and ω3FA-treated diabetic (▲) male Wistar rats. Day 1 represents day of either vehicle (control) or streptozocin (diabetic) injection. *n* = 11–12 animals per group.

treatment did not affect food or water consumption of either control or diabetic groups. Concentrations of plasma insulin and glucose collected at decapitation are shown in Table 1. The data clearly indicate that all animals given STZ were rendered diabetic. Insulin concentrations of STZ-D rats were significantly lower than those of controls.

Two-way ANOVAs indicated a significant group effect between control and diabetic groups when either insulin or glucose concentrations were considered. The data show that Promega treatment did not affect glucose homeostasis of controls and did not exacerbate the hyperglycemia of STZ-D. Plasma triglyceride concentrations of diabetic and treated diabetic groups were significantly elevated compared with nondiabetic control values. Promega treatment was associated with a hypotriglyceridemic response in the diabetic rats, although this effect was not significant. Table 1 also indicates that the plasma cholesterol concentration of untreated diabetic animals was higher than nondiabetic controls. In ω3FA-treated diabetic rats, there was a tendency for plasma cholesterol to be reduced compared with the untreated diabetic rats, although this effect was not significant. There was no effect of Promega on plasma cholesterol concentrations of the nondiabetic control treated animals.

The effect of diabetes and of ω3FA treatment on isolated working heart function is shown in Figs. 2–4. Peak LVDP of untreated diabetic rats was significantly lower than that of controls at each LAFP assayed (7.5–20 cm H_2O) (Fig. 2). Statistical analysis of the data by two-way ANOVA confirmed a significant group effect at each of these LAFP and also established that there was a significant group-treatment interaction at each LAFP >12.5 cm H_2O. Treating STZ-D rats with Promega for 4 wk reduced the degree to which the cardiac dysfunction developed. This is apparent in Fig. 2, in view of the fact that the cardiac function curve of the diabetic-treated animals is superior to that of the untreated diabetic animals. Fig. 2 also shows that Promega treatment did not affect peak LVDP of nondiabetic control Wistar rats.

The data in Figs. 3 and 4 illustrate the effect of diabetes and the ω3FA treatment protocol on the maximal rate of change of left ventricular pressure during systole ($+dP/dt_{max}$) and the maximal rate of change of left ventricular pressure during diastole ($-dP/dt_{max}$), respectively. Two-way ANOVA of the data in Figs. 3 and 4 indicates that there is a significant group-treatment effect at each LAFP >12.5 cm H_2O. The data in Figs. 3 and 4 suggest that the improved cardiac function of the treated diabetic animals is associated with increases in both systolic and diastolic function.

The SR is thought to play an important role in cardiac relaxation through active sequestration of myoplasmic calcium during diastole. Because diastolic function of Promega-treated diabetic rats was improved (Fig. 4), SR calcium transport activity was determined. The effect of the diabetic state and Promega treatment on SR calcium transport is shown in Table 2. The rate of calcium transport was determined for two $[Ca^{2+}]_{free}$, 0.1 and 5.3 μM. These calcium concentrations were used because 0.1 μM is in the range of the KCa^{2+} and 5.3 μM is a concentration at which V_{max} can be determined (data not shown). Cardiac SR Ca^{2+} transport at 0.1 μM $[Ca^{2+}]_{free}$ was significantly reduced in untreated diabetic rats. This depression in SR Ca^{2+} transport was prevented by the Promega treatment. Table 2 indicates that comparable

TABLE 1
Concentrations of plasma insulin, glucose, triglycerides, and cholesterol

Group	*n*	Insulin (μU/ml)	Glucose (mM)	Triglycerides (mM)	Cholesterol (mM)
Control	10	44.2 ± 3.2	5.95 ± 0.25	1.68 ± 1.02	1.32 ± 0.02
Control treated	12	58.3 ± 2.8	5.96 ± 0.23	1.45 ± 0.94	1.22 ± 0.06
Diabetic	8	22.2 ± 3.4*	20.31 ± 0.28*	5.51 ± 1.15	1.96 ± 0.34
Diabetic treated	10	19.7 ± 3.4*	20.60 ± 0.25*	2.16 ± 1.02	1.38 ± 0.11

Values are means ± SE.
*$P < .05$ vs. control.

results were obtained when Ca^{2+} transport was determined at 5.3 μM $[Ca^{2+}]_{free}$. The data in Table 2 also show that differences in calcium transport rates at 5.3 μM were not due to differences in nonspecific oxalate-independent calcium binding. These values averaged <3.5% of total oxalate-dependent calcium transport in all cases. It was also determined, that non-ATP-dependent calcium binding was negligible and did not contribute to the observed calcium transport rates (data not shown).

DISCUSSION

The results of the study indicate that the hypertriglyceridemia and hypercholesterolemia of the STZ-D rat are partially, although not significantly, reduced to levels seen in nondiabetic control rats. The study also showed that the Promega treatment reduced the degree to which the cardiac dysfunction developed in the diabetic rats. There was a significant improvement in the cardiac function of the ω3FA-treated diabetic rats when peak LVDP, $+dP/dt_{max}$, and $-dP/dt_{max}$ were assessed. However, the Promega treatment of the diabetic rats did not improve cardiac function to the level seen in the nondiabetic control group. The Promega treatment did not affect plasma lipids or isolated working heart function of nondiabetic control animals.

The effects of ω3FAs on lipid metabolism in diabetes are of considerable interest because of the established hyperlipidemia of the disease and the cardiovascular risk elevated serum lipids confer on the diabetic population. Results of studies investigating the effects of an ω3FA dietary supplement or enrichment on serum lipid parameters in diabetic patients are equivocal. Our results indicate that although Promega treatment tended to reduce plasma cholesterol and triglyceride concentrations, the hypolipidemic effect was not significant. Similarly, Promega treatment had no effect on either plasma cholesterol or triglyceride concentrations in the nondiabetic control rats. These results are at variance with studies showing that in nondiabetic conditions, fish-oil–enriched diets can significantly reduce plasma triglyceride and cholesterol levels (13,29,35). The reason for the variable results concerning the effect of ω3FA on plasma lipids is not known.

In previous studies, dietary fish oil has not been shown to affect isolated working heart function in nondiabetic control rats after 4 wk of supplementation (36). Our data confirm that ω3FA has no effect on heart function of control rats. In the STZ-D rat, ω3FA treatment was associated with an effect on isolated working heart function when compared to untreated diabetic rats. This improvement in isolated working heart performance of the Promega-treated diabetic rats was of particular interest. Our laboratory has previously reported that pharmacological treatments can improve cardiac performance of STZ-D rats (37,38). These studies suggest that improvements in diabetic cardiac performance are associated with restoration of distinct metabolic abnormalities induced by the diabetic state. The results of this study indicate that cardiac performance of diabetic rats can be improved despite the diabetic state. Because cardiac performance of the ω3FA-treated diabetic rats was not parallel to that of the

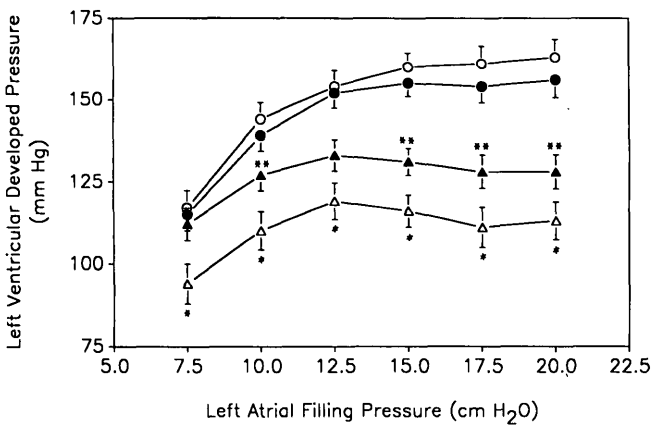

FIG. 2. Peak left ventricular developed pressure (mmHg) vs. left atrial filling pressure (cm H_2O) of control (○), omega-3 fatty acid (ω3FA)–treated control (●), diabetic (△), and ω3FA-treated diabetic (▲) male Wistar rats. *n* = 11–12 animals per group. *Significantly different from untreated control at $P < .05$. **Significantly different from untreated diabetic at $P < .05$.

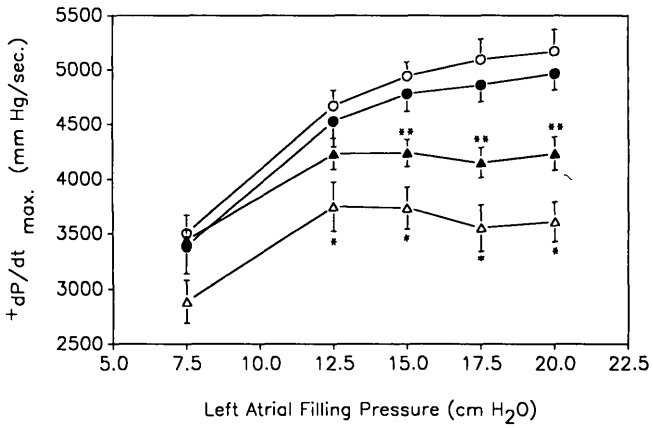

FIG. 3. Maximum rate of change of left ventricular pressure during systole ($+dP/dt_{max}$) vs. left atrial filling pressure of control (○), omega-3 fatty acid (ω3FA)–treated control (●), diabetic (△), and ω3FA-treated diabetic (▲) male Wistar rats. *n* = 11–12 animals per group. *Significantly different from untreated control at $P < .05$. **Significantly different from untreated diabetic at $P < .05$.

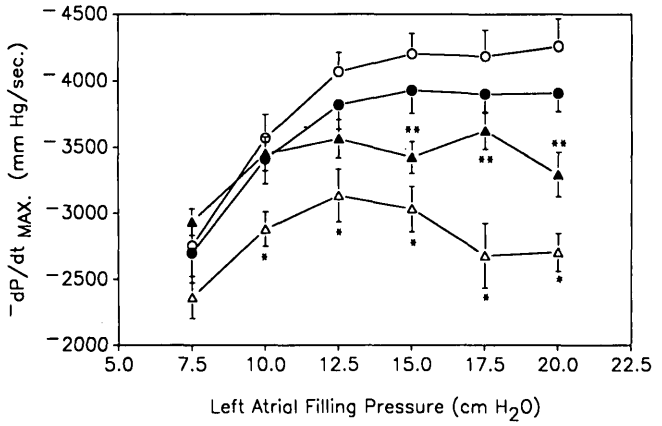

FIG. 4. Maximum rate of change of left ventricular pressure during diastole ($-dP/dt_{max}$) vs. left atrial filling pressure of control (○), omega-3 fatty acid (ω3FA)–treated control (●), diabetic (△), and ω3FA-treated diabetic (▲) male Wistar rats. n = 11–12 animals per group. *Significantly different from untreated control at $P < .05$. **Significantly different from untreated diabetic at $P < .05$.

nondiabetic control animals, this may indicate that ω3FA treatment affects only a discrete aspect of the cardiomyopathy. The limited effect of Promega on the development of diabetic cardiomyopathy indicates that more profound myocardial alterations exist that the treatment is incapable of preventing.

The direct effects of ω3FAs on the myocardium have been investigated in other disease states. Salutary effects of dietary fish oil have been associated with reductions in both ischemic damage (26) and the incidence of spontaneous arrhythmias (28) in rodent models of myocardial ischemia. In addition, in normal control rats, cardiac contractile responsiveness to the α-receptor agonist phenylephrine was reduced by ~50% after an ω3FA-enriched diet (36). The mechanism for these effects is not known. Determining the mechanisms by which ω3FAs affect the heart is complicated by results of studies with nonrodent models of myocardial ischemia, which have failed to demonstrate beneficial effects of ω3FA (29). This study has demonstrated that in the STZ-D rat, treatment with ω3FA can ameliorate the cardiac complications associated with this model of type I diabetes. The etiology of the diabetic cardiomyopathy is not known, and similarly, the mechanism whereby the ω3FA treatment confers a degree of protection against the development of cardiomyopathy is not known. The observation that isolated cardiac SR calcium transport activity was improved concomitant with improved diastolic function of ω3FA-treated diabetic rats suggests that the calcium transport activity of this organelle is involved in the cardiomyopathy, as has been previously shown (5,6). The diabetic cardiomyopathy is of complex etiology, and the improved SR calcium transport activity represents one possible mechanism involved in the improved cardiac function of the ω3FA-treated rats. Previously, findings from our laboratory have shown that under certain circumstances cardiac function is not improved despite improved SR calcium transport function (39). This may indicate that the Promega treatment has additional effects, possibly on sarcolemmal membrane function (i.e., Ca^{2+} transport and/or Na^+-K^+-ATPase activities) which, in addition to the alterations in SR calcium transport activity, contribute to the improved cardiac function. In addition to possible effects on membrane transport processes, it is possible that the effect of the Promega treatment on cardiac function may be substrate dependent because glucose was used in the working heart perfusion buffer and not FAs, which are the primary cardiac metabolic substrates in vivo. These potential mechanisms are the subject of current investigations.

Based on reported effects of ω3FA supplementation on alterations of cardiac phospholipid fatty acyl composition, it is conceivable that the Promega treatment is affecting diabetic heart function via a membrane-associated event. It is known that the activities of the Δ^5 (40), Δ^6, and Δ^9 (41) desaturase enzymes are impaired in the STZ-D rat. Cardiac desaturase enzyme activity is also affected as the arachidonic acid and product-precursor ratio of C22:6,n-3/C22:5,n-3 are decreased in diabetes, the latter alteration possibly indicative of impaired Δ^4 desaturase activity (42). The effect of these enzymatic changes induced by the diabetic state may result in a reduction in the fluidity of cardiac membranes. The nature of the lipid bilayer is considered to be an important factor in determining cardiac function (43) and the activity of membrane-associated enzymes (44), including the SR Ca^{2+}-pump ATPase (22,23,24). Therefore, because of a reduced potential to unsaturate FAs in the diabetic myocardium, and because the activity of the SR is dependent on the appropriate fluid state, it is conceivable that the Promega treatment is affecting SR function by altering the phospholipid fatty acyl structure. The sarcolemma may also be similarly affected, contributing to the improved cardiac performance of the treated diabetic rats. Studies are in progress to test this hypothesis by determining the phospholipid acyl-chain structure of cardiac, SR, and sarcolemma phospholipids of the diabetic and treated diabetic animals.

TABLE 2
Cardiac sarcoplasmic reticulum (SR) calcium transport activity (nmol · mg^{-1} SR protein · min^{-1})

Group	n	Free calcium concentration (μM)		
		0.1	5.3	5.3 (-oxalate)
Control	6	16.13 ± 0.76	59.22 ± 2.80	1.55 ± 0.14
Treated control	6	15.33 ± 0.76	60.82 ± 2.80	1.72 ± 0.14
Diabetic	6	11.65 ± 0.76*	50.26 ± 3.06*	1.55 ± 0.14
Treated diabetic	6	14.63 ± 0.76†	66.55 ± 2.80†	1.73 ± 0.14

Values are means ± SE.
*$P < .05$ vs. control.
†$P < .05$ vs. diabetic.

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