Alterations of heart function and Na\(^{+}\)-K\(^{+}\)-ATPase activity by etomoxir in diabetic rats

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Also, the signals responsible for impaired subcellular overall depression of heart function remains ill defined. Various disorders in subcellular organelles also occur during diabetes, but their contribution to the mechanism for this depression is poorly understood. The sarcosomal Na\(^{+}\)-K\(^{+}\)-ATPase is a key component in regulation of the ion homeostasis and resting potential in cardiac myocytes. It consists of \(\alpha\)- and \(\beta\)-subunits. The catalytic \(\alpha\)-subunit contains the binding site for ATP, Na\(^{+}\), K\(^{+}\), and ouabain. The \(\beta\)-subunit is a glycoprotein that is necessary for the functional activity of the Na\(^{+}\)-K\(^{+}\)-ATPase.

Diabetes is associated with elevated plasma levels of free fatty acids (FFAs) and a marked increase in their oxidation (5, 20, 23). Thus excessive utilization of FFAs by the diabetic myocardium could represent a signal for the defects in subcellular organelles. To support this suggestion, several lipid-lowering agents are known to exert beneficial effects on the diabetic heart (5, 20, 23). We previously reported that the carnitine palmitoyltransferase I (CPT I) inhibitor etomoxir (10) partially normalized the myosin isozyme population in diabetic rat hearts (23). Also, bypassing the CPT I block via administration of dietary medium-chain fatty acids did not blunt the normalization of myosin isozymes (23). Thus the major effect of etomoxir may be due to its lipid-lowering action that results from inhibition of de novo fatty acid synthesis (2, 27). Similarly, changes in the sarcoplasmic reticulum Ca\(^{2+}\)-pump ATPase activity were also prevented after treatment of diabetic rats with etomoxir (23).

The present study examined whether etomoxir treatment can reverse diabetes-induced alterations in sarcoplasmic function (Na\(^{+}\)-K\(^{+}\)-ATPase activity), plasma lipids, and heart function in rats with streptozotocin (Stz)-induced diabetes. Several recent studies reported changes in Na\(^{+}\)-K\(^{+}\)-ATPase subunit expression in pathological conditions such as pressure-overload hypertrophy (4) and diabetes (26). Ng et al. (16) found that the \(\alpha_1\)-subunit of the Na\(^{+}\)-K\(^{+}\)-ATPase was unaltered in diabetic rat hearts, but both the \(\alpha_2\) and \(\beta_1\)-subunits were decreased. Thus diabetes may cause a differential regulation of Na\(^{+}\)-K\(^{+}\)-ATPase subunits similar to that for myosin isozymes. Accordingly, this study utilizes \([\text{H}]\) ouabain binding and western blot analysis to examine the diabetes-induced alterations in Na\(^{+}\)-K\(^{+}\)-ATPase subunits and the ability of etomoxir to reverse these changes.

METHODS

Experimental model and hemodynamic measurements. Diabetes was produced in male Sprague-Dawley rats (175–200 g) via injection of Stz (65 mg/kg body wt) (18, 21, 23) into the tail...
vein, and sham-treated rats served as controls. Two weeks after injection, control rats and diabetic rats received either tap water or water containing (+j etomoxir (sodium salt; RBI, Natick, MA) for 4 wk. The etomoxir intake was adjusted to 8 mg/kg body wt by monitoring the water intake.

Cardiac performance was measured by insertion of a microtip pressure transducer (model SPR-249, Millar Instruments, Houston, TX) into the left ventricle from the arteria carotis dextra after anesthesia with ketamine (90 mg/kg body wt) and xylazine (10 mg/kg body wt). After stabilization for 30 min, heart rate, left ventricular systolic pressure, left ventricular end-diastolic pressure, and the rates of pressure development and its decline (dP/dt and –dP/dt, respectively) were measured to assess heart function. After the hemodynamic measurements were made, rats were decapitated. Hearts were removed and frozen in liquid nitrogen. Trunk blood (first 5–7 ml) was collected, and the serum was used for measuring lipids and hormones. Experimental procedures conformed with institutional animal care guidelines.

Measurements of Na+–K+–ATPase and [H]ouabain binding. Cardiac sarcolemmal membrane was isolated as previously described (19). Three hearts were pooled for each experiment. Marker enzyme activities (18) revealed that the membrane preparations from control, diabetic, and etomoxir-treated hearts contained minimal (3–5%) cross contamination with other subcellular organelles. The activity of Na+–K+–ATPase was assayed as hydrolysis of ATP as described previously (18). The K+-dependent n-nitrophenolphosphatase (KpNPPase) activity was measured as hydrolysis of n-nitrophenylphosphate (18).

Ouabain binding was performed as described by Dixon et al. (8). Sarcolemmal vesicles were resuspended in 10 mM Tris–HCl (pH 7.5) to a concentration of 1.0 mg/ml and were transferred to the reaction mixture (0.1 mg/ml, final protein concentration) containing 1.5 mM MgCl2, 1.0 mM phosphate, 10 mM Tris–HCl (pH 7.5), and 10–5,000 nM [H]ouabain (18.0 Ci/mmol; NEN Life Sciences Products, Boston, MA) in the absence or presence of 2.0 mM ouabain. This ouabain concentration inhibited >95% of the Na+–K+–ATPase activity. The final volume of the reaction mixture was 0.5 and 1.0 ml for measurement of the low-affinity and high-affinity site, respectively.

Sodium dodecyl sulfate (9 µg/ml) was added to the incubation medium to make the sarcolemmal vesicles permeable to ouabain. The reaction was terminated by filtration (0.45-µm pore; Millipore, Bedford, MA) after 1 h at 37°C. Filters were washed three times with 2.5 ml ice-cold buffered solution containing 50 mM Tris–HCl (pH 5.0), 0.1 mM ouabain, and 15 mM KCl. Radioactivity on the filters was checked by a manual method.

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RESULTS

Injection of Stz resulted in a general decrease in growth characteristics of diabetic rats after 6 wk (Table 1). Body weight decreased by 25%, and heart weight decreased by 16%, thereby increasing the heart weight-to-body weight ratio by 12%. Diabetic rats also exhibited increased serum concentrations of glucose, triglycerides, and FFAs, whereas insulin and T3 concentrations were decreased (Table 1). Treatment of diabetic and control rats with etomoxir did not influence body weight but increased heart weight by 16 and 27%, respectively. In diabetic rats, etomoxir significantly decreased serum triglycerides and FFA concentrations, but it did not alter the levels of insulin, glucose, or T3 (Table 1). Treatment of diabetic rats with etomoxir resulted in a decrease in growth characteristics of diabetic rats after 6 wk (Table 1).

Table 1. Characteristics of control and diabetic animals with or without etomoxir treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BW, g</th>
<th>HW, g</th>
<th>HW/BW, mg/g</th>
<th>SL proteins, mg/ml</th>
<th>Glucose, mg/dl</th>
<th>Triglycerides, mM</th>
<th>T3, nM</th>
<th>Insulin, pm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>513 ± 9</td>
<td>126 ± 0.03</td>
<td>82 ± 0.04</td>
<td>434 ± 8</td>
<td>0.98 ± 0.09</td>
<td>1.27 ± 0.54</td>
<td>13 ± 0.2</td>
<td>52 ± 6</td>
</tr>
<tr>
<td>Diabetic</td>
<td>384 ± 12*</td>
<td>1.06 ± 0.03</td>
<td>802 ± 62</td>
<td>802 ± 62</td>
<td>0.87 ± 0.05</td>
<td>3.56 ± 0.90</td>
<td>0.3 ± 0.1</td>
<td>31 ± 8</td>
</tr>
<tr>
<td>Diabetic + Etomoxir</td>
<td>374 ± 10*</td>
<td>1.35 ± 0.06†</td>
<td>733 ± 46†</td>
<td>424 ± 68†</td>
<td>1.68 ± 0.24†</td>
<td>1.68 ± 0.43†</td>
<td>0.24 ± 0.06†</td>
<td>25 ± 4†</td>
</tr>
<tr>
<td>Control + Etomoxir</td>
<td>508 ± 13†</td>
<td>1.46 ± 0.04†</td>
<td>733 ± 46†</td>
<td>424 ± 68†</td>
<td>2.22 ± 0.28*p</td>
<td>0.67 ± 0.11†</td>
<td>1.2 ± 0.2†</td>
<td>45 ± 6†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 12 animals in each group. Values for glucose, free fatty acids, T3, and insulin are serum levels. Diabetes was induced by injection of streptozotocin (65 mg/kg iv), and animals were used 6 wk later. Eтомoxir (80 mg/kg, daily) was started in the drinking water 2 wk after induction of diabetes, and animals were used 4 wk later. BW, body wt; HW, heart wt; SL, sarcolemma; T3, 3,3',5-triiodo-L-thyronine. *P < 0.05 compared with control group. †P < 0.05 compared with diabetic group.
weight by 193% in diabetic rats and 227% in control rats (Table 1).

In untreated diabetic rats, heart rate, left ventricular systolic pressure, \( +\text{dP/dt} \), and \(-\text{dP/dt}\) were decreased, whereas left ventricular end-diastolic pressure was increased (Table 2). Treatment with etomoxir partially prevented the depression in left ventricular systolic pressure, \( +\text{dP/dt} \), and \(-\text{dP/dt}\). Moreover, elevation of the left ventricular end-diastolic pressure was completely prevented by etomoxir. The etomoxir did not, however, reverse the decreased heart rate in diabetic rats. Etomoxir treatment had no significant effects on hemodynamic parameters in control rats (Table 2).

Diabetes produced a significant depression of sarcolemmal Mg\(^{2+}\)-ATPase activity (Fig. 1). Etomoxir treatment exerted no significant effect on Mg\(^{2+}\)-ATPase of diabetic rats. Diabetic rats also exhibited a significant depression of Na\(^{+}\)-K\(^{+}\)-ATPase activity (Fig. 2). Etomoxir treatment of diabetic rats prevented the decrease in Na\(^{+}\)-K\(^{+}\)-ATPase activity when expressed per gram heart weight but not when expressed per milligram sarcolemmal protein. Similarly, diabetic rats exhibited decreased K\(p\)NPPase activity (Fig. 3). Etomoxir reversed the depression of K\(p\)NPPase activity in diabetic rats when expressed per gram heart weight but not when expressed per milligram sarcolemmal protein. In control rats, etomoxir treatment decreased Mg\(^{2+}\)-ATPase and Na\(^{+}\)-K\(^{+}\)-ATPase activity when expressed per milligram sarcolemmal protein but not when expressed per gram heart weight. In contrast, etomoxir increased K\(p\)NPPase activity in the control rats when expressed per gram of heart weight.

Scatchard plot analysis of specific \[^3H\]ouabain binding to sarcolemmal membranes is shown in Fig. 4. The B\(_{\text{max}}\) of the high-affinity binding sites but not of low-affinity sites was decreased in untreated diabetic rats. Etomoxir treatment normalized B\(_{\text{max}}\) expressed per gram heart weight in diabetic rats but had no effect in control rats (Table 3). However, B\(_{\text{max}}\) was not normalized by etomoxir when expressed per milligram sarcolemmal protein (Table 3). Etomoxir treatment increased B\(_{\text{max}}\) of the low-affinity binding sites in both diabetic and control rats, even though B\(_{\text{max}}\) (expressed per g heart wt) was not significantly depressed in the diabetic rats. To examine any acute effects of etomoxir,

**Table 2. Hemodynamic responses of control and diabetic animals with or without etomoxir treatment**

<table>
<thead>
<tr>
<th>Heart rate, beats/min</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic+ Etomoxir</th>
<th>Control + Etomoxir</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>246 ± 3</td>
<td>207 ± 6*</td>
<td>203 ± 9*</td>
<td>228 ± 4†</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>125 ± 4</td>
<td>99 ± 3*</td>
<td>114 ± 3†</td>
<td>124 ± 3†</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>4.8 ± 0.3</td>
<td>9.8 ± 0.9*</td>
<td>5.1 ± 0.9†</td>
<td>5.1 ± 1.0†</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>1,988 ± 57</td>
<td>1,489 ± 36*</td>
<td>1,761 ± 43†</td>
<td>2,006 ± 74†</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>1,999 ± 51</td>
<td>1,179 ± 38*</td>
<td>1,489 ± 94†</td>
<td>1,875 ± 121†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6 animals in each group. Experimental interventions are the same as in Table 1. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt and −dP/dt, rates of pressure development and decline, respectively. *P < 0.05 compared with control group. †P < 0.05 compared with diabetic group.

DISCUSSION

The present study demonstrates that diabetic rat hearts exhibit a marked depression of left ventricular function involving both systolic pressure development and relaxation. The impaired heart function is associated with a reduction in Na\(^{+}\)-K\(^{+}\)-ATPase activity, a decrease in \(\alpha_2\) and \(\alpha_3\)-subunit content of the enzyme, and a depression of B\(_{\text{max}}\) for the high-affinity ouabain-
binding sites but not for the low-affinity sites. Because the high-affinity site is affected by the α2- and α3-subunits and the low-affinity site by the α1-subunit (16), our data are consistent with those of Ng et al. (16) indicating that Stz-induced diabetes results in a decrease in the α2-subunit but not in the α1-subunit. To examine possible metabolic links between the diabetic state and functional disorders, we treated diabetic rats with the CPT I inhibitor etomoxir. Inhibition of CPT I reduces FFA utilization and increases glucose oxidation in a compensatory manner (21). However, plasma FFAs are not elevated but are reduced on a long-term basis by CPT I inhibitors, which inhibit acetyl-CoA carboxylase (2, 27), the rate-limiting enzyme of de novo fatty acid synthesis. This mixed profile of decreasing both FFA utilization and FFA supply should exert beneficial effects on the deranged metabolic state of insulin-dependent diabetes mellitus.

We previously reported that etomoxir partially improves the population of myosin isozymes and prevents changes in the sarcoplasmic reticulum Ca2+-pump ATPase activity in Stz-induced diabetes and that a close correlation exists between myosin V3 and plasma concentrations of FFAs and triglycerides (23). The present data show that cardiac function is also partially normalized by etomoxir; this agrees with the findings of Schmitz et al. (25). It is noteworthy that the depressed function of the diabetic rat heart was improved, although hypoinsulinemia and the associated hyperglycemia were unaffected. Furthermore, etomoxir did not affect the reduced thyroid influence arising from decreased T3 levels, which depress heart function. Although CPT I inhibition should enhance glucose utilization, a previous study involving CPT I bypass with dietary medium-chain fatty acids suggests that FFA plays a major role in the metabolic disturbances of Stz-induced diabetes (23). A reduction in the elevated circulating FFA levels should blunt the inhibitory effect of FFA on glucose oxidation, which would already be depressed due to hypoinsulinemia (28).

One might also argue that the etomoxir-induced alterations in acylderivatives may mediate improved cardiac function. Etomoxir treatment is expected to reduce long-chain acylderivatives, which can cause heart dysfunction and decrease Na+-K+-ATPase activity (1, 6). However, Lopaschuk et al. (13) claimed that the protective effect of etomoxir in reperfused ischemic myocardium arises from enhanced glucose utilization and not from changes in long-chain acylderivatives levels. Other evidence also suggests that the improvement in heart function is attributable at least in part to the lipid-lowering action of etomoxir. The antihypertensive compound hydralazine also improves the heart function in diabetic rats by reducing the serum lipid concentrations (20). Our study supports the therapeutic concept of normalization of serum FFAs whenever...
Effects of etomoxir on rat heart sarcolemmal Mg$^{2+}$-ATPase and Na$^{+}$-K$^{+}$-ATPase activities in vitro

<table>
<thead>
<tr>
<th>Etomoxir, µM</th>
<th>Mg$^{2+}$-ATPase Activity, µmol Pi · mg SL protein⁻¹·h⁻¹</th>
<th>Na$^{+}$-K$^{+}$-ATPase Activity, µmol Pi · mg SL protein⁻¹·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>183.9±0.1</td>
<td>37.4±1.4</td>
</tr>
<tr>
<td>1</td>
<td>170.8±2.1†</td>
<td>40.3±1.8</td>
</tr>
<tr>
<td>3</td>
<td>170.1±2.3†</td>
<td>42.8±1.7</td>
</tr>
<tr>
<td>5</td>
<td>158.9±0.2†</td>
<td>44.8±2.8</td>
</tr>
<tr>
<td>10</td>
<td>169.2±1.0†</td>
<td>42.4±1.6</td>
</tr>
<tr>
<td>30</td>
<td>156.8±2.5†</td>
<td>38.1±2.3</td>
</tr>
<tr>
<td>100</td>
<td>159.4±0.2†</td>
<td>45.6±2.5</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 experiments. *P < 0.05 compared with 0 µM etomoxir.

Table 4. Effects of etomoxir on rat heart sarcolemmal Mg$^{2+}$-ATPase and Na$^{+}$-K$^{+}$-ATPase activities in vitro

Fig. 5. Representative Western blots depicting expression of α$_1$-, α$_2$-, and α$_3$-subunits of rat Na$^{+}$-K$^{+}$-ATPase subunits from control (C), diabetic (D), diabetic plus etomoxir (D + E) or control plus etomoxir (C + E) rat hearts. All lanes were loaded with 20 µg of sarcolemmal protein.
hearts. Etomoxir treatment of diabetic rat hearts produced a further reduction in the \(\alpha_2\) and \(\alpha_3\)-subunits as well as a decrease in \(\beta_2\)-subunit. However, etomoxir treatment increased sarcolemmal protein yield by 193% in diabetic rat hearts, which would counter the effects of the decreased subunit content on enzyme activity and high-affinity ouabain binding sites when expressed per gram heart weight. Etomoxir treatment also increased sarcolemmal protein in control hearts (227%) but did not reduce \(\alpha_2\)-subunit content. It did, however, produce a marked reduction in \(\beta_2\)-subunit content. Changes in \(\beta_2\)-subunit content would indirectly influence Na\(^{+}\)-K\(^{+}\)-ATPase activity because the \(\beta_2\)-subunit is required for functional integrity of the enzyme (26). Thus we believe that the changes in enzyme activity and ouabain binding in etomoxir-treated hearts may reflect overall changes in the subunit composition of the enzyme rather than relative changes in a specific subunit.

In the present study, etomoxir treatment markedly reversed the depression of Na\(^{+}\)-K\(^{+}\)-ATPase and KpNPPase activity when expressed per gram heart weight. Moreover, etomoxir normalized the depression of \(B_{\text{max}}\) for the high-affinity ouabain binding sites (expressed per g heart wt). Although \(B_{\text{max}}\) for the low-affinity sites (expressed per gram heart weight) was not significantly depressed in diabetic rats, etomoxir treatment increased \(B_{\text{max}}\) in diabetic as well as control rats. Therefore, etomoxir may exert differential effects on high-affinity vs. low-affinity binding sites in control rats. Sahin-Erdemli et al. (24) also postulated a differential regulation of high- and low-affinity binding sites. These authors showed that deoxycorticosterone acetate increased Na\(^{+}\)-K\(^{+}\)-ATPase activity by an increase in protein content of the \(\alpha_2\)-subunit, whereas the \(\alpha_2\) and \(\alpha_3\)-subunits were not affected. Our approach to express Na\(^{+}\)-K\(^{+}\)-ATPase data per gram heart weight is based on the findings of Gick et al. (11), who suggested that “expression of enzyme activity per unit protein is rendered difficult if protein content per unit weight is not constant among the tissues examined.” In our study, protein content of the tissue examined (heart) was increased in etomoxir-treated vs. untreated rats. Thus expression of enzyme activity per gram tissue weight may represent a more valid basis for intratissue comparisons under treatment conditions that alter tissue protein content.

In summary, our results indicate that the depressed cardiac function in Stz-induced diabetes in rats is associated with a decrease in Na\(^{+}\)-K\(^{+}\)-ATPase activity and high-affinity binding sites. Etomoxir partially improves the depressed heart function and increases the activity of Na\(^{+}\)-K\(^{+}\)-ATPase (expressed per g heart wt) as well as \(B_{\text{max}}\) for the high-affinity sites for ouabain. We conclude that improved glucose utilization or reduced FFA utilization associated with etomoxir treatment may play a role in maintenance of the long-term activity of Na\(^{+}\)-K\(^{+}\)-ATPase.

This work was supported by a grant from the Medical Research Council (MRC) of Canada (MRC Group in Experimental Cardiology). K. Kato was supported by a Fellowship from the Heart and Stroke Foundation of Canada. H. Rupp was a Visiting Professor from the University of Marburg and was supported by the Science and Technology Cooperation Germany/Canada (BMBF/HM4). A. Lukas was the recipient of the Myles Robinson Memorial Heart Scholarship.

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Received 19 June 1998; accepted in final form 17 November 1998.

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