Etomoxir, a Carnitine Palmitoyltransferase I Inhibitor, Protects Hearts From Fatty Acid-Induced Ischemic Injury Independent of Changes in Long Chain Acylcarnitine

Gary D. Lopaschuk, Stephen R. Wall, Peter M. Olley, and Norman J. Davies

Fatty acids are known to increase the severity of injury during acute myocardial ischemia. In this study, we determined the effects of a carnitine palmitoyltransferase I inhibitor, ethyl 2-[6-(4-chlorophenoxy)hexyl]oxiran-2-carboxylate (Etomoxir) on reperfusion recovery of fatty acid perfused hearts. Following a 25-minute period of global ischemia, isolated working hearts perfused with 1.2 mM palmitate, 11 mM glucose exhibited depressed function compared to hearts perfused with 11 mM glucose alone. A low dose of Etomoxir (10^-9 M) decreased long chain acylcarnitine and long chain acyl-coenzyme A (CoA) levels but did not prevent depressed function. In contrast, a high dose of Etomoxir (10^-6 M) prevented the palmitate-induced depression of function but did not decrease myocardial long chain acylcarnitine or long chain acyl-CoA levels. At this high dose of Etomoxir, oxygen consumption per unit work was decreased during reperfusion recovery, and ATP and creatine-phosphate levels were significantly higher after reperfusion. In aerobic hearts not subjected to ischemia, Etomoxir (10^-6 M) increased glucose oxidation both in the presence and absence of palmitate, while 10^-9 M Etomoxir had no effect. In these aerobic hearts, only the low dose of Etomoxir decreased long chain acylcarnitine and long chain acyl-CoA levels. These data demonstrate that Etomoxir (10^-6 M) increases functional recovery of fatty acid perfused ischemic hearts. This protection is unrelated to changes in levels of long chain acylcarnitines but may be due to increased glucose use by the reperfused heart, resulting in decreased oxygen consumption per unit work. (Circulation Research 1988;63:1036-1043)

In the early hours following acute myocardial infarction, plasma free fatty acid content is elevated— and is thought to contribute to both myocardial infarct size and mortality. Fatty acid potentiation of ischemic injury occurs in several experimental models, including the in situ pig and dog heart, as well as the isolated perfused rat heart. The detrimental effects of fatty acids have been attributed to an increased requirement of oxygen for catabolism or to the accumulation of potentially toxic intracellular intermediates of fatty acid metabolism. Two of these intermediates that accumulate in the ischemic myocardium are long chain acyl-coenzyme A (CoA) and long chain acylcarnitine. A host of studies in membrane vesicle and solubilized enzymes have demonstrated that these amphiphiles can alter the function of a number of critical membrane proteins. In intact tissue, a correlation between acylcarnitine levels and the degree of ischemic injury has been suggested, although this finding has not been consistent. We recently demonstrated in isolated working rat hearts that fatty acids adversely effect functional recovery after ischemia (G.D. Lopaschuk and M. Spafford, submitted manuscript). Decreased recovery of fatty acid perfused hearts was accompanied by a large increase in myocardial long chain acylcarnitine levels, although it could not be determined if this increase caused the depression of functional recovery.

Recently, studies have appeared which demonstrate that agents which block fatty acid oxidation can protect the ischemic myocardium. Carnitine palmitoyltransferase I (CPT 1) inhibitors, such as POCA (sodium 2-[5-(4-chlorophenyl)-pentyl]oxiran-2-carboxylate), TDGA (2-tetraglycidic acid), and...
Etomoxir (ethyl 2-[(6-(4-chlorophenoxy)hexyl)oxirane-2-carboxylate]) is a new CPT 1 inhibitor that is perhaps due to a decrease in myocardial long chain acylcarnitine levels. However, a switch from predominantly fatty acid oxidation to carbohydrate oxidation, with a resultant decrease in myocardial oxygen consumption, could also explain the salutary effects of this drug.

Ischemic Heart Perfusions

After 15 minutes of work, global ischemia was induced by clamping off left atrial and aortic flow. After 25 minutes of no-flow ischemia, left atrial and aortic flow was restored, and recovery of mechanical function was monitored for a further 30-minute period. The ischemic interval was chosen because it allowed complete recovery of function in glucose perfused hearts. Hearts were then frozen using Wollenberger clamps cooled to the temperature of liquid nitrogen. A number of hearts were also frozen following the 25-minute ischemic period. Etomoxir was administered either 5 minutes before ischemia or immediately before reperfusion of ischemic hearts.

Palmitate and Glucose Oxidation in Aerobic Hearts

Steady state oxidative rates of palmitate and glucose were performed as described previously. After a 10-minute washout perfusion, hearts were perfused in the working mode with recirculated Krebs-Henseleit buffer containing either 11 mM [1-14C]glucose, 1.2 mM palmitate, 11 mM [1-14C]glucose, or 1.2 mM [1-14C]palmitate with 11 mM glucose. All perfusions were performed in the presence of 3% albumin. Rates of [1-14C]palmitate or [1-14C]glucose oxidation were determined by measurement of steady state 14CO2 production. Perfusion was continued for 60 minutes under conditions of high cardiac work (15 cm H2O preload, 80 mm Hg afterload), and rates of palmitate oxidation were determined between 10 and 60 minutes, when rates of 14CO2 production had reached a steady state. At the end of the perfusion, hearts were freeze-clamped with Wollenberger clamps cooled to the temperature of liquid nitrogen.

Tissue Workup

Frozen ventricular tissue was weighed and powdered in a mortar and pestle cooled to the temperature of liquid nitrogen. A portion of the powdered tissue was used to determine the dry-to-wet weight ratio. With this ratio, as well as with the total frozen ventricular weight and the weight of the dried atrial tissue, total dry weight of the heart was determined. Extraction of ATP, creatine phosphate, long chain acyl-CoA, and long chain acylcarnitine was as described previously. Measurement of ATP and creatine phosphate levels from perchloric acid extracts was determined with standard enzymatic assays. Extracted long chain acyl-CoA was hydrolyzed and free CoA was measured fluorometrically. Extracted long chain acylcarnitine was also hydrolyzed and free carnitine was measured radiometrically.

Statistical Analysis

Statistical analysis was performed using analysis of variance, followed by comparison of group means using group t tests. Statistical significance was set at p<0.05.
Results

Effects of Etomoxir on Fatty Acid Perfused Ischemic Hearts

Heart function, as measured by the heart rate/peak systolic pressure product, was similar or slightly depressed during baseline aerobic perfusion if palmitate was present in the perfusion medium (Figures 1 and 2, Table 1). After 25 minutes of global no-flow ischemia, as we have previously demonstrated (G.D. Lopaschuk and M. Spafford, submitted manuscript), the presence of palmitate resulted in a significantly decreased postischemic recovery of heart rate and peak systolic pressure development (Table 1). A low concentration of Etomoxir (10⁻⁹ M) did not improve recovery in the palmitate-perfused hearts (Figure 1, Table 1). In fact, a significant decrease in heart rate was noted in Etomoxir-treated hearts, although the heart rate/peak systolic pressure product was not significantly different between the two groups. However, perfusion of hearts with this concentration of Etomoxir did decrease long chain acylcarnitine and long chain acyl-CoA levels in palmitate-perfused hearts (Table 2). The decreases, which would be expected to occur if CPT 1 were inhibited, approached the levels seen in glucose perfused hearts. This suggests that this low dose of Etomoxir was sufficient to inhibit CPT 1 activity, yet no beneficial effect on reperfusion recovery of ischemic hearts was seen.

The higher concentration of Etomoxir (10⁻⁶ M) significantly increased recovery of both heart rate and peak systolic pressure development in palmitate-perfused hearts, although the heart rate/peak systolic pressure product was not significantly different between the two groups. However, perfusion of hearts with this concentration of Etomoxir did decrease long chain acylcarnitine and long chain acyl-CoA levels in palmitate-perfused hearts (Table 2). The decreases, which would be expected to occur if CPT 1 were inhibited, approached the levels seen in glucose perfused hearts. This suggests that this low dose of Etomoxir was sufficient to inhibit CPT 1 activity, yet no beneficial effect on reperfusion recovery of ischemic hearts was seen.

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Table 2. Effect of Etomoxir on Tissue Levels of Long Chain Acyl-CoA, Long Chain Acylcarnitine, ATP, and Creatine Phosphate Levels Following Reperfusion of Ischemic Hearts

<table>
<thead>
<tr>
<th>Perfusion condition</th>
<th>Long chain acyl-CoA (nmol/g dry wt)</th>
<th>Long chain acylcarnitine (nmol/g dry wt)</th>
<th>ATP (µmol/g dry wt)</th>
<th>Creatine phosphate (µmol/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 mM glucose</td>
<td>82±6</td>
<td>206±47</td>
<td>17.3±0.7</td>
<td>24.2±2.1</td>
</tr>
<tr>
<td>1.2 mM palmitate,</td>
<td>105±12</td>
<td>1,228±298*</td>
<td>13.2±0.8*</td>
<td>14.5±2.3*</td>
</tr>
<tr>
<td>+10⁻⁶ M Etomoxir</td>
<td>59±9*†</td>
<td>345±28*†</td>
<td>11.2±1.7*</td>
<td>9.3±0.6*†</td>
</tr>
<tr>
<td>+10⁻⁶ M Etomoxir</td>
<td>198±17*†</td>
<td>1,648±432*†</td>
<td>23.1±3.5*†</td>
<td>17.9±1.9*†</td>
</tr>
</tbody>
</table>

Tissue levels of long chain acyl-CoA, long chain acylcarnitine, ATP, and creatine phosphate were measured at the end of the 30-minute reperfusion period of ischemic hearts. Values are the mean±SEM of five to eight hearts in each group.

*Significantly different from glucose control.
†Significantly different from palmitate control.

Etomoxir Effects on Myocardial Oxygen Consumption

As shown in Figures 3B and 4B, use of palmitate as a metabolic substrate results in a small increase in oxygen consumption corrected for work. Oxygen consumption, however, was significantly different at only one time point in each perfusion group. Measurements of absolute levels of oxygen consumed (as based on micromoles per gram dry weight per minute) were lower in palmitate-perfused hearts compared with control (a reflection of the decreased work performed by these hearts). Addition of 10⁻⁹ M Etomoxir to the perfusate resulted in a decrease in total oxygen consumption although no changes in oxygen consumption were noted when corrected for workload differences. Addition of 10⁻⁶ M Etomoxir resulted in a small, but significant, increase in total oxygen consumption in palmitate-perfused hearts. When adjusted for the increased work performed during reperfusion, a significant decrease in oxygen consumption was noted. Thus, at this dose, Etomoxir effectively decreases oxygen consumption/unit work in palmitate perfused hearts to levels similar to those seen in glucose perfused hearts.

Etomoxir Effects on High Energy Phosphates

The effects of Etomoxir on recovery of high energy phosphate levels was determined after 30 minutes of reperfusion recovery of ischemic hearts. Following ischemia, levels of ATP in glucose- and palmitate-perfused hearts were 10.0±0.9 and 12.0±0.9 µmol/g dry wt, respectively (normal levels are 25 µmol/g dry wt). Following 30 minutes of reperfusion with glucose, a significant increase in ATP levels from immediate postischemic values was seen (Table 2). In contrast, no increase was seen...
Etomoxir also had no effect on palmitate oxidation. As shown in Table 3, palmitate results in a marked decrease in steady state glucose oxidation. In steady state aerobic hearts not subjected to ischemia, levels were 3.7±0.6 and 5.1±0.7 μmol/g dry wt in glucose and palmitate perfused hearts, respectively (normal levels are 35 μmol/g dry wt). Reperfusion with glucose increased creatine phosphate levels to a greater extent than palmitate perfused hearts. At the lower dose (10⁻⁹ M) Etomoxir to palmitate-perfused hearts resulted in lower creatine phosphate levels following reperfusion, while 10⁻⁶ M Etomoxir significantly increased creatine phosphate levels compared with palmitate alone.

**Etomoxir Effects on Myocardial Glucose and Palmitate Oxidation**

Ideally, it would be desirable to determine directly the effect of Etomoxir on glucose and palmitate oxidation in postischemic hearts during the reperfusion period. However, measured oxidative rates under non–steady state conditions are difficult to interpret due to the contribution of intracellular glucose and fatty acid to overall oxidative metabolism (i.e., the labeled substrate is not in equilibrium with intracellular unlabeled substrate). Similarly changes in work performed by the heart under non–steady state conditions can markedly alter oxidative metabolism. During ischemia and initial reperfusion, the heart is not in a steady state. Therefore, we looked at the effect of both low and high concentrations of Etomoxir on palmitate and glucose oxidation in steady state aerobic hearts not subjected to ischemia. As shown in Table 3, palmitate results in a marked decrease in steady state glucose oxidation. At the low dose of Etomoxir (10⁻⁹ M) no significant effect on glucose oxidation was seen in either the presence or absence of palmitate. At this dose, Etomoxir also had no effect on palmitate oxidation (Table 4). At a high concentration (10⁻⁶ M) Etomoxir results in a small increase in palmitate oxidation. This dose of Etomoxir results in a much larger increase in glucose oxidation, which occurred both in the presence and absence of palmitate (Table 3). Under these perfusion conditions, neither dose of Etomoxir had a major effect on long chain acylcar- nitine levels in either palmitate- or glucose-perfused hearts. At the lower dose (10⁻⁹ M) a significant decrease in long chain acyl-CoA was seen in both glucose- and palmitate-perfused hearts. This is probably a reflection in lower mitochondrial long chain acyl-CoA levels due to some inhibition of CPT 1.

**Discussion**

Recent studies have demonstrated beneficial effects of CPT 1 inhibitors in ischemic hearts. Paulson et al showed that POCA could protect isolated perfused rat hearts from ischemia. Molaparast-Saless et al demonstrated a protective effect of oxefencine in situ pig hearts. In contrast, Seitelberger et al failed to demonstrate a protect-
et and Etomoxir's effect on myocardial acylcarnitine levels. At a very low dose (10^{-9} M), Etomoxir appears to inhibit CPT 1 in ischemic hearts, as measured by a decrease in long chain acylcarnitine levels. However, at this dose no protective effect is seen during reperfusion with fatty acids. At higher doses (10^{-6} M) in which protection occurs, Etomoxir resulted in a small increase in long chain acylcarnitine levels and a significant increase in long chain acyl-CoA levels. This possibly results from CPT 2 inhibition, which would explain the observed increase in these fatty acid intermediates. If CPT 2 were inhibited, cytosolic long chain acyl-CoA should increase and the decrease in long chain acylcarnitine due to CPT 1 inhibition would be overcome. No studies have yet been published that determine if higher doses of Etomoxir will inhibit CPT 2 in vitro.

Etomoxir is presently being tested as an antidiabetic agent due to its ability to increase glucose utilization.20 The increase in myocardial glucose oxidation we observed in aerobic hearts may explain the beneficial effects of Etomoxir in ischemic hearts. We suggest that decreased fatty acid transport into mitochondria by Etomoxir results in a lowering of TCA cycle intermediates such as citrate and acetyl-CoA. Lowering citrate would result in a decreased inhibition of phosphofructokinase, while lowering acetyl-CoA should result in increased glycolytic flux and glucose oxidation.19,26 We are presently measuring these metabolites following similar perfusion protocols.

In isolated mitochondrial preparations, CPT 1 inhibition decreases fatty acid oxidation.28 However, in our intact hearts, even a high dose of Etomoxir does not significantly decrease palmitate oxidative rates. To our knowledge, no other study has looked at the effect of POCA or Etomoxir on palmitate oxidation in intact hearts. In nonischemic perfused hearts, a decrease in myocardial lipolysis was observed with POCA,29 although these hearts were not perfused with fatty acids. Oxfencine has been shown to inhibit palmitate oxidation28 although as of yet we have not tried this agent under our perfusion conditions. In our perfusions, a high concentration of palmitate was used since circulat-

<table>
<thead>
<tr>
<th>Perfusion condition</th>
<th>^14CO_2 production (µmol palmitate/g dry wt/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 mM palmitate, 11 mM glucose</td>
<td>0.45±0.05</td>
</tr>
<tr>
<td>+10^{-3} M Etomoxir</td>
<td>0.50±0.08</td>
</tr>
<tr>
<td>+10^{-4} M Etomoxir</td>
<td>0.60±0.06*</td>
</tr>
</tbody>
</table>

Palmitate oxidation was determined by measuring ^14CO_2 production in hearts perfused with 11 mM glucose [1-14C]palmitate (1.2 mM). Hearts were perfused for 60 minutes at a 1.5 cm H_2O left atrial filling pressure and 80 mm Hg hydrostatic afterload. Steady state ^14CO_2 production was determined between 20 and 60 minutes. Values are the mean±SEM of six to eight hearts in each group.

*Significantly different from glucose control.

### Table 5. Effect of Etomoxir on Tissue Levels of Long Chain Acyl-CoA and Long Chain Acylcarnitine in Aerobic Hearts

<table>
<thead>
<tr>
<th>Perfusion condition</th>
<th>Long chain acyl-CoA (nmol/g dry wt)</th>
<th>Long chain acylcarnitine (nmol/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 mM glucose</td>
<td>54±1</td>
<td>642±120</td>
</tr>
<tr>
<td>+10^{-3} M Etomoxir</td>
<td>47±2*</td>
<td>738±163</td>
</tr>
<tr>
<td>+10^{-4} M Etomoxir</td>
<td>76±8*</td>
<td>761±110</td>
</tr>
<tr>
<td>1.2 mM palmitate, 11 mM glucose</td>
<td>139±13*</td>
<td>1,671±266*</td>
</tr>
<tr>
<td>+10^{-3} M Etomoxir</td>
<td>76±9*†</td>
<td>1,221±157*</td>
</tr>
<tr>
<td>+10^{-4} M Etomoxir</td>
<td>98±11*†</td>
<td>1,580±264*</td>
</tr>
</tbody>
</table>

Tissue levels of long chain acyl-CoA and long chain acylcarnitine were measured at the end of the 60-minute perfusion period of aerobic hearts. Values are the mean±SEM of five to seven hearts in each group.

*Significantly different from glucose controls.
†Significantly different from palmitate controls.
ing fatty acid increases during ischemia.\textsuperscript{1,26} It would be expected that this high concentration of fatty acid could compete with Etomoxir at the level of acyl-CoA synthetase. Although oxidative metabolism is not inhibited, it is conceivable that intramitochondrial levels of fatty acid intermediates are decreased by Etomoxir treatment. This possibility has not been addressed in our study.

In these studies, \textsuperscript{30} M Etomoxir increases glucose oxidation in aerobic hearts. An important question is what effect this dose of Etomoxir has on glucose oxidation during reperfusion of ischemic hearts. Reperfusion of ischemic myocardium will enhance glucose uptake and utilization and decrease fatty acid utilization.\textsuperscript{30} During ischemia, the decrease in oxidative metabolism will decrease glucose utilization, but not to the same degree as the decrease in free fatty acid utilization.\textsuperscript{31-33} This relative increase in glucose utilization in ischemic and reperfused myocardium is thought to benefit the cell by decreasing oxygen consumption at a given workload. In our hearts, \textsuperscript{30} M Etomoxir did result in a decrease in oxygen consumption per unit work, although whether this was due to an increase in glucose utilization remains to be determined. Since oxidative rates are in large part dependent on heart work, maintenance of heart work in a steady state is necessary to accurately measure non-work dependent changes in glucose oxidation. This could not be achieved during the 30-minute reperfusion period used in our studies, although longer periods of reperfusion may have approximated a steady state and facilitated detection of non-work dependent changes in glucose oxidation.

In summary, we demonstrate that Etomoxir significantly improves myocardial function during reperfusion of palmitate perfused ischemic hearts. This effect was concentration dependent and was not correlated with a decrease in myocardial long chain acylcarnitine levels. A decrease in oxygen consumption per unit work, due to an increase in glucose utilization, may account for the beneficial effect of Etomoxir.

Acknowledgment

We wish to acknowledge the technical assistance of Mrs. Marguerite Spafford.

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**Key Words** • etomoxir • fatty acids • ischemia • long chain acylcarnitine • carnitine palmitoyltransferase
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Circ Res. 1988;63:1036-1043
doi: 10.1161/01.RES.63.6.1036

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