Malonyl Coenzyme A Decarboxylase Inhibition Protects the Ischemic Heart by Inhibiting Fatty Acid Oxidation and Stimulating Glucose Oxidation

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Abstract—Abnormally high rates of fatty acid oxidation and low rates of glucose oxidation are important contributors to the severity of ischemic heart disease. Malonyl coenzyme A (CoA) regulates fatty acid oxidation by inhibiting mitochondrial uptake of fatty acids. Malonyl CoA decarboxylase (MCD) is involved in the decarboxylation of malonyl CoA to acetyl CoA. Therefore, inhibition of MCD may decrease fatty acid oxidation and protect the ischemic heart, secondary to increasing malonyl CoA levels. Ex vivo working rat hearts aerobically perfused in the presence of newly developed MCD inhibitors showed an increase in malonyl CoA levels, which was accompanied by both a significant decrease in fatty acid oxidation rates and an increase in glucose oxidation rates compared with controls. Using a model of demand-induced ischemia in pigs, MCD inhibition significantly increased glucose oxidation rates and reduced lactate production compared with vehicle-treated hearts, which was accompanied by a significant increase in cardiac work compared with controls. In a more severe rat heart global ischemia/reperfusion model, glucose oxidation was significantly increased and cardiac function was significantly improved during reperfusion in hearts treated with the MCD inhibitor compared with controls. Together, our data show that MCD inhibitors, which increase myocardial malonyl CoA levels, decrease fatty acid oxidation and accelerate glucose oxidation in both ex vivo rat hearts and in vivo pig hearts. This switch in energy substrate preference improves cardiac function during and after ischemia, suggesting that pharmacological inhibition of MCD may be a novel approach to treating ischemic heart disease. (Circ Res. 2004; 94:e78-e84.)

Key Words: metabolism ■ fatty acids ■ glucose ■ ischemia ■ malonyl CoA decarboxylase

The heart has a high-energy demand that is primarily met by the oxidation of fatty acids in the mitochondria. The oxidation of fatty acids is a highly regulated process ensuring that energy supply matches energy demand. Carnitine palmitoyltransferase 1 (CPT-1) is the key enzyme involved in the mitochondrial uptake of fatty acids and is inhibited by endogenous malonyl coenzyme A (CoA). Therefore, malonyl CoA acts as physiologic regulator of fatty acid oxidation in the heart through inhibition of CPT-1, which decreases fatty acid transport into the mitochondria and its subsequent oxidation. Malonyl CoA has a rapid turnover in the heart and is produced primarily by the carboxylation of acetyl CoA by acetyl CoA carboxylase (ACC). Studies from our laboratory and others have implicated malonyl CoA decarboxylase (MCD) as being responsible for malonyl CoA degradation back to acetyl CoA. Although it has been suggested that MCD modulates malonyl CoA and subsequently regulates fatty acid oxidation in the heart, the evidence supporting this has only been correlative. Although many mechanisms contribute to ischemic injury, there is clear evidence that contractile dysfunction during and after myocardial ischemia is mediated, at least in part, by the type of energy substrate metabolized by the heart. For instance, an excessive use of fatty acids by the heart during and after ischemia contributes to contractile dysfunction and ischemic injury. These detrimental effects of high fatty acid oxidation appear to occur secondary to an inhibition of glucose oxidation in the heart. During ischemia, glycolysis is stimulated, and as a result, low rates of glucose oxidation increase the production of protons and lactate.
When this occurs, there is a decrease in cardiac efficiency as energy produced by the heart, in the form of ATP, is redirected away from contractile function and toward re-establishment of $H^+$, $Na^+$, and $Ca^{2+}$ ionic homeostasis. After ischemia, fatty acid oxidation also dominates as a source of energy by the heart. This results in a continued low rate of glucose oxidation and a continued decrease in cardiac efficiency during the critical period of reperfusion.

The primary reasons why fatty acid oxidation rates are high during and after ischemia are attributable to the fact that circulating plasma levels of fatty acids are dramatically elevated during and after ischemia and that there are direct alterations in the subcellular control of fatty acid oxidation in the heart. One of these changes in fatty acid oxidation control is a dramatic decrease in malonyl CoA levels during and after ischemia. This is because of an activation of AMP-activated protein kinase, which phosphorylates and inhibits ACC activity and subsequent malonyl CoA production. The combination of high-circulating fatty acid levels and a decrease in malonyl CoA control of mitochondrial fatty acid uptake results in the preferential use of fatty acids as an oxidative substrate over glucose during and after ischemia.

This phenomenon is based on the Randle cycle, in which fatty acid-derived acetyl CoA can decrease the production of glucose-derived acetyl CoA via inhibition of the pyruvate dehydrogenase complex. Because the regulation of malonyl CoA levels is central to the control of fatty acid oxidation rates in the heart, MCD may play a major role in controlling the extent of ischemic injury by promoting glucose oxidation.

The purpose of this study was to directly determine the importance of MCD in controlling malonyl CoA and fatty acid oxidation in the heart and to determine whether inhibition of MCD could increase malonyl CoA levels, decrease fatty acid oxidation, increase glucose oxidation, and protect the ischemic heart. This was achieved by using novel MCD inhibitors (MCDs) in both the in vivo and ex vivo heart.

Materials and Methods

Animal Care

The University of Alberta adheres to the principles for biomedical research involving rats developed by the Council for International Organizations of Medical Sciences and complied with the Canadian Council on Animal Care guidelines. Pig studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, revised 1985) and the Institutional Animal Care and Use Committee at Case Western Reserve University.

In Vitro MCD Inhibitory Activity Assay

The decarboxylase activity of recombinant human MCD (hMCD) was measured spectrophotometrically by monitoring the acetyl CoA formation using the malate dehydrogenase/citrate synthase–coupling system. The conversion of acetyl CoA from malonyl CoA was assayed using a modified protocol as described previously. Assays were initiated by the addition of malonyl CoA, and the rates were corrected for the background rate determined in the absence of hMCD. Kinetic data analysis was performed as described (see online data supplement available at http://circres.ahajournals.org).

MCD Inhibitors

MCDs were synthesized at Chugai Pharma USA, LLC. The detailed account of discovery and development of small molecule MCDs will be published in separate articles.

Rat Heart Perfusion

Sprague-Dawley rats (300 to 350 g) were anesthetized with sodium pentobarbital (60 mg/kg i.v.). Hearts were then excised and placed in ice-cold Krebs–Henseleit bicarbonate solution and perfused as described (see online data supplement) with either [5-3H/14C]-labeled glucose or [9,10-3H]-labeled palmitate for glycolysis, glucose oxidation, and palmitate oxidation measurements, respectively. Spontaneously beating hearts were perfused for: (1) a 60-minute aerobic period with the vehicle or 20 $\mu$mol/L CBM-300864 (MCDi) present for the entire 60 minutes; (2) a 30-minute aerobic period followed by 30 minutes of mild ischemia (35% reduction in coronary flow) with the vehicle or 20 $\mu$mol/L CBM-300864 (MCDi) present for the entire 60 minutes; or (3) 30 minutes of aerobic perfusion followed by 30 minutes of global no-flow ischemia and 60 minutes of aerobic reperfusion with the vehicle or 1 $\mu$mol/L CBM-301940 added, either at the onset of the perfusion (preischemic) or at the onset of aerobic reperfusion after ischemia (postischemic). For the global ischemia/reperfusion protocols, coronary flow was reduced to zero for a 30-minute period, after which hearts were aerobically reperfused as described previously. For the mild ischemia model, the perfusion apparatus contained a 1-way valve in the aortic outflow line that does not impede systolic ejection but restricts diastolic perfusion of the coronary arteries. Coronary flow, measured continuously from the difference in flow between cardiac output (left atrial inflow) and aortic flow, was controlled by a back-flow controller that permitted ejected perfusate to bypass the 1-way valve and return to enter the coronary circulation. At the end of the aerobic period (60 minutes), mild ischemic periods, or reperfusion periods (60 minutes), the ventricle from the heart was frozen immediately in liquid nitrogen (N$_2$) and the mass recorded.

Measurement of Fatty Acid Oxidation, Glucose Oxidation, and Glycolysis

Rates of glucose oxidation, glycolysis, and palmitate oxidation were measured in 2 separate series of heart perfusions as described (see online data supplement).

Calculated Proton Production

Proton production from glucose metabolism is calculated as 2(rate of glycolysis−rate of glucose oxidation), which takes into consideration the net production of 2 protons per molecule of glucose that passes through glycolysis that is not subsequently oxidized. An increase in proton production indicates increased uncoupling of glycolysis from glucose oxidation.

Determination of CoA Esters

Detection and quantification of CoA esters were performed by extracting CoA esters from powdered tissue into 6% perchloric acid and measuring with a modified high-performance liquid chromatography (HPLC) as described. (see online data supplement).

Enzymatic Assays

Either the mitochondrial or cytosolic fractions of rat heart tissue were assayed, as described for MCD, ACC, CPT-1, and long-chain 3-ketoacyl CoA thiolase activities.

Model of Demand-Induced Ischemia in Pigs

Experiments were performed on 16 domestic pigs (vehicle n=8, mean weight 35.9±1.8 kg; CBM-300864 n=8, mean weight 37.3±1.0 kg). Pigs were fasted overnight, sedated, and the left anterior descending coronary artery (LAD) was perfused via a roller pump with blood supplied from the femoral artery as described in detail previously. This preparation allowed the LAD perfusion bed to be subjected to demand-induced ischemia by decreasing LAD flow by 20% and infusing dobutamine to increase heart rate and cardiac contractility. Animals were heparinized and given an intravenous infusion of 20% triglyceride emulsion to increase fatty acid concentration to levels observed in angina patients (~0.8 mmol/L). After completion of the instrumentation, a continuous infusion of...
[U-14C] glucose (0.2 μCi/min) was introduced into the proximal end of the coronary perfusion line. After 30 minutes of tracer infusion, an infusion of either vehicle or CBM-300864 was initiated directly into the coronary perfusion circuit at a rate set to result in a step increase of 100 μmol/L CBM-300864 in the coronary arterial blood. Arterial and interventricular venous samples were drawn at 20 and 27 minutes after treatment. Thirty minutes after starting treatment, demand-induced ischemia was initiated with an infusion of dobutamine (15 μg · kg⁻¹ · min⁻¹) to increase myocardial oxygen demand and by reducing LAD blood flow by 20% for a period of 15 minutes. Arterial and anterior interventricular venous blood samples were then taken at 3, 6, 10, and 15 minutes after demand-induced ischemia. Blood samples were analyzed for the concentrations of oxygen, lactate, and glucose in blood and plasma-free fatty acids. In addition, samples were analyzed for 14C-glucose and 14CO₂ calculations of the rates of glucose oxidation, as described previously.27 Heart rate, left ventricular pressure (LVP), peak positive and negative rates of developed pressure, and segment length were continuously recorded using a commercial online data acquisition system. After 18 minutes of ischemia, myocardial biopsies from the LAD perfusion bed (~2 g) were freeze clamped (3 to 5 seconds) on aluminum blocks precooled in N₂ and stored at −80°C for subsequent analysis of tissue malonyl CoA content. The LVP-segment-length loop area was used as an index of external wall work of the anterior-free wall as described previously.27 Vehicle and treatment groups were compared using an unpaired t test with significance set at P<0.05.

Results

MCD Inhibitors
To understand the role of MCD in the regulation of fatty acid oxidation in the heart and to evaluate the potential therapeutic value of MCD inhibition, an MCD discovery program was initiated using human recombinant MBP fusion MCD protein. A high throughput screening for MCD inhibitory activity was performed on an in-house small molecule compound library at Chugai, which led to the discovery of several weak inhibitors. Structural optimization of 1 of these series led to the development of 2 potent inhibitors: CBM-300864 (Ki=98 nM) and CBM-301940 (Ki=28 nM) (Table 1). In addition, CBM-301940 and CBM-300864 significantly inhibited both rat and pig heart MCD in an in vitro assay to the same extent with CBM-300864, inhibiting rat heart MCD from 8472±333 to 416±23 nmol · g dry wt⁻¹ · min⁻¹ and pig heart MCD from 4167±278 to 250±23 nmol · g dry wt⁻¹ · min⁻¹. Neither of these compounds inhibited other key enzymes that control malonyl CoA or fatty acid oxidation in the heart, including ACC, CPT-1, or long-chain 3-ketoacyl CoA thiolase activities (data not shown).

MCD Inhibition Increases Glucose Oxidation in Isolated Working Rat Hearts
With potent MCDs in hand, it became possible to directly examine the importance of MCD in regulating fatty acid oxidation. Ex vivo working rat hearts were aerobically perfused for 60 minutes with Krebs–Henseleit solution containing either 5 mmol/L [U-3H]glucose and 1.2 mmol/L [9,10-3H]palmitate or 5 mmol/L [5-14C]glucose and 1.2 mmol/L [1,4-14C]palmitate.17 The concentration of fatty acid used is equivalent to plasma concentrations that have been observed clinically in patients experiencing myocardial ischemia.21,28 Rates of glycolysis, glucose oxidation, and fatty acid oxidation were measured directly from the production of H₂O and 14CO₂ as described.18 In all instances, either 20 μmol/L CBM-300864 dissolved in 0.1% dimethyl sulfoxide (DMSO; drug treatment group) or 0.1% DMSO alone (control group) was added at the beginning of the perfusion. The mechanical function of the MCD-treated hearts was not significantly different from control hearts (data not shown), demonstrating that MCD inhibition has no adverse effects on cardiac function or coronary flow. At the end of the perfusion, hearts were frozen and malonyl CoA levels were determined by HPLC analysis.29 Treatment with the MCD, CBM-300864 effectively blocked malonyl CoA degradation, as demonstrated by a 7-fold increase in myocardial malonyl CoA content (Figure 1A). In addition, inhibition of MCD significantly suppressed fatty acid oxidation (Figure 1B) and increased glucose oxidation compared with control hearts (Figure 1C) without altering the rate of glycolysis (Figure 1D). MCD inhibition resulted in a dramatic switch in energy substrate preference such that glucose metabolism became the predominant source of energy in the heart (Figure 1E). Therefore, our data show that MCD controls intracellular malonyl CoA levels and is a major regulator of cardiac fatty acid oxidation rates. These data also demonstrate that MCD inhibition can significantly increase glucose oxidation secondary to decreasing palmitate oxidation while having no effect on glycolytic rates.

MCD Inhibition Decreases Proton Production During Mild Ischemia in Rat Hearts
Because it is well established that there is a benefit to partially suppressing myocardial fatty acid oxidation and increasing glucose oxidation during myocardial ischemia, we evaluated the effects of CBM-300864 using a model of mild global ischemia. Ex vivo working rat hearts were subjected to a mild global ischemia by reducing coronary flow by 35% for a 30-minute period, which reduced cardiac function to 40% to 50% of normal aerobic values (Table 2). Despite the high levels of palmitate in

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**Table 1. Structures and Inhibitory Activity of CBM-300864 and CBM-301940**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Ki (nM)</th>
</tr>
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<tbody>
<tr>
<td>CBM-300864</td>
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<td>98</td>
</tr>
<tr>
<td>CBM-301940</td>
<td><img src="image2" alt="Structure" /></td>
<td>28</td>
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</tbody>
</table>

*The decarboxylase activity of hMCD was measured spectrophotometrically, and the inhibitory constants (Ki) of MCD inhibitors were measured as described in Materials and Methods.*
the perfusate, MCD inhibition significantly reduced fatty acid oxidation in the ischemic period (484 ± 55 versus 328 ± 50 nmol·g dry wt\(^{-1}\)·min\(^{-1}\), control and MCDi, respectively) and accelerated glucose oxidation during both the aerobic and the ischemic periods (Figure 1F). Because accelerated glucose oxidation rates improve the coupling of glucose oxidation to glycolysis, the drug-treated group demonstrated a significant reduction in calculated proton production from anaerobic glucose metabolism compared with control hearts during the ischemic period (Figure 1G).

**MCD Inhibition Improves Cardiac Function in Demand-Induced Ischemia in Pigs**

Increasing glucose oxidation without affecting glycolytic rate reduces myocardial lactate production during ischemia. A reduction in lactate production may be the mechanism by which partial inhibitors of myocardial fatty acid oxidation improve cardiac function during demand-induced ischemia. Rat hearts were perfused in working mode in the presence (closed bars) or absence (open bars) of 20 μmol/L CBM-300864 (MCD) dissolved in 0.1% DMSO. Control hearts were perfused with an equal concentration of DMSO (vehicle). Malonyl CoA levels (A) in the perfused heart tissue were quantified using HPLC. Rates of palmitate oxidation (B), glucose oxidation (C), and glycolysis (D) were determined during aerobic perfusion. The overall ATP contribution from palmitate oxidation (black bars), glucose oxidation (white bars), and glycolysis (gray bars) is shown for both groups of hearts (E). Low-flow ischemia was produced by lowering coronary flow by 35%, and glucose oxidation rates (F) and proton production (G) were also determined in the presence or absence of the MCD. Values are the mean ± SE of n = 8 hearts in each group. The asterisk indicates significant difference from the control group; \( P < 0.05 \).

**TABLE 2. Effect of MCD Inhibition With CBM-300864 on Mechanical Function in Hearts Aerobically Perfused for 30 min, then Subjected to 30-min Period of Mild Ischemia**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Aerobic</th>
<th>Demand Low Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate pressure product (beats·min(^{-1})·mm Hg·10(^{-3}))</td>
<td>37 ± 1 Vehicle</td>
<td>38 ± 1 MCDi</td>
</tr>
<tr>
<td>Cardiac output (ml·min(^{-1}))</td>
<td>47 ± 1 Vehicle</td>
<td>49 ± 2 MCDi</td>
</tr>
<tr>
<td>Aortic flow (ml·min(^{-1}))</td>
<td>21 ± 1 Vehicle</td>
<td>25 ± 1 MCDi</td>
</tr>
<tr>
<td>Coronary flow (ml·min(^{-1}))</td>
<td>26 ± 2 Vehicle</td>
<td>26 ± 1 MCDi</td>
</tr>
<tr>
<td>Cardiac power (ml·mm Hg·min(^{-1})·10(^{-3}))</td>
<td>75 ± 3 Vehicle</td>
<td>78 ± 3 MCDi</td>
</tr>
</tbody>
</table>

Values are the mean ± SE of 8 untreated and 8 MCDi-treated hearts. Hearts were subjected to a 30-min aerobic perfusion followed by a 30-min demand low-flow ischemia. A concentration of 20 μmol/L CBM-300864 was added at the onset of the perfusion.

**Figure 1.** Effects of MCD inhibition on cardiac energy metabolism, malonyl CoA levels, and proton production in the aerobic and ischemic ex vivo working rat heart. Rat hearts were perfused in working mode in the presence (closed bars) or absence (open bars) of 20 μmol/L CBM-300864 (MCDi) dissolved in 0.1% DMSO. Control hearts were perfused with an equal concentration of DMSO (vehicle). Malonyl CoA levels (A) in the perfused heart tissue were quantified using HPLC. Rates of palmitate oxidation (B), glucose oxidation (C), and glycolysis (D) were determined during aerobic perfusion. The overall ATP contribution from palmitate oxidation (black bars), glucose oxidation (white bars), and glycolysis (gray bars) is shown for both groups of hearts (E). Low-flow ischemia was produced by lowering coronary flow by 35%, and glucose oxidation rates (F) and proton production (G) were also determined in the presence or absence of the MCD. Values are the mean ± SE of n = 8 hearts in each group. The asterisk indicates significant difference from the control group; \( P < 0.05 \).

**MCD Inhibition Improves Cardiac Function During Reperfusion of Severely Ischemic Rat Hearts**

To determine whether MCD inhibition can improve cardiac function during reperfusion of severely ischemic hearts, the effects of MCD inhibition on functional recovery of reperfused ischemic hearts were determined. To lessen the likelihood that our MCD, was inhibiting another enzyme, a more potent novel MCD (CBM-301940) was identified and used in ex vivo aerobically perfused rat hearts. At 1 μmol/L, CBM-301940 was equally efficacious in stimulating glucose oxidation as CBM-300864 (Figure 3B, aerobic period) but was significantly more potent. The MCD, CBM-301940 (1 μmol/L) was examined in ex vivo working rat hearts aerobically perfused for 30 minutes, followed by 30 minutes symptoms during demand-induced ischemia in patients with chronic stable angina. Therefore, we tested the effects of MCD inhibition in a swine model of demand-induced ischemia and measured rates of glucose oxidation, lactate production, and cardiac work. This model produces ischemia as a result of flow restriction and dobutamine stimulation of heart rate and contractility, with no change in myocardial oxygen consumption. Treatment with CBM-300864 (100 μmol/L in arterial blood) during the normal flow period had no effect on heart rate, peak systolic LVP, peak positive or negative LVDp/dt, or LV external work (as measured from the segment length-LVP loop area). Pig hearts treated with the MCD, CBM-300864 showed a significant increase in malonyl CoA levels during the demand-induced ischemic period compared with vehicle-treated hearts (Figure 2A). During this period, the percentage of glucose taken up by the myocardium that underwent immediate oxidation to CO\(_2\) was doubled in the MCD-treated animals, whereas lactate production was significantly reduced (Figure 2B and 2C). Accompanying this decrease in lactate production was a significant increase in external work in the ischemic region of the left ventricle (Figure 2D), demonstrating that MCD inhibition improves cardiac function during demand-induced ischemia.
of global no-flow ischemia and 60 minutes of aerobic reperfusion, as described previously.18 Hearts were treated with the MCDi, either before ischemia or at the onset of reperfusion to determine whether MCD inhibition was protective during or after ischemia. Hearts treated with the MCDi before ischemia had no significant alterations in mechanical function. However, cardiac function on aerobic reperfusion after ischemia was significantly improved in hearts treated with CBM-301940 when added either before or after ischemia compared with control hearts (Table 3, Figure 3A and 3C). In addition, MCD inhibition significantly improved rate pressure product, cardiac output, aortic flow, coronary flow, and cardiac power on aerobic reperfusion of ischemic hearts (Table 3). Moreover, MCD inhibition stimulated glucose oxidation rates both before and after ischemia compared with vehicle-treated hearts (Figure 3B and 3D). Similar effects of CBM-301940 on recovery of cardiac function (Table 3, Figure 3C) and glucose oxidation (Figure 3D) were seen when the drug was added in reperfusion, suggesting that the primary beneficial effect was occurring during reperfusion. These data show that the alteration in cardiac substrate preferences produced by MCD inhibition leads to improved cardiac function during reperfusion of severely ischemic hearts.

Discussion

High fatty acid oxidation rates inhibit glucose oxidation, which can decrease cardiac efficiency and contractile function during and after ischemia. Although fatty acid oxidation does not increase during ischemia, it does predominate as the major residual source of oxidative metabolism. During ischemia, mitochondrial oxidative metabolism decreases in proportion to the decrease in oxygen consumption.32 This includes both a decrease in fatty acid oxidation and a decrease in glucose oxidation.31,33,34 However, the residual fatty acid oxidation predominates over glucose oxidation as the main source of residual oxidative metabolism (which is attributable, in

Figure 2. Effects of MCD inhibition on malonyl CoA levels, glucose oxidation rates, lactate production, and cardiac work during demand-induced ischemia in the in vivo perfused pig heart. Pig hearts were infused with either 100 μmol/L CBM-300864 or vehicle for 45 minutes. After 30 minutes of normal perfusion, demand-induced ischemia was initiated with an infusion of dobutamine (15 μg · kg⁻¹ · min⁻¹) to increase myocardial oxygen demand and by reducing LAD blood flow by 20% for a period of 15 minutes. Malonyl CoA levels were determined in the LAD perfusion bed (A). Glucose oxidation rates (B) were measured as the percentage of glucose uptake (μmol/min) that was oxidized to produce ¹⁴CO₂, during both aerobic conditions and during demand-induced ischemia. Net myocardial lactate production (C) is plotted as a function of time for control (open circles) and MCDi (CBM-300864) treated hearts (closed circles) during normal conditions and demand-induced ischemia. Cardiac work (D) was calculated as a percentage of values obtained during the pre-ischemic period. Values are the mean±SE of n=8 hearts in each group. The asterisk indicates significant difference from the control group; P<0.05.

Figure 3. Effects of MCD inhibition on glucose oxidation rates and functional recovery of aerobically reperfused ischemic rat hearts. Rat hearts were perfused in working mode in the presence (closed bars) or absence (open bars) of 1 μmol/L CBM-301940 (MCDi) dissolved in 0.1% DMSO. Control hearts were perfused with an equal concentration of DMSO. Recovery of cardiac function in aerobically reperfused ischemic hearts was determined when the MCDi was added before (A) or after (C) ischemia (arrow indicates the time period of drug or vehicle administration). Glucose oxidation rates were determined in the aerobic and reperfusion periods for groups of hearts treated with the MCDi either before (B) or after ischemia (D). hr, heart rate; PSP, peak systolic pressure. Values are the mean±SE of n=8 hearts in each group. The asterisk indicates significant difference from the control group; P<0.05.
TABLE 3. Effect of MCD Inhibition With CBM-301940 on Recovery of Mechanical Function in Hearts Reperfused for 60 min After 30 min of global no-flow ischemia

<table>
<thead>
<tr>
<th>Addition</th>
<th>Pre-Ischemic Addtion</th>
<th>Post-Ischemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>MCDi</td>
</tr>
<tr>
<td>Rate pressure product (beats · min⁻¹ · mm Hg · 10⁻²)</td>
<td>12±2</td>
<td>22±2*</td>
</tr>
<tr>
<td>Cardiac output (ml · min⁻¹)</td>
<td>17±3</td>
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</tr>
<tr>
<td>Aortic flow (ml · min⁻¹)</td>
<td>3±1</td>
<td>13±3*</td>
</tr>
<tr>
<td>Coronary flow (ml · min⁻¹)</td>
<td>14±2</td>
<td>19±1*</td>
</tr>
<tr>
<td>Cardiac power (ml · mm Hg · min⁻¹ · 10⁻²)</td>
<td>15±3</td>
<td>38±4*</td>
</tr>
</tbody>
</table>

*Values are means±SE of 8 untreated and 16 MCDi-treated hearts (8 preischemia, 8 postischemia). Hearts were subjected to 30 min of aerobic perfusion, 30 min of global no-flow ischemia, and 60 min of aerobic reperfusion. A concentration of 1 μmol/L CBM-301940 was added either at the onset of the perfusion (preischemic) or at the onset of aerobic reperfusion after ischemia (postischemic). Values were determined at the end of the 60-min reperfusion period.

*Significantly different from vehicle-treated hearts of the same group.

part, to the high levels of fatty acids to which the ischemic myocardium is exposed). As originally shown by Neely et al.,33 in isolated working rat hearts perfused with 1.2 mmol/L palmitate, although oxidative rates decrease during ischemia, oxidation of palmitate accounts for the largest proportion of oxygen consumed during moderate ischemia (70%) and severe ischemia (90%). This finding has been confirmed in the in vivo pig heart.33,34 This predominance of fatty acid oxidation to residual oxidative metabolism, at the expense of glucose oxidation, occurs during a period when glycolysis has increased to compensate for the decrease in ATP supply. As a result, an uncoupling of glycolysis from glucose oxidation occurs, resulting in lactate production and acidosis (Figure 1G). For this reason, we believe that inhibition of “residual” fatty acid oxidation during ischemia will increase glucose oxidation, improve the coupling of glycolysis to glucose oxidation, lessen lactate and proton production, and improve cardiac efficiency. Therefore, a therapeutic goal in the treatment of ischemia would be to inhibit fatty acid oxidation, thereby stimulating glucose oxidation.

In this report, we provide evidence that MCD is a major regulator of cardiac fatty acid oxidation, secondary to modifying intracellular malonyl CoA levels. Rat hearts perfused in the presence of the MCDi (CBM-300864) showed a significant increase in intracellular malonyl CoA levels and a corresponding decrease in palmitate oxidation rates. This was accompanied by large increases in glucose oxidation rates. Because palmitate provides 8-acetyl CoA molecules to the Krebs cycle compared with 2-acetyl CoA molecules from glucose, a decrease in the oxidation of 1 palmitate molecule will result in an increase in the oxidation of 4 glucose molecules. As a result, inhibition of fatty acid oxidation by MCD inhibition resulted in dramatic increases in glucose oxidation (Figures 1, 2, and 3). Even in the presence of high levels of fatty acids, in which glucose oxidation provided only 13% of total ATP production under control conditions (Figure 1E), the presence of an MCD increased the contribution of glucose oxidation to ATP production by 3.8-fold. This resulted in glucose oxidation becoming a major source of energy (49% of total ATP). These data suggest the importance of MCD in energy substrate selection in the heart.

During and after ischemia, rates of glycolysis are high and glucose oxidation rates are low.15,19,20 This uncoupling of glucose oxidation from glycolysis increases the production of protons and lactate and decreases cardiac efficiency.17 Using a model of mild global ischemia, we show that MCD inhibition can decrease fatty acid oxidation and increase glucose oxidation during ischemia and improve the coupling of glucose oxidation to glycolysis, thus reducing proton production during the ischemic period. Furthermore, using a demand-induced ischemia model in pigs, we show that MCD inhibition increases malonyl CoA levels, increases glucose oxidation, reduces lactate production, and increases external work in the ischemic region of the left ventricle. Together, these data demonstrate that MCD inhibition improves cardiac function during mild ischemia.

We also show that MCD inhibition improves cardiac function during reperfusion of severely ischemic hearts. Hearts treated with MCDis before ischemia had significantly improved cardiac function compared with control hearts, which were accompanied by an increase in glucose oxidation during reperfusion. Although the energetics during ischemia are extremely important, it has been shown that after ischemia, fatty acid oxidation also dominates as a source of energy by the heart.4,5,15,19,20 Although we did not measure fatty acid oxidation rates during reperfusion in this study, it has already been shown that during reperfusion of ischemic hearts, there is a close relationship between fatty acid oxidation and glucose oxidation, with high rates of fatty acid oxidation resulting in stoichiometric decreases in glucose oxidation.15 This results in a continued decrease in cardiac efficiency during the critical period of reperfusion. We have observed previously that stimulation of glucose oxidation during reperfusion can improve function recovery.4,15-18 an effect we also observed with MCD inhibition. For this reason, MCDs also appear to be beneficial when added after ischemia and before reperfusion. These data suggest that the alteration in cardiac substrate preferences produced by MCD inhibition leads to improved cardiac function during reperfusion of severely ischemic hearts.

In this study, we show that MCD inhibition increases malonyl CoA levels and switches energy substrate preference from fatty acid oxidation to glucose oxidation. We propose that this increase in glucose oxidation is responsible for the beneficial effect of malonyl CoA on contractile function and cardiac efficiency. However, it cannot be ruled out that malonyl CoA may be acting by altering other mechanisms, including calcium handling, pH, altering redox potential or ATP levels. To our knowledge, there are no reports of malonyl CoA directly impacting either calcium or pH regulatory enzymes in the heart. However, stimulation of glucose oxidation in the heart will decrease proton production and acidosis in the heart.17 These beneficial effects on pH have the potential to decrease calcium accumulation, secondary to a decrease in Na⁺/H⁺ exchange and Na⁺/Ca²⁺ exchange activity. It is also possible that increasing malonyl CoA alters...
redox state or ATP levels. However, alterations in these parameters would be expected to occur because of an increase in energy supply, as opposed to a switch in energy substrate preference. Alternatively, redox state and ATP could be increased but secondary to an increase in cardiac function (which was observed in this study). As a result, further studies are needed to examine the relationship between alterations in malonyl-CoA levels and calcium handling, pH, redox state, and ATP levels.

In summary, this report describes the effects of novel MCDs on energy metabolism in the heart and provides evidence that MCD is a major regulator of cardiac fatty acid oxidation, secondary to modifying intracellular malonyl-CoA levels. Our data also show that MCD inhibition accelerates glucose oxidation in both ex vivo and in vivo hearts, and that MCD inhibition improves both functional recovery during reperfusion of an ischemic rat heart and cardiac work during demand-induced ischemia. Although MCDs require further evaluation, our data suggest that pharmacological inhibition of MCD may be a viable approach to the treatment of clinical pathologies associated with myocardial ischemia.

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Malonyl Coenzyme A Decarboxylase Inhibition Protects the Ischemic Heart by Inhibiting Fatty Acid Oxidation and Stimulating Glucose Oxidation

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ONLINE DATA SUPPLEMENT

Expanded Material and Methods:

**Kinetic Data Analysis.** Data were fitted to the appropriate rate equations using Grafit computer software (Erithacus Software). Initial velocity data conforming to Michaelis-Menton kinetics were fitted to eq. 1. Inhibition patterns conforming to apparent competitive, un-competitive, and non-competitive inhibition were fitted to eq. 2 - 7, respectively.

\[
v = \frac{VA}{K_a + A} \quad (1)
\]

\[
v = \frac{VA}{K_a(1 + I/K_{iS}) + A} \quad (2)
\]

\[
v = \frac{VA}{K_a + A(1 + I/K_{ii})} \quad (3)
\]

\[
v = \frac{VA}{K_a(1 + I/K_{iS}) + A(1 + I/K_{ii})} \quad (4)
\]

In eqs 4 - 7, \(v\) is the initial velocity, \(V\) is the maximum velocity, \(K_a\) is the apparent Michaelis constant, \(I\) is the inhibitor concentration, and \(A\) is the concentration of variable substrates. The nomenclature used in the rate equations for inhibition constants is that of Cleland \(^1\), in which \(K_{iS}\) and \(K_{ii}\) represent the apparent slope and intercept inhibition constants, respectively.

**Rat Heart Perfusions.** Sprague-Dawley rats (300 to 350 g) were anaesthetized with sodium pentobarbital (60 mg.kg\(^{-1}\)). Hearts were then excised and placed in ice-cold Krebs-Henseleit bicarbonate solution (118 mM NaCl, 25 mM NaHCO\(_3\), 4.7 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 2.5 mM CaCl\(_2\), and 5 mM glucose). Following excision and rinsing, the hearts were cannulated and perfused at a 11.5 mm Hg left atrial preload and 80 mm Hg aortic afterload with Krebs-Henseleit solution containing 100 µU/ml insulin, 5 mM glucose and 1.2 mM palmitate pre-bound to fatty acid free bovine serum albumin. Either \([5-^3H/U-^{14}C]\) labeled glucose or \([9,10-^3H]\) labeled palmitate were used for glycolysis, glucose oxidation and palmitate oxidation measurements, respectively. Spontaneously beating hearts were perfused for either: 1) a 60 min aerobic period with the vehicle or 20
µM CBM-300864 (MCDi) present for the entire 60 minutes, 2) a 30 min aerobic period followed by 30 min of mild ischemia (35% reduction in coronary flow) with the vehicle or 20 µM CBM-300864 (MCDi) present for the entire 60 minutes, or 3) 30 min or aerobic perfusion followed by 30 min of global no flow ischemia and 60 min of aerobic reperfusion with the vehicle or 1 µM CBM-30194 added either at the onset of the perfusion (pre-ischemic) or at the onset of aerobic reperfusion following ischemia (post-ischemic). For the global ischemia/reperfusion protocols, coronary flow was reduced to zero for a 30 min period, following which hearts were aerobically reperfused as previously described. For the mild ischemia model, the perfusion apparatus contained a one-way valve in the aortic outflow line that does not impede systolic ejection, but restricts diastolic perfusion of the coronary arteries. Coronary flow, measured continuously from the difference in flow between cardiac output (left atrial inflow) and aortic flow, was controlled by a backflow controller that permitted ejected perfusate to bypass the one-way valve and return to enter the coronary circulation.

For all perfusions, heart rate and systolic pressure measurements were recorded using a pressure transducer in the aortic outflow line (Harvard Apparatus). Data was collected using an MP100 system from AcqKnowledge (BIOPAC Systems, Inc USA). Cardiac output and aortic flows were obtained by monitoring the flow into the left atria and from the afterload line using Transonic flow probes. Coronary flow was calculated from the difference of the cardiac output and aortic flows. While cardiac work was calculated as the product of peak systolic pressure and cardiac output.

At the end of either the aerobic period (60 min), mild ischemic periods, or reperfusion periods (60 min), the ventricle from the heart was frozen immediately in liquid N\textsubscript{2} and the mass recorded. Hearts were then ground using a mortar and pestle cooled to the temperature of liquid N\textsubscript{2}. A portion of this powdered tissue (~20 mg) was weighed (wet wt) and then dried at 60 °C overnight to remove all water (dry wt). The ratio of this sample (dry /wet wt) was used to calculate the total dry mass of the heart. Metabolic rates were calculated using total dry mass of the heart to correct for variations in heart size.
**Measurement of fatty acid oxidation, glucose oxidation and glycolysis.** Rates of glucose oxidation, glycolysis and palmitate oxidation were measured in two separate series of heart perfusions. Steady state metabolic rates were calculated as the mean values from perfusate samples removed from the buffer of the working heart apparatus. Values obtained for the metabolic pathways were normalized for heart mass (dry wt). Glucose and palmitate oxidation were measured simultaneously by perfusing hearts with $[\text{U-}^{14}\text{C}]$ glucose and $[9,10-^{3}\text{H}]$ labeled palmitate, respectively. The total myocardial $^{3}\text{H}_{2}\text{O}$ production and $^{14}\text{CO}_{2}$ production were determined at 10 min intervals in the aerobic period(s). In a separate group of perfusions, glycolysis was measured by the quantitative collection of $^{3}\text{H}_{2}\text{O}$ production every 10 min from hearts perfused with buffer containing $[5-^{3}\text{H}]$ labeled glucose.

**Determination of CoA Esters.** Detection and quantification of CoA esters were performed by extracting CoA esters from powdered tissue into 6% perchloric acid and measuring with a modified HPLC as described. Essentially, each sample (100 µl) was run through a precolumn cartridge (C18, size 3 cm, 7 µm) and a Microsorb Short-one column (type C18, particle size 3 µm, size 4.6 Å~ 100 mm) on a Beckman System Gold HPLC. Absorbance was set at 254 nm and flow rate at 1 ml/min. A gradient was initiated using buffer $A$ (0.2 M NaH$_2$PO$_4$, pH 5.0) and buffer $B$ (0.25 M NaH$_2$PO$_4$ and acetonitrile, pH 5.0) in a ratio of 80:20 (vol/vol). Conditions were maintained at initial levels for 2.5 min (97% buffer $A$ and 3% buffer $B$) and were changed thereafter to 18% buffer $B$ over 5 min using Beckman's curve no. 3. The gradient was changed linearly at 15 min to 37% buffer $B$ over 3 min and subsequently to 90% buffer $B$ over 17 min. At 42 min the composition was returned linearly back to 3% buffer $B$ over 0.5 min, and at 50 min column equilibration was complete. Peaks corresponding to malonyl CoA and acetyl CoA were integrated by the Beckman System Gold software package.

**Enzymatic Assays.** Either the mitochondrial or cytosolic fractions of rat heart tissue were assayed, as described for MCD, ACC, carnitine palmitoyltransferase 1, and long-chain 3-ketoacyl CoA thiolase activities.
**Model of Demand-Induced Ischemia in Pigs.** Experiments were performed on sixteen domestic pigs (vehicle, n=8 mean weight 35.9 ± 1.8 kg; CBM-300864, n=8, mean weight 37.3 ± 1.0 kg). Pigs were fasted overnight, sedated and the left anterior descending coronary artery (LAD) was perfused via a roller pump with blood supplied from the femoral artery as previously described in detail. This preparation allowed the LAD perfusion bed to be subjected to demand-induced ischemia by decreasing LAD flow by 20% and infusing dobutamine to increase heart rate and cardiac contractility. Animals were heparinized and given an i.v. infusion of 20% triglyceride emulsion to increase fatty acid concentration to levels observed in angina patients (~0.8 mM). After completion of the instrumentation, a continuous infusion of [U-14C] glucose (0.2 µCi/min) was introduced into the proximal end of the coronary perfusion line. After 30 min of tracer infusion an infusion of either vehicle or CBM-300864 was initiated directly into the coronary perfusion circuit at a rate set to result in a step increase of 100 µM CBM-300864 in the coronary arterial blood. Arterial and interventricular venous samples were drawn at 20 and 27 minutes of treatment. Thirty min after starting treatment, demand-induced ischemia was initiated with an infusion of dobutamine (15 µg•kg⁻¹•min⁻¹) to increase myocardial oxygen demand and by reducing LAD blood flow by 20% for a period of 15 min. Arterial and anterior interventricular venous blood samples were then taken at 3, 6, 10 and 15 min of demand-induced ischemia. Blood samples were analyzed for the concentrations of oxygen, lactate and glucose in blood and plasma free fatty acids. In addition, samples were analyzed for 14C-glucose, and 14CO₂ calculation of the rates of glucose oxidation, as previously described. Heart rate, left ventricular pressure (LVP), peak positive and negative dP/dt, and segment length were continuously recorded using a commercial on-line data acquisition system. At 18 min of ischemia myocardial biopsies from the LAD perfusion bed (~2 g) were freeze-clamped (3-5 sec) on aluminum blocks pre-cooled in liquid nitrogen and stored at -80°C for subsequent analysis of tissue malonyl-CoA content. The LV pressure (LVP) - segment length loop area was used as an index of external wall work of the anterior free wall as previously described. Vehicle and treatment groups were compared using an unpaired t-test with significance set at p<0.05.
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