The Antianginal Agent Trimetazidine Does Not Exert Its Functional Benefit via Inhibition of Mitochondrial Long-Chain 3-Ketoacyl Coenzyme A Thiolase

Alan MacInnes, David A. Fairman, Peter Binding, Jo ann Rhodes, Michael J. Wyatt, Anne Phelan, Peter S. Haddock, Eric H. Karran

Abstract—Trimetazidine acts as an effective antianginal clinical agent by modulating cardiac energy metabolism. Recent published data support the hypothesis that trimetazidine selectively inhibits long-chain 3-ketoacyl CoA thiolase (LC 3-KAT), thereby reducing fatty acid oxidation resulting in clinical benefit. The aim of this study was to assess whether trimetazidine and ranolazine, which may also act as a metabolic modulator, are specific inhibitors of LC 3-KAT. We have demonstrated that trimetazidine and ranolazine do not inhibit crude and purified rat heart or recombinant human LC 3-KAT by methods that both assess the ability of LC 3-KAT to turnover specific substrate, and LC 3-KAT activity as a functional component of intact cellular β-oxidation. Furthermore, we have demonstrated that trimetazidine does not inhibit any component of β-oxidation in an isolated human cardiomyocyte cell line. Ranolazine, however, did demonstrate a partial inhibition of β-oxidation in a dose-dependent manner (12% at 100 μmol/L and 30% at 300 μmol/L). Both trimetazidine (10 μmol/L) and ranolazine (20 μmol/L) improved the recovery of cardiac function after a period of no flow ischemia in the isolated working rat heart perfused with a buffer containing a relatively high concentration (1.2 mmol/L) of free fatty acid. In summary, both trimetazidine and ranolazine were able to improve ischemic cardiac function but inhibition of LC 3-KAT is not part of their mechanism of action. The full text of this article is available online at http://www.circresaha.org.

Key Words: cardiac metabolism ischemia trimetazidine

Myocardial metabolic modulation is a new approach for the treatment and management of ischemic heart disease (IHD) and angina pectoris. In the healthy heart, fatty acids are the primary fuel source for the generation of ATP, with the balance of energy provision coming from glucose and lactate oxidation.

Myocardial ischemia radically alters the balance of cardiac fuel metabolism. The primary consequence of moderate ischemia (ie, a 30% to 60% reduction in coronary blood flow as experienced by stable angina patients) is a reduction in oxygen delivery to the tissue resulting in a decrease in ATP production. During ischemia, fatty acid and pyruvate oxidation both decrease as glycolysis becomes the predominant route for ATP production. However, as oxygen provision declines, the pyruvate produced from glycolysis cannot be fully oxidized by the tricarboxylic acid (TCA) cycle and is reduced to lactate that can further uncouple glycolysis from pyruvate oxidation. Glycolytic regeneration of ATP leads to intracellular acidosis via the production of cytosolic protons that in turn activate the sodium-hydrogen exchanger and sodium-calcium exchanger leading to intracellular calcium overload and contractile dysfunction. Ischemic injury elevates plasma catecholamine concentrations leading to activation of hormone sensitive lipase (HSL) resulting in an increase in plasma free fatty acid (FFA) concentrations (typically 0.8 to 1.4 mmol/L). On reperfusion, fatty acid oxidation becomes the predominant source of ATP production and the concomitant increase in reduced nicotinamide adenine dinucleotide (NADH):oxidized nicotinamide adenine dinucleotide (NAD⁺) and acetyl Coenzyme A (CoA):CoA ratios negatively feedback and inhibit the pyruvate dehydrogenase complex (PDC). This further uncouples glycolysis from pyruvate oxidation and exacerbates the intracellular calcium overload and contractile dysfunction.

Hypothetically, therefore, therapeutic benefit in IHD and stable angina may derive from agents that can recouple glycolysis and pyruvate oxidation and hence normalize myocardial metabolism. Several agents have been discovered that achieve this via different mechanisms: eg, PDC activation using dichloroacetate (DCA), modifiers of the mitochondrial acetyl CoA:CoA ratio, and carnitine palmitoyltransferase I (CPT-I) inhibitors.

The most recently discovered group of agents proposed to modulate myocardial metabolism are the substituted piperazines.
zine compounds trimetazidine (1-[2,3,4-trimethoxybenzyl]piperazine dihydrochloride) and ranolazine ((±)-N-(2,6-dimethylphenyl)-4-[2-hydroxy-3-(2-methoxyphenoxy)-propyl]-1-piperazineacetamide). Trimetazidine reduces intracellular acidosis during low-flow simulated ischemia and is cardioprotective in in vitro models of ischemic injury. It has been proposed that these effects derive from inhibition of the mitochondrial LC 3-KAT, consequent inhibition of β-oxidation and re-coupling of glucose metabolism.

β-Oxidation consists of four enzymatic steps. An initial acyl CoA dehydrogenase produces substrate for entry into the trifunctional protein complex (TFP). TFP consists of a multimeric α- and β-subunit complex. The α-subunit contains the enoyl CoA hydratase and L-3-hydroxyacyl CoA dehydrogenase activities, whereas the β-subunit contains the LC 3-KAT activity (Figure 1).

Several double-blinded clinical trials have shown a substantial benefit with trimetazidine in patients with stable angina, at least equivalent to propranolol and nifedipine and additive in combination with diltiazem. The efficacy of trimetazidine is considered different from traditional anti-anginal therapy in that benefit is achieved without any hemodynamic effects.

Trimetazidine’s mechanism of action remains ill-defined, with (1) direct effects on cardiac sodium current, (2) reducing the production of superoxide free radicals, (3) synthesis and turnover of complex lipids, and (4) binding to a mitochondrial transition pore binding site all having been subject to investigation. However, the present view is that trimetazidine directly inhibits LC 3-KAT and acts as a metabolic modulator.

The antianginal agent ranolazine, described as a partial fatty acid oxidation inhibitor (pFOXi), also appears to act as a metabolic modulator. Ranolazine exerts beneficial effects in reperfused ischemic hearts by stimulating glucose oxidation as a consequence of reducing fatty acid oxidation. More recently, ranolazine has demonstrated antiangiial efficacy alone and in combination with existing therapy, without any deleterious alterations in rate pressure product or coronary blood flow. Despite being termed a pFOXi, the exact mechanism of action of ranolazine has yet to be determined.

The purpose of this study was to assess directly whether trimetazidine and ranolazine exert their beneficial effects on mitochondrial substrate oxidation and cardiac function by selectively inhibiting LC 3-KAT. The effects of these agents were assessed on (1) the enzymatic activities of rat LC 3-KAT (purified from tissue) and human recombinant LC 3KAT (expressed and purified), (2) β-oxidation in a human myocardial cell line, and (3) the isolated working heart after a period of no-flow ischemia.

Materials and Methods

Protein Expression, Characterization, and Purification

TFP containing LC 3-KAT was purified from frozen rat heart tissue by a modification of the method described by Carpenter et al.

The mature forms of TFP-α and TFP-β were amplified by polymerase chain reaction using clones 8123703 and 4767040 (Incyte Corporation), respectively, as templates and the following primer pairs: α-forward, GGTCCGACAAAACGAGACCCATT, and α-reverse, TGCTCGAAGCTGTCCTTCGTTGTCATAGGCT; β-forward, CTGGGCCTGCCCCAGCTTGACACAAAA, and β-reverse, CGTCACTTGTCTTTTACTGGTTGATGACTTGTCTTAAGCCCT. PCR products were sequenced validated and cloned into pET bacterial expression vectors (Novagen) to include N-terminal affinity tags; S-tag for TFP-α and (His), tag for TFP-β (in pET14b) and (His), tag for TFP-β (in pET14b). BL21 (DE3) pLysS cells transformed with the TFP expression constructs were grown in LB medium supplemented with 34 μg/mL chloramphenicol plus 50 μg/mL kanamycin (pET29a_TFP-α construct) or 50 μg/mL carbenicillin (pET14b_TFP-β construct). Cells were grown at 37°C on an orbital shaker at 225 rpm, to an OD<sub>600</sub> value of 0.6. Cells were harvested by centrifugation at 10,000 g for 30 minutes at 4°C, and were resuspended in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L β-mercaptoethanol, 1% Tween-20, pH 7.6). After sonication (3×10 seconds, 4°C), insoluble material was pelleted by centrifugation at 27,000 g for 30 minutes at 4°C. The supernatant was processed using a single-step batch purification approach according to manufacturers’ instructions. TFP-α was purified with S-protein resin (Novagen) and TFP-β with NiNTA agarose (Qiagen). Proteins were exchanged into storage buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L β-mercaptoethanol, 0.5% Tween-20, 30% glycerol, pH 7.0) using PD-10 columns (Amersham Biosciences). Expression of TFP subunits was confirmed through analysis using 4% to 20%
SDS-PAGE followed by Western analysis using antibodies immuno-
reactive with the affinity tags. Reconstitution of the recombinant hu-
mant human TFP-α/β complex was achieved by mixing equal amounts of
each protein and incubation at 4°C overnight.

**Biochemical Analysis**

The enzymatic activity of LC 3-KAT was assayed using two
methods from (1) crude rat heart mitochondrial homogenate (crTFP),
(2) purified rat heart trifunctional protein (prTFP), or (3) recombi-
nant human trifunctional protein (rhTFP).

Method 1 assayed the turnover by LC 3-KAT of specific
3-ketoacyl CoA substrate that was produced in situ (Figure 1). Buffer
constituents were as described by Wanders et al. for the determi-
nation of thiolase (EC 2.3.1.16) activity without KCl and at pH 9.0.
Reactions were performed in 5-mL batches at 30°C and absorbance
changes detected using a Molecular Devices SPECTRmax PLUS
system. Palmitoyl CoA (50 μmol/L) was converted to the trans-D-enoyl form
using 0.1U/mL acyl CoA oxidase (EC 1.3.3.6) (detected as an
increase in absorption at 280 nm). RhTFP-α subunit was added, and
the enoyl CoA hydratase (EC 4.2.1.7) activity measured as a
decrease in absorption at 280 nm. NAD (2 mmol/L) was added and
the 1-3-hydroxyacyl CoA dehydrogenase (EC 1.1.1.35) activity
measured as an increase in absorbance at 340 nm until the change
in absorbance became asymptotic (typically equivalent to 40 to 45
μmol/L 3-ketoacyl CoA). The mixture was boiled for 30 seconds to
denature proteins and centrifuged (10 minutes at 13 000g) to recover the
3-ketoacyl CoA in the supernatant. Ninety microliters of the
supernatant was added to each well of a 96-well plate. Vehicle (10
μL 1% DMSO) or compound (either trimetazidine, ranolazine, or the
positive controls, acetyl CoA or benzotript) were added after plateau of the oxidase reaction. Dehydrogenase activity
was allowed to plateau (due to product inhibition) before CoA was
added. The increase in dehydrogenase activity induced by the
addition of CoA represented LC 3-KAT activity.

All measurements for methods 1 and 2 were made when the rates
of change in OD were linear over time.

**Whole Cell-Based β-Oxidation**

Cardiomyocytes (Girardi cells: CCL 27) (ECACC) were cultured as
described previously except that cells were seeded into 12-well
plates 72 hours before use and 1 mmol/L l-carnitine was added. Plates
were washed twice with PBS (with calcium and magnesium).
Compounds (either trimetazidine, ranolazine, or oxfenicine) were
detected and preincubated for 15 minutes before the addition of
13 C-palmitate (6 mmol/L in 15% BSA) to give a final concentration
of 1.2 mmol/L palmitate. At 60 minutes, the medium was aspirated,
200 μL 0.5 mol/L perchloric acid (PCA) added to each well, and the
plates frozen at –80°C until analysis. Acetyl-CoA derived from
β-oxidation of the labeled palmitate is utilized by the cells for citrate
synthesis or incorporated into acetyl-L-carnitine. The accumulation
of 13 C-acetyl-L-carnitine was quantitated as a measure of the rate of
β-oxidation. Acetyl-L-carnitine, L-carnitine, and labeled acetyl-L-
carnitine were measured in neutralized PCA extracts using a Shi-
madzu QP8000 single quadruple LCMS. Injections (5 μL) of each
sample were analyzed for the corresponding positive parent ions of
the three species (m/z 204.1, 162.1, and 206.1, respectively) using
single ion monitoring and quantified using the area under the curve
for each ion current.

**Working Rat Heart**

All animal procedures were conducted in accordance with the
Animals (Scientific Procedures) Act 1986 and were evaluated by the
local ethical review process. Male rats at 325 to 375 g (Charles
River, UK) were anesthetized with sodium pentobarbitone (Sagatal,
Rhône Mérieux) (10 mg/100 g body weight, IP). Hearts were
excised, immersed in Krebs (4°C), and cannulated for isolated
working heart perfusions. Briefly, spontaneously beating hearts
were perfused at a 11.5 mm Hg left atrial preload and an 80 mm Hg
afterload with a modified Krebs-Henseleit solution containing
2.5 mmol/L calcium, 5.5 mmol/L glucose, 100 μU/mL insulin, and
3% BSA in the presence or absence of 1.2 mmol/L palmitate (pH
7.4), and gassed with 95% O₂/5% CO₂. Heart rate, aortic pressure, aortic flow, and coronary flow were measured.

Hearts were normally perfused for 20 minutes (37°C) for baseline recording, followed by a 20-minute global no-flow ischemic period (33°C) and a 40-minute reperfusion (37°C) period. Hearts (n = 8/group) were randomly allocated to be perfused by media containing either (1) 5.5 mmol/L glucose, (2) 5.5 mmol/L glucose and 1.2 mmol/L palmitate, (3) 5.5 mmol/L glucose and 1.2 mmol/L palmitate \(10^{-5}\)mol/L trimetazidine, (4) \(120^{-5}\)mol/L ranolazine, or (5) 1 mmol/L DCA.

**Statistical Analysis**

All data are presented as the group mean ± SEM. For the biochemical analysis, \(t\) tests were only performed at the highest compound concentration. For the cell-based assay analysis of variance (ANOVA) was used to compare treatments to vehicle using a blocked experimental design. For the working heart preparation, ANOVA was used to compare treatments while the average of the three baseline values was used as a covariate.

All reagents were purchased from Sigma Aldrich Company Ltd. Trimetazidine, ranolazine, and oxfenicine were supplied by PGRD synthetic services, whereas benzotript was purchased from ICN Biomedicals Inc.

**Results**

**Trimetazidine Does Not Inhibit LC 3-KAT**

The TFP of mitochondrial \(\beta\)-oxidation contains an \(\alpha\) and \(\beta\) subunit. The enoyl CoA hydratase and L-3 hydroxyacyl CoA dehydrogenase activities are contained in the \(\alpha\)-subunit, whereas the LC 3-KAT activity is contained in the \(\beta\)-subunit. Our aim was to assess the specific inhibition by agents of LC 3-KAT. Therefore, only fractions of crTFP, prTFP, and rhTFP that demonstrated activity for the long-chain substrate (palmitoyl CoA) but were devoid of short chain substrate (butyryl CoA) -dependent hydratase, dehydrogenase, and thiolase activity were used (data not shown).

The addition of CoA to a reaction mixture containing 3-ketoacyl CoA and either crTFP, prTFP, or rhTFP caused a decrease in absorbance at 303 nm, representing a thiolase-dependent decrease in 3-ketoacyl CoA (data not shown): the effects of compounds to inhibit LC 3-KAT was expressed as the percentage change in absorbance compared with vehicle control (Figures 3A through 3C). Trimetazidine (0.1 to 100 \(\mu\)mol/L) and ranolazine (0.1 to 100 \(\mu\)mol/L) failed to inhibit any of the preparations of LC 3-KAT. The concentration ranges for trimetazidine and ranolazine were taken from Kantor et al\(^{12}\) who report the IC \(50\) of trimetazidine as 75 \(\mu\)mol/L. In our studies, the highest concentrations used for trimetazidine and ranolazine were limited by their solubility. Acetyl CoA, however, a known inhibitor of LC 3-KAT,\(^{32}\) dose-dependently inhibited all three LC 3-KAT activities with equal potency (\(P<0.05\) at 2 \(\mu\)mol/L).

\(\beta\)-Oxidation is a highly adaptive and tightly regulated process sensitive to small changes in concentrations of all components of the pathway. Because our first experiments focused on the thiolase and final step of the pathway, we also assessed the ability of trimetazidine and ranolazine to inhibit LC 3-KAT as a functional component of intact \(\beta\)-oxidation (Figure 4). The addition of CoA caused a shift in the equilibrium of the dehydrogenase reaction by removing the product inhibition of 3-ketoacyl CoA on the L-3-hydroxyacyl CoA dehydrogenase enzyme. LC 3-KAT activity was assayed as an increase in dehydrogenase activity, and the effects of compounds expressed as a percentage of the control group. Trimetazidine (0.1 to 100 \(\mu\)mol/L) and ranolazine (0.1 to 100 \(\mu\)mol/L) did not inhibit LC 3-KAT; however, acetyl CoA (0.03 to 2 \(\mu\)mol/L) did significantly inhibit LC 3-KAT in a dose-dependent manner (\(P<0.05\) at 2 \(\mu\)mol/L). Benzotript
(0.3 to 2 mmol/L), a known inhibitor of \(\beta\)-oxidation\(^3\) used as a positive control to validate the methodology, dose-dependently inhibited LC 3-KAT (\(P<0.05\) at 2 mmol/L) (Figure 4).

**Trimetazidine Does Not Inhibit \(\beta\)-Oxidation in Cardiomyocytes**

The effects of compounds on flux through \(\beta\)-oxidation was assessed using intact human cardiomyocytes and quantified by analysis of the \(^{13}\)C-palmitate–dependent accumulation of \(^{13}\)C-acetyl-L-carnitine (Figure 5 and Table 1). The formation of \(^{13}\)C-acetyl-L-carnitine was shown to be solely dependent on \(^{13}\)C-palmitate by demonstrating zero production of \(^{13}\)C-acetyl-L-carnitine in the presence of \(^{12}\)C-palmitate (data not shown). The addition of 1.2 mmol/L \(^{13}\)C-palmitate caused an accumulation of 235±6 pg/\(\mu\)L \(^{13}\)C-acetyl-L-carnitine after 60 minutes. Preincubation with trimetazidine (10 to 300 \(\mu\)mol/L)

**Figure 4.** Effect of trimetazidine, ranolazine, and acetyl CoA on LC 3-KAT activity from purified rat heart TFP using method 2. *\(P<0.05\) vs control; \(n=3\) for all determinations.

**Figure 5.** Effect of trimetazidine (TMZ), ranolazine, and oxfenicine (OX) on \(^{13}\)C-acetyl-L-carnitine accumulation in Girardi cells. *\(P<0.05\) vs vehicle; \(n=9\) for vehicle and \(n=3\) for compounds.

**TABLE 1.** Effects of Trimetazidine, Ranolazine, and Oxfenicine on \(^{13}\)C-Acetyl-L-Carnitine Accumulation in Girardi Cardiomyocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean, pg/(\mu)L</th>
<th>Difference to Vehicle, pg/(\mu)L</th>
<th>95% Confidence Intervals</th>
<th>(P) Value Versus Vehicle</th>
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</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>235</td>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>100 (\mu)mol/L trimetazidine</td>
<td>258</td>
<td>23</td>
<td>(-3, 49)</td>
<td>0.077</td>
</tr>
<tr>
<td>30 (\mu)mol/L ranolazine</td>
<td>233</td>
<td>(-2)</td>
<td>(-28, 23)</td>
<td>0.843</td>
</tr>
<tr>
<td>100 (\mu)mol/L ranolazine</td>
<td>207</td>
<td>(-28)</td>
<td>(-53, 2)</td>
<td>0.036</td>
</tr>
<tr>
<td>300 (\mu)mol/L ranolazine</td>
<td>163</td>
<td>(-72)</td>
<td>(-97, 46)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10 (\mu)mol/L oxfenicine</td>
<td>77</td>
<td>(-158)</td>
<td>(-184, -133)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are for comparison of treatments to vehicle using one-way ANOVA adjusted for 3 data blocks; \(n=3\) for all treatments and \(n=9\) for vehicle.
Figure 6. Effect of 1.2 mmol/L palmitate: trimetazidine/ranolazine/DCA on recovery of cardiac function in isolated working rat heart. Values are mean±SEM with 8 hearts per treatment group. Hearts were subjected to 20-minute global ischemia followed by reperfusion. All compounds had a significant effect (*P<0.001) on cardiac work after ischemia compared with the control group glucose+palmitate.

had no effect on the accumulation of 13C-acetyl-L-carnitine. Preincubation with ranolazine (10 to 300 μmol/L), however, caused a dose-dependent inhibition of the accumulation of 13C-acetyl-L-carnitine. Ranolazine (100 μmol/L) reduced β-oxidation of 13C-palmitate by ~12% (*P<0.05), whereas 300 μmol/L ranolazine caused a ~30% attenuation (*P<0.001). The selective CPT-I inhibitor, oxfenicine, shown previously to inhibit β-oxidation,34 was used as a positive control to validate the assay and detection systems. Oxfenicine (10 μmol/L) significantly attenuated the accumulation of 13C-acetyl-L-carnitine by ~67% (*P<0.001), confirming that this assay system is sensitive to inhibitors of mitochondrial fatty acid metabolism.

**Trimetazidine Improves Cardiac Function**

Isolated working rat hearts were subjected to a 20-minute period of global no-flow ischemia. The recovery of cardiac function in the presence of trimetazidine (10 μmol/L), ranolazine (20 μmol/L), and DCA (1 mmol/L) was followed during a 40-minute reperfusion period (Figure 6 and Table 2). There was no significant difference (*P=0.926) in cardiac function during the preischemic baseline period between any of the treatment groups (data not shown). After a 20-minute period of no-flow ischemia, isolated hearts that were perfused with a buffer containing 5.5 mmol/L glucose recovered to values similar to those before ischemia. However, the addition of 1.2 mmol/L palmitate significantly depressed the posts ischemic recovery of cardiac function by ~70%. None of the compounds significantly changed heart rate, peak systolic pressure, developed pressure, cardiac output, or cardiac work during the preischemic period (data not shown). All three agents, however, significantly improved the recovery of cardiac function during the reperfusion period in the presence of 1.2 mmol/L palmitate (*P<0.001) for area under the curve [AUC] values) (Table 2). At the end of the reperfusion period, hearts perfused with trimetazidine, ranolazine, or DCA recovered to a degree that was not significantly different to control hearts perfused in the absence of 1.2 mmol/L palmitate (*P>0.05) (Figure 6). These data are in agreement with previous literature for trimetazidine9 and ranolazine35 under similar conditions, confirming that both trimetazidine and ranolazine are cardioprotective after exposure to a period of ischemia even under conditions of high circulating concentrations of FFA.

**Discussion**

The effectiveness of trimetazidine as an antianginal agent is undisputed: trimetazidine demonstrates equivalent antianginal efficacy to current therapy and additive efficacy in combination with current antianginal agents. Trimetazidine is also different from traditional antianginal therapy in that efficacy is not associated with any hemodynamic effects.13–16 However, the mechanism of trimetazidine’s action is unresolved. Recently, Kantor et al12 suggested that trimetazidine selectively inhibited LC 3-KAT and thereby reduced fatty acid oxidation. The aim of this study was to assess whether LC 3-KAT is the pharmacological target for trimetazidine, and to ascertain whether ranolazine, also referred to as a metabolic modulator or pFOXi, acts similarly. Importantly, our data demonstrate that trimetazidine and ranolazine are not inhibitors of LC 3-KAT.

**TABLE 2. Effects of Trimetazidine, Ranolazine, and DCA on the Recovery of Cardiac Function in Isolated Working Rat Hearts After a 20-Minute Period of Global Ischemia**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean AUC</th>
<th>Difference to Vehicle</th>
<th>95% Confidence Intervals</th>
<th>P Value Versus Vehicle</th>
</tr>
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<tr>
<td>Vehicle control</td>
<td>2788</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Palmitate control</td>
<td>872</td>
<td>−1916</td>
<td>−2589,−1243</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10 μmol/L trimetazine</td>
<td>2313</td>
<td>−475</td>
<td>−1150, 200</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>20 μmol/L ranolazine</td>
<td>2158</td>
<td>−630</td>
<td>−1302, 42</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1 mmol/L DCA</td>
<td>2475</td>
<td>−313</td>
<td>−986, 360</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are expressed as the AUC for the recovery period in comparison to a vehicle group in the absence of palmitate using one-way ANOVA. Average of the 3 baseline values was used as a covariate; n=8 for all groups.
We used recombinant human trifunctional protein complex in addition to a crude mitochondrial homogenate and protein isolated and purified from frozen rat heart tissue to assess the ability of these agents to inhibit LC 3-KAT. The assay systems used were confirmed to be measuring LC 3-KAT activity because they were CoA- and 3-ketoacyl CoA-dependent. We were unable to inhibit LC 3-KAT in any form with either trimetazidine or ranolazine over a wide concentration range (Figures 3 and 4). All forms of LC 3-KAT were, however, dose-dependently inhibited by acetyl CoA. Acetyl CoA serves as a sensor for the β-oxidation pathway that will inhibit the continued flux through this system when products are plentiful. Thus, our ability to demonstrate a dose-dependent inhibition of all three forms of LC 3-KAT with acetyl CoA and the fact that this inhibition is dependent on the presence of CoA, confirms the assay is sensitive to agents that inhibit this particular enzyme. Additionally, we were able to detect inhibition of flux through β-oxidation by using the known inhibitor of β-oxidation, benzotript, further confirming that our assay system was sensitive to inhibition. Therefore, we have demonstrated (and in more than one species) that neither trimetazidine nor ranolazine exert their cardioprotective effects by selectively inhibiting LC 3-KAT. This directly contrasts with the published findings of Kantor et al.12

We therefore examined whether trimetazidine or ranolazine act to inhibit β-oxidation via a different mechanism in a whole human cardiomyocyte assay. Girardi human atrial cells preferentially utilize glycolytic substrates. However, by incubating these cells with t-carnitine for 72 hours, we demonstrated, in a similar manner to Molstad et al.28 that substrate preference is shifted from a glycolytic to a fatty acid source. The assay assessed the overall flux through β-oxidation by monitoring the linear oxidation of [1-13C]palmitate to [1-13C]-acetyl-l-carnitine over a 60-minute time period. We used the standard CPT-I inhibitor, oxfenicine, to demonstrate the assay system was capable of detecting inhibitors of β-oxidation. As illustrated in Figure 5 and Table 1, trimetazidine did not inhibit the accumulation of [U-13C]-acetyl-l-carnitine up to concentrations of 300 μmol/L, demonstrating that in addition to not inhibiting LC 3-KAT, trimetazidine was unable to inhibit any other component of the β-oxidation pathway as present in these cells. The pFOXi ranolazine did, however, demonstrate a partial inhibition of this process in a dose-dependent manner, albeit at higher concentrations than those associated with improvement in cardiac function in isolated organ preparations.36

Having demonstrated a lack of effect in whole cells, we then used the isolated working rat heart model to assess cardiac function in the presence of these agents. By using a modest period of global no-flow ischemia, coupled with a perfusion media that contains a high concentration of FFA (1.2 mmol/L palmitate), we have reproduced key elements of the pathway associated with cardiac ischemia in vivo. As a result, recovery of cardiac function, in this case expressed as cardiac work, was reduced by ≈70% compared with that of hearts exposed to buffer containing zero FFA. In this model, therefore, cardiac dysfunction is driven by the presence of high concentrations of FFA. As such, it provides an excellent system for investigating agents thought to improve function by affecting fatty acid metabolism. This experimental paradigm was validated by demonstrating that DCA, a metabolic modulator known to increase pyruvate oxidation via activation of PDC, improved the recovery of function to control levels (in the absence of FFA). We have demonstrated that trimetazidine and ranolazine significantly improve the recovery of function to a similar extent as DCA, with no deleterious impact on heart rate or coronary blood flow. These data, therefore, suggest that trimetazidine and ranolazine are exerting their benefit by some mechanism other than causing coronary vasodilatation. It is acknowledged that concentrations of trimetazidine used in our working heart studies were in excess of free plasma concentrations necessary to achieve anti-anginal benefit in the clinic. These concentrations, however, are in agreement with previously published studies.9,35

Our data suggest, therefore, that the clinical efficacy associated with trimetazidine is associated with a mechanism of action unrelated to inhibition of any part of the β-oxidation pathway, although various hypotheses exist in the literature. For example, others have suggested effects on intracellular calcium homeostasis through binding of trimetazidine to the mitochondrial permeability transition pore,26 although there is very little data to support this.

In summary, we demonstrate that although ranolazine may assist in the recovery of cardiac function after ischemia by acting as a pFOXi, it is not via inhibition of LC 3-KAT. Additionally, and in contrast to the findings of Kantor et al,12 we demonstrate that although trimetazidine does improve the recovery of cardiac function after a period of ischemia, it does not achieve this through inhibition of LC 3-KAT nor does it interfere with any component of β-oxidation that we have assayed: pure and crude enzyme preparations and whole cell β-oxidation. Trimetazidine and ranolazine failed to demonstrate inhibitory activity of LC3-KAT at concentrations 10-fold greater than the concentrations used to demonstrate functional benefit in the working heart model. Further studies will be required to establish molecular targets for the significant antianginal effects of trimetazidine and ranolazine.

Acknowledgments

This work was funded by Pfizer Global Research and Development. The authors are grateful to Dr Bruce Middleton for his helpful, practical advice on purifying the trifunctional protein and discussion about its physiological role, Stephen Ballard for advice with all aspects of the biochemical analysis, Diane Harbison for her assistance with identification of trifunctional protein Incyte clones, David Fox, David Bull, Mel Glossop, and Dannielle Roberts for synthesis of reagents, and to Katrina Todd for her statistical planning and analysis of all experimental data.

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The Antianginal Agent Trimetazidine Does Not Exert Its Functional Benefit via Inhibition of Mitochondrial Long-Chain 3-Ketoacyl Coenzyme A Thiolase

Alan MacInnes, David A. Fairman, Peter Binding, Jo ann Rhodes, Michael J. Wyatt, Anne Phelan, Peter S. Haddock and Eric H. Karran

_Circ Res._ published online July 17, 2003;

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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