Response to Research Commentary

Beneficial Effects of Trimetazidine in Ex Vivo Working Ischemic Hearts Are Due to a Stimulation of Glucose Oxidation Secondary to Inhibition of Long-Chain 3-Ketoacyl Coenzyme A Thiolase

Gary D. Lopaschuk, Rick Barr, Panakkezhum D. Thomas, Jason R.B. Dyck

Abstract—High rates of fatty acid oxidation in the heart and subsequent inhibition of glucose oxidation contributes to the severity of myocardial ischemia. These adverse effects of fatty acids can be overcome by stimulating glucose oxidation, either directly or secondary to an inhibition of fatty acid oxidation. We recently demonstrated that trimetazidine stimulates glucose oxidation in the heart secondary to inhibition of fatty acid oxidation. This inhibition of fatty acid oxidation was attributed to an inhibition of mitochondrial long-chain 3-ketoacyl CoA thiolase (LC 3-KAT), an enzyme of fatty acid β-oxidation. However, the accompanying Research Commentary of MacInnes et al suggests that trimetazidine does not inhibit cardiac LC 3-KAT. This discrepancy with our data can be attributed to the reversible competitive nature of trimetazidine inhibition of LC 3-KAT. In the presence of 2.5 μmol/L 3-keto-hexadecanoyl CoA (KHC0A), trimetazidine resulted in a 50% inhibition of LC-3-KAT activity. However, the inhibition of LC 3-KAT could be completely reversed by increasing substrate (3-keto-hexadecanoyl CoA, KHCoA) concentrations to 15 μmol/L even at high concentrations of trimetazidine (100 μmol/L). The study of MacInnes et al was performed using concentrations of 3K-HCoA in excess of 16 μmol/L, a concentration that would completely overcome 100 μmol/L trimetazidine inhibition of LC 3-KAT. Therefore, the lack of inhibition of LC 3-KAT by trimetazidine in the MacInnes et al study can easily be explained by the high concentration of KHCoA substrate used in their experiments. In isolated working hearts perfused with high levels of fatty acids, we found that trimetazidine (100 μmol/L) significantly improves functional recovery of hearts subjected to a 30-minute period of global no-flow ischemia. This occurred in the absence of changes in oxygen consumption resulting in an improved increase in cardiac efficiency. Combined with our previous studies, we conclude that trimetazidine inhibition of LC 3-KAT decreases fatty acid oxidation and stimulates glucose oxidation, resulting in an improvement in cardiac function and efficiency after ischemia. The full text of this article is available online at http://www.circresaha.org. (Circ Res. 2003;93:e33-e37.)

Key Words: 3-ketoacyl coenzyme A thiolase ■ glucose oxidation ■ fatty acid oxidation ■ ischemia ■ trimetazidine

High rates of fatty acid oxidation are an important contributor to myocardial ischemic injury. A combination of high levels of circulating fatty acids1 and alterations in the subcellular control of fatty acid oxidation2-3 can result in 80% to 100% of the mitochondrial oxidative metabolism during and after ischemia originating from fatty acid oxidation.4-6 Unfortunately, this occurs at the expense of glucose oxidation. An ischemic-induced acceleration of glycolysis combined with this decrease in glucose oxidation results in an imbalance between glycolysis and glucose oxidation.7-8 If glycolysis is not coupled to glucose oxidation, the metabolic by-products are lactate and cytoplasmic protons.7-9 Contrasting this, if glycolysis is coupled to glucose oxidation, neither lactate or protons are produced.7-9 Because an imbalance between glycolysis and glucose oxidation increases the production of protons in the ischemic heart, and because coronary flow is diminished at this time, protons accumulate resulting in an increase in intracellular acidosis.9 These protons exchange for other cations, and can lead to increases in intracellular Na+ and Ca2+ (see review10). The need to use ATP to reestablish H+, Na+, and Ca2+ homeostasis leads to a decrease in cardiac efficiency, as ATP is used to re-establish ion homeostasis instead of supporting contractile function. Intracellular proton accumulation also directly decreases the efficiency of the contractile proteins, which also contributes to a decrease in cardiac efficiency.11 This decrease in cardiac efficiency occurs at a time when the heart is starved of energy. As a result of these two actions, proton accumulation can be an important contributor to contractile failure and myocardial injury during ischemia.8,9
Because fatty acid inhibition of glucose oxidation decreases cardiac efficiency and contributes to ischemic injury, a therapeutic goal of metabolic modulation is to either inhibit fatty acid oxidation and/or stimulate glucose oxidation. This can be achieved with a number of agents, including trimetazidine,12 ranolazine,13 dichloroacetate,7–9 etomoxir,6 and oxefunicine.14

Trimetazidine is the first clinically used antiangiinal agent that has a mechanism of action that can be attributed to an optimization of energy metabolism. Several clinical studies have also shown that trimetazidine has clinical efficacy in the treatment of various clinical forms of ischemia, including angina pectoris,15–16 and acute coronary syndromes.19,20 The beneficial effects of this agent have been attributed to an inhibition of fatty acid oxidation. Fantini et al21 has shown in isolated rat heart mitochondria that trimetazidine is a potent inhibitor of palmitoyl carnitine oxidation, with no significant effect on pyruvate oxidation. We have also reported that trimetazidine inhibits fatty acid oxidation and stimulates glucose oxidation in isolated working rat hearts.22 During and after ischemia, stimulation of glucose oxidation would be expected to decrease proton production, which is supported by the observation that trimetazidine reduces intracellular acidosis during ischemia.12

Our earlier studies have shown that trimetazidine inhibits the β-oxidative enzyme, long-chain 3-ketoacyl CoA thiolase (LC 3-KAT), and have suggested that this is the mechanism by which trimetazidine inhibits fatty acid oxidation.22 However, the research commentary by MacInnes et al23 (see accompanying article) suggests that trimetazidine is neither a LC 3-KAT inhibitor, nor an inhibitor of fatty acid oxidation in isolated cardiac cells and concludes that trimetazidine is not a metabolic modulator. Although the work by MacInnes et al supports the data demonstrating that trimetazidine improves cardiac function after ischemia, it raises doubt as to the role trimetazidine plays in the inhibition of the β-oxidation pathway.

In this article, we provide data to support our original conclusion that trimetazidine is a LC 3-KAT inhibitor, and suggest that the lack of inhibition of LC 3-KAT by trimetazidine in the MacInnes et al study23 can easily be explained by the high concentration of 3-keto-hexadecanoyl CoA (KHC0A) substrate used in their experiments.

Materials and Methods

Mitochondrial Membrane Preparation
Male Sprague-Dawley rats were provided by BioScience Animal Services (Edmonton, Alberta, Canada). The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with National Institutes of Health animal care guidelines. Mitochondria were prepared from freshly excised rat hearts by differential centrifugation. Briefly, rat hearts were minced, rinsed with the 5 mmol/L tris buffer (pH 7.4) containing 0.25 mol/L sucrose and 1 mmol/L EDTA (buffer A), and homogenized with a Polytron homogenizer. The homogenate was centrifuged at 800 g for 10 minutes, and the supernatant was centrifuged again at 6000 g for 15 minutes. The resulting mitochondrial pellet was resuspended in buffer A, frozen in liquid nitrogen, thawed, sonicated for 15 seconds, and centrifuged at 100 000 g for 1 hour. The pellet containing the mitochondrial membrane was resuspended in buffer A and was used in subsequent LC 3-KAT assays. Protein was assayed using Bio Rad Protein Assay Reagent.

Spectrophotometric Assay of LC 3-KAT Activity

Long-chain 3-ketoacyl CoA thiolase in the mitochondrial membrane fraction was measured using 3-kettohexadecanoyl CoA as a substrate.22,24 The assay contained either 2.5 μmol/L, 5 μmol/L, 10 μmol/L, or 15 μmol/L 3-keto-hexadecanoyl CoA, sonicated mitochondrial membrane fractions and 75 μmol/L coenzyme A in 100 mmol/L tris buffer, pH 8.0, containing 25 mmol/L MgCl₂ and 50 mmol/L KCl. The reaction was started by the addition of coenzyme A after a 3-minute preincubation (with the rest of the assay components) and followed by monitoring the decrease in absorbance at 303 nm at 25°C. The reaction rate for the first 10 or 15 seconds was used for the calculation of kinetic parameters.

Isolated Working Heart Perfusions

Hearts were excised from pentobarbital (60 mg/kg) anesthetized male Sprague-Dawley rats (250 to 300 g) and cannulated as working hearts as described previously.2,22 After equilibration, hearts were switched to the working mode and perfused with a modified Krebs-Henseleit solution containing 5 mmol/L glucose, 2.5 mmol/L free calcium, 100 μU/mL insulin, 3% fatty acid free bovine serum albumin in the presence of 1.2 mmol/L palmitate. Hearts were allowed to beat spontaneously and were perfused at a constant left atrial preload (11.5 mm Hg) and a constant aortic afterload (80 mm Hg). Hearts were subjected to a 30-minute period of aerobic perfusion, followed by 30 minutes of global no-flow ischemia, and 60 minutes of aerobic reperfusion. Mechanical function (heart rate, peak systolic pressure, and maximum developed pressure), heart output, aortic flow, and coronary flow were measured with either in-line pressure transducers or flow probes, as described previously.22 Myocardial O₂ consumption (MV0₂) was determined with in-line oxygen probes in the preload and the cannulated pulmonary arterial line. Cardiac efficiency was defined as the ratio of cardiac work to O₂ consumption. Hearts were maintained at 37°C during the entire perfusion period.

Statistical Analysis

All data are represented as the group mean ± SEM. Data were analyzed using the statistical program Instat. One-way ANOVA was used to compare values between control and trimetazidine-treated hearts, as well as between different control groups. A Tukey-Grammar post hoc test was used to confirm intergroup differences. A value P < 0.05 was considered significant.

Results

Effects of Trimetazidine on Long-Chain 3-Ketoacyl CoA Thiolase Activity

In a previous study,22 we demonstrated that trimetazidine resulted in an approximately 40% inhibition of LC 3-KAT activity, with an IC₅₀ of 75 nmol/L. However, the recent research commentary of MacInnes et al23 reported that trimetazidine was unable to inhibit crude and purified rat heart or recombinant human LC 3-KAT activity. This lack of effect was observed even in the presence of 100 μmol/L trimetazidine. It should be noted, however, that the concentration of the 3-keto-hexadecanoyl CoA (3-KHC0A) substrate used in their study was in excess of 16 μmol/L. Because the relationship between trimetazidine, LC 3-KAT, and 3-KHC0A is unknown, we determined what effect 100 μmol/L trimetazidine had on rat heart mitochondrial membrane LC 3-KAT activity at varying concentrations of 3-KHC0A (Figure 1). Confirming our earlier results, we demonstrated that at 100 μmol/L trimetazidine, inhibition of LC 3-KAT could be overcome by increasing substrate con-
centration of the enzyme to 15 μmol/L. This finding is consistent with trimetazidine being a competitive inhibitor of LC 3-KAT. It should be noted in the MacInnes et al study that 16 μmol/L of the substrate was used, which is well above the concentration needed for complete reversal of 100 μmol/L trimetazidine inhibition of LC 3-KAT activity.

Effect of Trimetazidine on Fatty Acid Oxidation
In an earlier study, we reported that trimetazidine inhibits fatty acid oxidation in isolated working rat hearts, and that this is accompanied by an increase in glucose oxidation.22 This contrasts the data in the Research Commentary by MacInnes et al,23 in which trimetazidine did not alter fatty acid oxidation in cultured Girardi cells. Because MacInnes et al did not measure fatty acid oxidation rates in the working heart, a direct comparison to our earlier data cannot be performed. However, we can comment on the potential problems with the MacInnes et al report. For example, one of the problems with measuring fatty acid oxidation rates in cultured cells is that there is considerably lower metabolic demand in isolated cells compared with the intact working heart. In addition, the technique used by MacInnes et al23 to measure fatty acid oxidation followed the accumulation of 13C-acetylcarnitine, which is an indirect approach to measure fatty acid oxidation. Because acetylcarnitine production by the cell is dependent on factors other than just fatty acid oxidation rates, this technique may provide spurious results. As a result, we do not agree with the conclusions of MacInnes et al that trimetazidine does not inhibit fatty acid oxidation.

Cardioprotective Actions of Trimetazidine in the Ischemic/Reperfused Heart
We have previously shown that stimulation of glucose oxidation can overcome the detrimental effects of high levels of fatty acids in the ischemic heart. We therefore examined what effect trimetazidine has on functional recovery of ischemic hearts subjected to a 30-minute period of global ischemia (Figure 2). A significant improvement in the recovery of cardiac work after ischemia was observed in the presence of trimetazidine. DCA also resulted in an improved recovery of cardiac work. These data are consistent with previous studies,6-9 as well as the attached study of MacInnes et al,23 in which both trimetazidine and DCA could reverse the detrimental effects of high levels of fatty acids.

Consistent with our previous studies, in the presence of high levels of fatty acids, cardiac efficiency was markedly decreased during reperfusion (Figure 2C) due to a maintained...
Discussion

Inhibition of LC 3-KAT by Trimetazidine

We have previously shown that trimetazidine directly inhibits fatty acid oxidation in the heart, secondary to an inhibition of mitochondrial long-chain 3-ketoacyl CoA thiolase. Data to support this mode of action of trimetazidine included the following: (1) demonstration that trimetazidine inhibits mitochondrial membrane LC 3-KAT at clinically relevant concentrations; (2) trimetazidine inhibits fatty acid oxidation in isolated working rat hearts at similar concentrations; (3) trimetazidine does not inhibit medium- or short-chain fatty acid oxidation, which is consistent with a specific inhibition if LC 3-KAT. We also observed that inhibition of long-chain fatty acid oxidation was accompanied by an increase in pyruvate dehydrogenase activity, resulting in a significant increase in glucose oxidation rates. Although these observations are challenged by MacInnes et al (see accompanying Research Commentary), we show that the lack of trimetazidine inhibition of LC 3-KAT observed in their studies could be explained by the high concentrations of substrate used in their LC 3-KAT assay. Using similar concentrations of trimetazidine as MacInnes et al, we confirm that trimetazidine inhibits LC 3-KAT at low concentrations of LC 3-KAT substrate (KHCoA), but that this inhibition of LC 3-KAT is reversed at increasing concentration of KHCoA. This is entirely consistent with trimetazidine being a reversible competitive inhibitor of LC 3-KAT. It is noteworthy that MacInnes et al used a substrate concentration in excess of 16 μmol/L, a concentration that would be expected to completely reverse trimetazidine inhibition of LC 3-KAT. At this high concentration of substrate, we have never been able to inhibit LC 3-KAT with any concentration of trimetazidine. This is well above the Km for LC 3-KAT. Although the actual concentration of LC 3-KAT substrate in vivo is not known, what is clear is that it is much lower than 16 μmol/L (in most cases is undetectable).

Fatty Acid Oxidation Inhibition by Trimetazidine

Our demonstration that trimetazidine inhibits long-chain fatty acid oxidation, but not medium- or short-chain fatty acid oxidation, in the intact heart supports LC 3-KAT being the mechanism of action of trimetazidine, because shorter chain fatty acids are primarily metabolized by the mitochondrial matrix medium- and short-chain 3-KAT. Trimetazidine inhibition of fatty acid oxidation is also consistent with the studies of Fantini et al using isolated mitochondrial preparations. These authors demonstrated that trimetazidine could markedly reduce mitochondrial respiratory activity, but that this effect was dependent on the substrate offered to the mitochondria (ie, palmitoylcarnitine oxidation was markedly reduced, but not that of pyruvate, glutamate, or citrate). This suggests that trimetazidine is not inhibiting either TCA cycle activity or mitochondrial oxidative phosphorylation, but rather directly inhibiting fatty acid β-oxidation.

Inhibition of Fatty Acid Oxidation as an Approach to Treating Ischemic Heart Disease

In this study, we show that trimetazidine also has beneficial effects on the recovery of mechanical function and cardiac efficiency during reperfusion of ischemic hearts (Figure 2). The detrimental effects of high levels of fatty acids in the ischemic heart are prevented by trimetazidine, due to an inhibition of fatty acid oxidation, which increase glucose oxidation and improve cardiac efficiency. Stimulation of glucose oxidation improves the coupling of glycolysis to glucose oxidation during this critical period, resulting in a decreased proton production, a decrease in tissue acidosis and an improvement in cardiac efficiency. As a result, selective stimulation of glucose oxidation by trimetazidine can explain the anti-ischemic effects of this agent, probably by increasing the coupling between glycolysis and glucose oxidation, thereby decreasing proton production. Indeed, in rat hearts perfused with high levels of fatty acids, trimetazidine has been shown to attenuate the degree of acidosis during ischemia. As a result, our observations and those of MacInnes et al in the intact heart are consistent with trimetazidine, DCA, and ranolazine acting as metabolic modulators that stimulate glucose oxidation in hearts perfused with high levels of fatty acids.

Limitations of Study

Our studies examining trimetazidine effects on cardiac mitochondrial LC 3-KAT activity, as well as cardiac function and fatty acid and glucose oxidation in isolated working hearts, involved the acute administration of trimetazidine. As a result, it cannot be ruled out that the long-term administration of trimetazidine, as seen in the treatment of angina pectoris, may involve additional cardioprotective mechanisms not determined by short-term administration. This possibility remains to be determined.
The actual in vivo concentration of the substrate for mitochondrial LC 3-KAT is not known. As a result, it cannot be said with certainty as to what concentration of substrate or trimetazidine that LC 3-KAT sees in vivo. However, the low concentrations of substrate measurable in vivo support the likelihood that the concentration of substrate is low enough that it would not always override trimetazidine inhibition of LC 3-KAT. It is also possible that ischemic-induced changes in the intramitochondrial milieu (i.e., pH, ionic alterations) could alter the response of LC 3-KAT to trimetazidine inhibition. These possibilities remain to be determined.

**Summary**

Trimetazidine is a clinically effective antianginal agent that acts independent of hemodynamics. The beneficial effects of trimetazidine can be explained by an inhibition of fatty acid oxidation, secondary to an inhibition of mitochondrial long-chain 3-ketoacyl CoA thiolase. This results in an increase in glucose oxidation and an improved coupling of glycolysis with glucose oxidation, which has previously been shown to protect the ischemic heart. As a result, switching energy substrate preference from fatty acid oxidation to glucose oxidation can explain the antiischemic properties of trimetazidine, and trimetazidine can be considered a “metabolic modulator.”

**Acknowledgments**

G.D.L. is a Medical Scientist, Alberta Heritage Foundation for Medical Research (AHFMR). J.R.B.D. is a scholar of the AHFMR.

**References**

Beneficial Effects of Trimetazidine in Ex Vivo Working Ischemic Hearts Are Due to a Stimulation of Glucose Oxidation Secondary to Inhibition of Long-Chain 3-Ketoacyl Coenzyme A Thiolase
Gary D. Lopaschuk, Rick Barr, Panakkezhum D. Thomas and Jason R.B. Dyck

Circ Res. 2003;93:e33-e37; originally published online July 17, 2003;
doi: 10.1161/01.RES.000086964.07404.A5

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
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:
http://circres.ahajournals.org/content/93/3/e33

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/