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SHORT COMMUNICATION

Teriflunomide treatment exacerbates cardiac ischemia reperfusion injury in isolated rat hearts

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Abstract

Purpose Previous work suggests that Dihydroorotate dehydrogenase (DHODH) inhibition via teriflunomide (TERI) may provide protection in multiple disease models. To date, little is known about the effect of TERI on the heart. This study was performed to assess the potential effects of TERI on cardiac ischemia reperfusion injury.

Methods Male and female rat hearts were subjected to global ischemia (25 min) and reperfusion (120 min) on a Langendorff apparatus. Hearts were given either DMSO (VEH) or teriflunomide (TERI) for 5 min prior to induction of ischemia and during the reperfusion period. Left ventricular pressure, ECG, coronary flow, and infarct size were determined using established methods. Mitochondrial respiration was assessed via respirometry.

Results Perfusion of hearts with TERI led to no acute effects in any values measured across 500 pM–50 nM doses. However, following ischemia–reperfusion injury, we found that 50 nM TERI-treated hearts had an increase in myocardial infarction ($p < 0.001$). In 50 nM TERI-treated hearts, we also observed a marked increase in the severity of contracture ($p < 0.001$) at an earlier time-point ($p = 0.004$), as well as reductions in coronary flow ($p = 0.037$), left ventricular pressure development ($p = 0.025$), and the rate-pressure product ($p = 0.008$). No differences in mitochondrial respiration were observed with 50 nM TERI treatment ($p = 0.24$–0.87).

Conclusion This study suggests that treatment with TERI leads to more negative outcomes following cardiac ischemia reperfusion, and administration of TERI to at-risk populations should receive special considerations.

Keywords Dihydroorotate dehydrogenase · Infarct · Ischemia reperfusion · Teriflunomide

Introduction

Cardiac ischemia–reperfusion (I/R) injury is a major cause of death and disability worldwide, with no current effective treatment or therapeutic strategies readily available. While we understand some of the mechanisms involved in cardiac I/R injury, few strategies beyond reperfusion have been developed to decrease myocardial infarction [1, 2]. Drugs that have targeted mitochondrial pathways (e.g., ATP depletion, intracellular calcium overload, and generation of reactive oxygen species) have shown promise in animal models, suggesting that mitochondria may be a hub for decreasing I/R injury by effecting multiple signaling cascades that lead to infarction, and our previous work supports this hypothesis [3, 4].

Dihydroorotate dehydrogenase (DHODH) is an inner mitochondrial membrane protein that plays a role in linking pyrimidine biosynthesis and the electron transfer of dihydroorotate to ubiquinone in the electron transport chain (ETC) [5]. DHODH has been thought to stimulate the ETC, and subsequent ATP production, by superficially binding to the ubiquinone pocket, reducing it, and decreasing the thermodynamic requirement for electron flow to Complex III. Inhibitors of DHODH have been used successfully to treat autoimmune diseases such as rheumatoid arthritis and multiple sclerosis through a drug called teriflunomide (TERI). In addition, TERI has been explored as a potential treatment in models of pediatric epilepsy, as well as influenza and SARS-COV2 infection [6, 7]. Proposed mechanisms of protection in these models suggest a combination of antiviral, immune regulation, regulation of intracellular calcium, down-regulation of anti-apoptotic proteins, disruption of mitochondrial

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processes, and modulating cell survival signaling [8–12]. To date, little is known about the effect of DHODH inhibition within the heart or its potential effects on cardiac I/R injury. Given that similar pathways discussed above play a role in I/R injury, we sought to investigate the effects of TERI administration on cardiac I/R injury. Our findings are important in establishing that forethought regarding a patient's cardiovascular risk factors may need to be considered prior to treatment with TERI.

Methods

All animal procedures were approved by the Institutional Animal Care and Usage Committee of East Tennessee State University and in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996). Male and female Sprague–Dawley rats (175–199 g; Envigo) were used in this study.

Langendorff Preparation

Langendorff heart preparations were done similarly to the previously described [4]. Following heart excision, hearts were retrograde perfused at a constant pressure between 70 and 80 mmHg. Hearts were perfused for at least 20 min followed by 5 min of stable recordings, and 5 min–teriflunomide (TERI; N = 20) or vehicle (VEH; N = 8). Global ischemia was then induced for 25 min followed by 2-h reperfusion with either TERI or VEH. Infarct size was determined via TTC stain. Hemodynamic data was averaged into 30-s blocks for analysis. Inflection point for contracture during ischemia was defined when the rate of minimum left ventricular (LV) pressure increased by more than 1 mmHg/min for at least 90 s. Assessment of arrhythmias was performed by two independent, blinded reviewers on a scale of 0–9, with higher scores correlating with more severe arrhythmias.

Mitochondrial Oxygen Consumption

Freshly isolated mitochondria from 3 male rats were prepared from rat LV similar to established protocols [13, 14]. Briefly, samples were placed in mitochondrial isolation medium (MIM) and minced. Following homogenization, samples were then brought to volume with MIM + BSA. The homogenates were centrifuged at 800 × g for 10 min (4 °C) and supernatants subjected to centrifugation at 12,000 × g for 10 min (4 °C). The remaining pellets were washed and resuspended with MIM. 75-µg mitochondria were added to parallel chambers of an Oxygraph O2k (Oroboros). A substrate-uncoupler-inhibitor titration (SUIT) protocol was used to investigate mitochondrial oxygen consumption under specific respiratory states. After addition of mitochondria, but prior to onset of the SUIT protocol, 50 nM TERI or DMSO was added to the chamber to assess the direct effect of TERI on respiration. Subsequent additions of substrates were added to assess the contributions of Complex I and Complex II respiration followed by the uncoupler Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) to assess maximal rate of respiration.

Statistical Analysis

Data collected were first subjected to a 2-way ANOVA to determine differences between drug treatment and sex. Since there were no significant differences between sexes (p-value range 0.113–0.942) for any of the primary outcomes, data from male and female animals were pooled. A one-way ANOVA, with Fisher's LSD post-hoc was performed to determine differences between TERI doses and VEH group. For analysis of reperfusion hemodynamics, a 2-way repeated measures ANOVA (group × time) was used with Fisher's LSD post hoc to determine which time points were different. For mitochondrial respiration data, a Student's t-test was performed to determine the effect of TERI under each respiration state. $p < 0.05$ was considered significant.

Results

Prior to ischemia onset, hearts were perfused for 5 min with TERI (50 pM, 500 pM, 5 nM, 50 nM doses) or VEH to determine (1) If there are acute effects of TERI on cardiac function and (2) ensure TERI is active in hearts at reperfusion onset. Baseline data comparing VEH and 50 nM TERI are summarized in Fig. 1A. At all concentrations TERI had no effect on baseline performance (p-values 0.21–0.93). Finally, acute perfusion of TERI did not lead to the formation of cardiac arrhythmias during the baseline period ($p = 0.54$ for 50 nM TERI vs. VEH).

Within the first minute of global ischemia, the development of left ventricular pressure ceased. Although pressure development halted, the ECG signal continued to persist minutes into ischemia. The duration that the ECG signal persisted during ischemia was significantly shorter in 50 nM TERI-treated hearts (Fig. 1B; $p = 0.004$). Typically following the cessation of the ECG signal, it is common to observe a rise in the diastolic pressure, termed ischemic contracture. In the 50 nM TERI-treatment group, the maximum amount of contracture was ~ fourfold larger (19.99 vs. 60.36 mmHg; Fig. 1D; $p < 0.001$) compared to VEH treated groups. This difference was significant for 3 min during ischemia (between 6 and 9 min). In addition, the inflection point in which pressures began to rise in this dose was significantly

A

Acute effects of TERI perfusion at baseline.			
	VEH (N=8)	50 nM TERI (N=10)	p-value
Coronary Flow (mL/min)	14.9 ± 2.0	13.3 ± 1.3	0.45
LVDP (mmHg)	65.6 ± 6.6	69.0 ± 7.5	0.76
Heart Rate (bpm)	244 ± 10	228 ± 9	0.21
QT (ms)	0.042 ± 0.011	0.048 ± 0.009	0.61
QRS (ms)	0.020 ± 0.005	0.023 ± 0.005	0.57

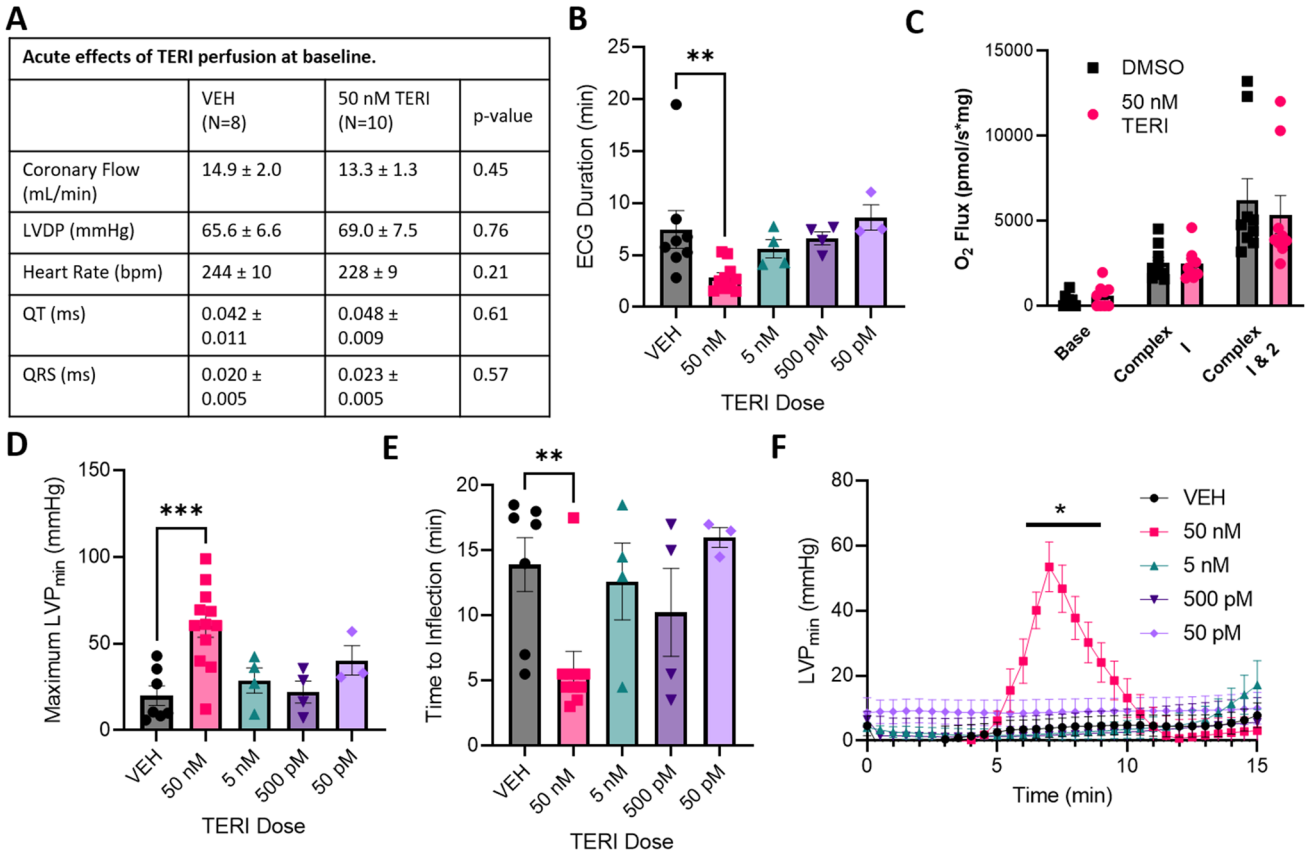

Fig. 1 Baseline and values during ischemia. There were no differences following TERI administration in any of the values measured. 50 nM data presented (**A**). The duration into ischemia that the ECG signal persisted across all doses (**B**). No differences in mitochondrial oxygen consumption were observed with the addition of 50 nM TERI (**C**). Ischemic contracture, over time, as measured by minimum left ventricular pressure (**D**). The maximal amount of contracture (**E**) and time to inflection point (**F**). *, $p \leq 0.05$ vs vehicle; **, $p \leq 0.01$; ***, $p \leq 0.001$. N's = 8 (VEH), 10 (50 nM), 4 (5 nM, 500 pM) 3 (50 pM)

earlier (Fig. 1E; $p = 0.004$). This effect on pressure during ischemia was only observed in the 50 nM TERI group.

While there was no effect on coronary flow by TERI prior to ischemia, we noticed a significant reduction in the average flow rate during the first 5 min of reperfusion in the 50 nM TERI-treated group (Fig. 2A; $p = 0.037$). At individual time points at the onset of reperfusion, this was significantly depressed between 3 and 7 min into reperfusion (Fig. 2B; $p = 0.04$). Reduction in coronary flow was only observed in the 50 nM TERI-treated group.

In 50 nM and 5 nM TERI-treated hearts, there was a significant reduction in the development of left ventricular pressures. When looking at individual time points during reperfusion ($p = 0.025$), there was significance between 9- and 29 min during reperfusion in both treatment groups (Fig. 2C). There were no significant differences in the 500 pM or 50 pM TERI groups. In addition to changes in pressure and flow, heart rate was increased at 15 min into reperfusion in 50 nM TERI-treated hearts (Fig. 2D; $p = 0.048$). Since cardiac output is a function of pressure and rate, the cardiac rate-pressure product (LVDP x HR) is a correlate measure of cardiac output and cardiac oxygen demand. Despite the significant increase in heart rate, the rate pressure product was still significantly lower in the 50 nM ($p = 0.008$) and 5 nM ($p = 0.024$) groups (Fig. 2E), suggesting that the increased heart rate was not sufficient to account for the diminished left ventricular developed pressure.

Following 2 h of reperfusion, hearts were removed, and infarct size was assessed. We found that the effects of TERI on infarct size was dose-dependent. Hearts treated with 50 nM TERI had a significantly larger amount of myocardial infarction compared to vehicle hearts ($p < 0.001$). Infarct size was not significantly increased in the 5 nM ($p = 0.15$), 500 pM ($p = 0.39$), or 50 pM ($p = 0.87$) groups (Fig. 2G).

Given the potential effects of TERI on cellular excitability, we also investigated if DHODH inhibition influenced cardiac arrhythmias. As opposed to our other measures, in the 50 nM TERI group, we observed a significant decrease in the severity of cardiac arrhythmias during the

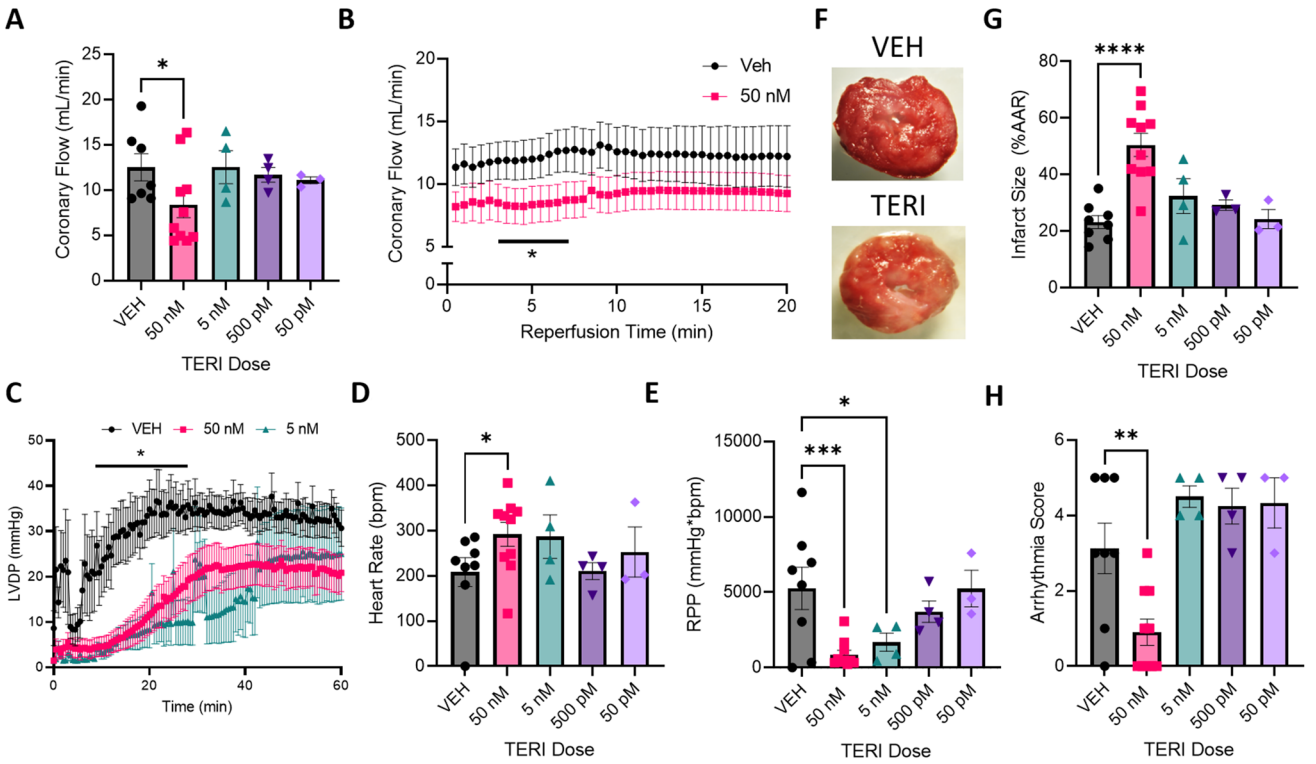

Fig. 2 Reperfusion values and myocardial infarction. Reperfusion coronary flow averaged over the first 5 min (**A**) and over time (**B**) demonstrates that coronary flow is depressed only in the 50 nM TERI-treated group. In addition, left ventricular developed pressure was depressed in the 50 nM and 5 nM TERI-treated groups (**C**), while heart rate was only increased in the 50 nM group (**D**). The rate-pressure product (**E**) remained depressed in the 50 nM and 5 nM groups. (**F**) Representative images of myocardial infarction from VEH and 50 nM TERI-treated hearts. The infarct size is significantly greater in hearts given 50 nM TERI (**G**). Arrhythmias during the first 15 min of reperfusion were significantly lower in the TERI-treated group (**H**). *, $p \leq 0.05$ vs vehicle; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. N's = 8 (VEH), 10 (50 nM), 4 (5 nM, 500 pM) 3 (50 pM)

first 15 min of reperfusion (Fig. 2H; $p = 0.0017$). In addition, the development of ventricular arrhythmias over the entire reperfusion period remained lower in this treatment ($p = 0.026$). This protection from ventricular arrhythmias was only seen in the 50 nM TERI group.

To determine if TERI had any effect on cardiac mitochondrial respiration, we performed respiration analysis on cardiac mitochondrial preparations (Fig. 1C). In the absence of any substrate addition, 50 nM TERI did not alter the rate of oxygen consumption ($p = 0.79$). Similarly, upon subsequent addition of electron chain substrates, there were no significant differences in states targeting Complex I–driven respiration (addition of pyruvate, malate, glutamate, and ADP; $p = 0.87$) or Complex II respiration (addition of succinate; $p = 0.46$, followed by rotenone; $p = 0.49$). Finally, maximal rate of oxygen consumption was assessed after addition of the uncoupler CCCP and was not significantly different in TERI-treated mitochondria ($p = 0.24$).

Discussion

This study was performed to assess the effects of DHODH (Dihydroorotate dehydrogenase) inhibition on cardiovascular outcomes during ischemia–reperfusion. Specifically, we investigated the effects of TERI, a clinically approved drug that is being explored as treatment in a variety of other diseases, using the isolated Langendorff heart model of global ischemia. This model provides the advantage of allowing us to remove potential autonomic effects and administer specific doses directly to the heart in an efficient manner, without concerns of drug absorption and metabolism. To date, there is scarce data available on the effects of TERI on cardiovascular function, underscoring the importance of these findings. Notably, we found that DHODH inhibition by TERI led to an exacerbation of the injury following global cardiac ischemia reperfusion at two different doses. Myocardial infarct size was increased

by approximately 2.2-fold (51% vs. 23%) in the highest concentration of TERI used. This increase in myocardial infarction declined as TERI dose decreased, with 50 nM being the first dose to have a significant rise in infarction. In addition, during reperfusion, we saw reductions in coronary flow, left ventricular pressure development, and the rate-pressure product across the 50 nM treatment group. The only measure where TERI improved outcomes was in our quantification of cardiac arrhythmias, suggesting that the effects of TERI may be different between cell death and cell excitability. Together this suggests that treatment with TERI may lead to more negative outcomes following cardiac ischemia reperfusion, and consideration should be given to a patient's risk of myocardial infarction.

Multiple physiological models for diseases such as multiple sclerosis (MS) and breast cancer have shown positive results from targeting DHODH because of its linkage between pyrimidine synthesis and the ETC [11, 12, 15]. DHODH inhibition in the central nervous system (CNS) models has displayed an overall diminished inflammatory response, mitigation of lymphocyte infiltration, reduced axonal loss, and preservation of neurological function [8, 11]. Many of these findings have utilized an active metabolite of the DHODH inhibitor TERI for their DHODH inhibition. The full inhibition mechanism is not completely understood, but TERI has been noted to be an uncompetitive inhibitor, and that most abundantly binds in the ubiquinone pocket [7–9].

A recent discovery by Styr et al. [7] demonstrated that the preservative effects of 50 nM TERI in neurons may provide protection in epilepsy. Their results suggest that TERI may curb neurodegeneration and reduce seizure susceptibility, potentially by regulating intracellular calcium. Given the role of both calcium and mitochondria in cardiac I/R injury, we wondered if DHODH inhibition, with a lower dose of TERI, would lead to differences in cardiac function of animals subjected to I/R injury. An important distinction of this study is that we chose doses (50 pM–50 nM) significantly lower than has been shown to inhibit mitochondrial complex V (IC50 of 35 μM) [15]. The higher doses of TERI reported by others can lead to direct inhibition of Complex V and increased burden of ER stress, but our study suggests that negative outcomes may be independent of direct inhibition of mitochondrial respiration.

Our data on mitochondrial respiration further support this conclusion, as 50 nM TERI had no effect on basal, complex I, complex II, or maximal states of respiration in the heart. Our results suggest that even in the absence of mitochondrial inhibition, TERI can lead to more adverse outcomes in cardiac I/R injury. It should be noted that our results were obtained under baseline conditions and the effect of TERI on mitochondrial respiration during the reperfusion phase was not assessed. Further analysis of mitochondrial ionic fluxes and respiration during reperfusion may provide further insights into how TERI alters cardiac performance during I/R injury.

In addition to differences in infarct size, we also saw a reduction in coronary flow during the reperfusion period in the 50 nM group. However, this reduced flow was not observed in the 5 nM group, which still had an elevated amount of tissue damage. While our previous work suggests that there is often a correlation between infarct size and coronary flow, this is not always the case [3, 16, 17]. Our results suggest that while a reduction in coronary flow may explain some of our negative outcomes (as there are reductions in the 50 nM TERI dose, with highest infarction), it is not sufficient to explain all of the mechanisms behind an increase in infarction (as no reductions in flow were seen in 5 nM group, while there was still a trend toward increased infarction).

Finally, differences were also observed in cardiac parameters during the ischemic period in the 50 nM TERI group. We observed that 50 nM treated hearts had a significantly higher and earlier rise in diastolic pressure as the heart contracted during ischemia. This ischemic contracture is thought to be triggered by a combination of low ATP levels and rising calcium, leading to contraction of cardiac muscle. After the onset of contracture, the heart "relaxed" back to baseline and no differences were observed between groups immediately prior to the onset of reperfusion. Similar to the findings of others (7), this suggests that cellular calcium handling may be affected starting at 50 nM TERI.

Conclusion

Taken together, our results demonstrate that low dose (50 nM) of TERI in isolated hearts can lead to an exacerbation of infarction and reduced performance. This is an important consideration as treatments targeting DHODH may begin expanding to a population that could possibly be at higher risk of myocardial infarction. While no baseline differences were observed with TERI treatment, once the heart experienced a challenge (global ischemia), differences emerged. This suggests that the effects of TERI on I/R injury are likely multi-factorial and involve changes in intracellular signaling in both cardiac and smooth muscle cells within the heart. The exact mechanism by which TERI affects cardiac performance in the setting of ischemia and reperfusion thus warrants a fuller investigation, as modulation of this pathway has the potential to lead to novel therapeutic avenues.

Author contribution C.R.F and E.D.A. planned and designed the work. Material (solutions) preparation, Langendorff collection, and data analysis were performed by all authors. Mitochondrial isolation and respiration experiments were performed by J.L.A. Blinded analysis of data was performed by E.D.A., J.L.A., and T.S.B. The first draft of

the manuscript was prepared by E.D.A and C.R.F. C.R.F. prepared the resubmitted manuscripts. All authors read and gave comments on all versions of the manuscript.

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Data availability Request for data used in this manuscript should be addressed to the corresponding author.

Code availability Not applicable.

Declarations

Ethics approval and consent to participate All animal procedures were approved by the Institutional Animal Care and Usage Committee of East Tennessee State University and in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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