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SHORT COMMUNICATION

Temporal association of elevations in serum cardiac troponin T and myocardial oxidative stress after prolonged exercise in rats

Jinlei Nie · Graeme Close · Keith P. George · Tom K. Tong · Qingde Shi

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Abstract The objective of this study was to determine if prolonged exercise resulted in the appearance of cardiac troponin T (cTnT) in serum and whether this was associated with elevated levels of myocardial oxidative stress. Forty-five male Sprague–Dawley rats were randomized into four groups and killed before (PRE-EX), immediately (0HR), 2 (2HR) and 24 h (24HR) after a 3-h bout of swimming with 5% body weight attached to their tail. In all animals serum cTnT was assayed using 3rd generation electrochemiluminescence. In homogenized heart tissue myocardial malondialdehyde (MDA), a marker of lipid peroxidation, glutathione (GSH), and a non-enzymatic estimate of total antioxidant capacity (T-AOC) were assessed spectrophotometrically. At PRE-EX cTnT was undetectable in all animals. At 0HR (median, range: 0.055, 0.020–0.100) and 2HR post-exercise (0.036, 0.016–2.110) cTnT was detectable in all animals ($P < 0.05$). At 24HR post-exercise cTnT was undetectable in all animals. An elevation in MDA was observed 0HR (mean ± SD: 1.7 ± 0.2 nmol mgpro^{-1}) and 2HR (1.6 ± 0.3 nmol mgpro^{-1}) post-exercise compared with PRE-EX (1.3 ± 0.2 nmol mgpro^{-1}; $P < 0.05$). The antioxidant response to this challenge was a significant ($P < 0.05$) decrease in GSH 2HR and 24HR post-exercise. Despite this T-AOC did not alter across the trial ($P > 0.05$). The results indicated that prolonged and strenuous exercise in rats resulted in an elevation in cTnT, a biomarker of cardiomyocyte damage, in all animals 0HR and 2HR after exercise completion. The time course of cTnT elevation was temporally associated with evidence of increased lipid peroxidation in the rat heart.

Keywords Cardiac biomarker · Exercise · Myocardium · Oxidative stress

Introduction

Cardiac troponins (cTnT and cTnI) are highly specific and sensitive laboratory markers of myocardial insult and are considered as the gold standard for biochemical detection of myocardial injury (Alpert et al. 2000). Numerous reports on animals (Chen et al. 2000) and humans (Nie et al. 2008; Scharhag et al. 2008; Shave et al. 2007) have observed elevated serum cTnT/cTnI after prolonged exercises that can exceed clinical cut-off value for acute myocardial infarction (Nie et al. 2010; Shave et al. 2007). The appearance of cTnT/cTnI in apparently healthy, young people participating in prolonged exercise has raised concerns about the cardiovascular health consequences of such exercise (La Gerche and Prior 2007), and this predicates the need to determine why and how these elevations occur.

The underlying mechanism(s) responsible for exercise-induced cTnT/cTnI release are unknown. Previous authors have speculated that cTnT/cTnI release during prolonged

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J. Nie (✉) · Q. Shi
School of Physical Education and Sports,
Macao Polytechnic Institute,
Rua de Luis Gonzaga Gomes, Macao, China
e-mail: jnie@ipm.edu.mo

G. Close · K. P. George
Research Institute for Sport and Exercise Sciences,
Liverpool John Moores University, Liverpool, UK

T. K. Tong
Department of Physical Education, Dr. Stephen Hui Research Centre for Physical Recreation and Wellness,
Hong Kong Baptist University, Hong Kong, China

exercise is mediated through myocardial stunning (Starnes and Bowles 1995), the ischemic development of blebs (Hickman et al. 2010; Lippi and Banfi 2010), and/or transient changes in membrane permeability (Neumayr et al. 2002). It is possible that any or all of these potential mechanisms could be related to elevated reactive oxygen species (ROS) production that occurs with prolonged exercise (Sahlin et al. 2010). The heart may be vulnerable to peroxidative damage due to oxidative stress (Kakarla et al. 2005) because it is both highly aerobic (Chance et al. 1979) whose metabolic processes produce ROS at rest and during exercise (Di Meo and Venditti 2001), and it has reduced antioxidant enzyme activity compared with other tissues (Kakarla et al. 2005). Increased levels of ROS could be harmful to the cells (Sen 2001) specifically by triggering lipid cell membrane peroxidation, which may result in myocyte cell membrane dysfunction and/or damage (Ji 2001).

Whilst it has been speculated that ROS may contribute to the cTnT/cTnI release observed following prolonged endurance exercise, to date, no studies have examined directly the role of ROS in the exercise-induced cTnT/cTnI release. Studying the impact of ROS generation on biomarker release in humans who perform prolonged exercise is clearly limited by the ability to assess tissue-specific changes (i.e. myocardial) rather than infer purely from serum biomarkers (Whyte et al. 2005). Therefore, the purpose of this study was to observe the myocardial oxidative stress and serum cTnT levels following prolonged exercise in an animal model.

Materials and methods

Animals

Male Sprague–Dawley (SD) rats ($n = 45$), weighing 210–230 g (body weight on the day before swimming), were used in these experiments. The rats were housed in cages in rooms regulated for temperature (21–23°C), humidity (40–55%), and light cycle (07:00–17:00) and provided laboratory rat chow and water ad libitum. The research involving rodents in this study conforms with the *Guidelines for Care and Use of Laboratory Animals* and was approved by the Institutional Animal Care and Use Committee.

Experimental design

To attempt to minimize the general stress response of forced swimming, all rats were familiarized with swimming for 25 min, without body weight attached to the rats, 3 days before formal swimming protocols. Ten rats were used as controls (PRE-EX) and killed at rest, and 35 rats swam for 3 h in a swimming tank with 5% body weight (workload) attached to the tail. This is similar to a protocol used by Chen et al. (2000) that resulted in elevated serum levels of cTnT. A total of five rats were removed from the data analysis due to an inability to complete the exercise protocol. After exercise, the rats were towel-dried and killed immediately (0HR, $n = 10$), 2 h (2HR, $n = 10$), and 24 h (24HR, $n = 10$) after swimming. The water temperature was 35°C, and the water depth was 35 cm.

Animal death and tissue sampling

Rats were anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg ip). The abdominal cavity was quickly opened, and about 5 ml of blood was drawn out from the abdominal aorta. Serum samples were collected and stored at −80°C for cTnT measurements. The chest cavity was then quickly opened, and the heart excised. Left ventricular tissues were isolated, immediately frozen in liquid nitrogen, and stored at −80°C.

Heart tissue was sliced and homogenized in cold 0.9% NaCl to give 10% homogenate (w/v). The homogenates were centrifuged at 3,000 rpm for 10 min at 0°C in a cold centrifuge. The supernatants were separated and used for biochemical analyses. Protein content was measured according to the method of Lowry et al. (1951) using bovine serum albumin as the standard for the determination of oxidative stress parameters.

Serum biochemical assays

The analysis of serum cTnT (third-generation) employed electrochemiluminescence technology employed by the Elecsys 2010 automated batch analyzer (Roche Diagnostics, Basel, Switzerland). The detection limit of the third-generation cTnT assay was 0.01 ng ml^{-1}. All cTnT analyses were performed with the same assay kit. Before the assays were performed, the analyzers were calibrated with standard calibrators according to the manufacturer-recommended protocols. Precision expressed as the percent coefficient of variation at 0.03 ng ml^{-1} for cTnT was <10%. Furthermore, this assay has been previously validated for use in several laboratory animals, including SD rats (Fredericks et al. 2002).

Myocardial lipid peroxidation and antioxidant potential

The levels of myocardial malondialdehyde (MDA), glutathione (GSH), and an estimate of non-enzymatic antioxidative capacity (T-AOC) were determined using commercial assay kits (Nanjing Jiancheng Institute, China)

in a spectrophotometer (DU7400, Beckman Co., USA) according to the manufacturer's instructions. Briefly, lipid peroxidation was evaluated by the thiobarbituric acid reactive substances method (TBARS) and was expressed as MDA concentration. The method used obtained a spectrophotometric measurement of the color produced during the reaction of thiobarbituric acid (TBA) with MDA at 535 nm. The MDA level was expressed as nmol mgpro^{-1}. Levels of GSH were determined colorimetrically at 412 nm with the spectrophotometer following reaction with 5,5-dithiobes(2-ni-trobenzoic acid) (DTNB), and was expressed as mg gpro^{-1}. T-AOC was measured based on the method of Benzie and Strain (1996). In the reaction mixture ferric ion was reduced by antioxidant reducing agents and to form the complex ferrous-tripyridyltriazine, which can be estimated by colorimetric assay to reflect T-AOC which was expressed as U mgpro^{-1}.

Statistical analyses

The Kolmogorov–Smirnov test was used to evaluate the normality of the data. Due to the undetectable levels in some serum samples the distribution of cTnT was skewed. Thus, we utilized an arbitrary figure of 0.005 ng ml^{-1} for cTnT when below the detection limit and then applied a non-parametric Kruskal–Wallis *H* test to compare the serum cTnT across time points (PRE-EX, 0HR, 2HR and 24HR). A Mann–Whitney *U* test used *post hoc* to assess pairwise comparisons. Data for cTnT were expressed as median and range as well as mean ± SD. One-way ANOVA was computed to examine the differences in MDA, GSH, and T-AOC across assessment time points. *Post hoc* analyses using Newman–Keuls were performed when a main effect was significant. Data for these variables were presented as mean ± SD. All tests for statistical significance assumed a critical alpha level of $P < 0.05$. Statistical analyses were performed using Statistica Software (Statsoft, Tulsa, USA).

Results

Data for serum cTnT as well as myocardial tissue measures of free-radical production/damage and antioxidant capacity are given in Table 1. Serum cTnT was undetectable in all rats at PRE-EX. Serum samples 0HR and 2HR post-exercise were elevated ($P < 0.05$) in all rats and undetectable in all rats at 24HR post-exercise.

Levels of MDA, a representation of lipid peroxidation, were elevated at 0HR and 2HR post-exercise compared with baseline data ($P < 0.05$). At 24HR post-exercise MDA concentrations were at baseline levels. As a consequence of this oxidant challenge the specific antioxidant GSH was significantly ($P < 0.05$) depressed at 2HR and 24HR post-exercise compared with baseline. Data for T-AOC were unchanged across all time points ($P > 0.05$).

Discussion

The key outcome of the current study was that the completion of 3 h of strenuous swimming exercise resulted in the appearance of detectable levels of cTnT, a biomarker of cardiomyocyte damage, in all animals. The exercise-related elevation of cTnT has disappeared by 24 h post-exercise. Further, the temporal change in serum cTnT was matched by an increase in myocardial tissue concentration of MDA a marker of free-radical related lipid peroxidation. The temporal association of cTnT and MDA suggests a role for exercise-induced increases in ROS in the mediation of cTnT release from human cardiomyocytes that is commonly witnessed after prolonged exercise.

The appearance of the biomarker cTnT in the systemic circulation after prolonged exercise has been described earlier in animal swimming models (Chen et al. 2000) as well as numerous human exercise studies (Scharhag et al. 2008; Shave et al. 2007). Previous human studies have reported that between 0 and 78% of participants had a

Table 1 Serum cardiac troponin T (cTnT), and myocardial malondialdehyde (MDA), glutathione (GSH), and an estimate of non-enzymatic anti-oxidant capacity (T-AOC) before (PRE-EX), and immediately (0HR), 2 (2HR) and 24 (24HR) h after 3-h swim with 5% bodyweight in SD rats are shown

	PRE-EX ($n = 10$)	0HR ($n = 10$)	2HR ($n = 10$)	24HR ($n = 10$)
cTnT (ng ml^{-1})	0.005[a] ± 0	0.053 ± 0.023*	0.329 ± 0.678*	0.005[a] ± 0
Median (range)	–	0.055 (0.020–0.100)	0.036 (0.016–2.110)	–
n Above detection limit	0	10	10	0
MDA (nmol mgpro^{-1})	1.3 ± 0.3	1.7 ± 0.2*	1.6 ± 0.3*	1.5 ± 0.2
GSH (mg gpro^{-1})	41.6 ± 2.5	43.1 ± 1.8	38.9 ± 2.6*	37.9 ± 1.6*
T-AOC (U mgpro^{-1})	1.3 ± 0.3	1.4 ± 0.2	1.2 ± 0.1	1.4 ± 0.3

* Significantly different from corresponding Pre-ex value $P < 0.05$

[a] Arbitrary value for undetectable cTnT samples

cTnT positive blood sample after bouts of prolonged exercise (Shave et al. 2007). This discrepancy may relate to the limited number of blood sampling points in past work. In support of this argument, a recent study by Middleton et al. (2008) observed cTnT elevations in all subjects both during and after a treadmill marathon using multiple time point measurements. The fact that cTnT was present in the circulation of all animals at 0HR and 2HR post-exercise in the present study is also interesting and supports Middleton et al. (2008) study. Whether the same is true of the animal study performed by Chen et al. (2000) cannot be determined from the data as only mean and SD values were presented. The fact that all animals in the current study had elevated serum cTnT can be interpreted in two different ways: (1) as this happens in every animal performing a similar exercise bout this is likely a normal and physiological response, or (2) elevated levels of serum cTnT in these animals provide evidence that prolonged exercise provides a significant insult to the myocardium that at the very least disrupts myocardial membrane permeability. In an effort to determine which of these scenarios is more likely it is imperative that we attempt to determine the mechanism by which cTnT is released from within the cardiomyocyte to the intravascular space during prolonged exercise and recovery.

Whilst numerous theories have been proposed to explain the appearance of cardiac cell proteins in the blood stream during or after prolonged exercise (Scharhag et al. 2008; Shave et al. 2007), there is no substantive evidence to support a direct role for ischemia, stunning, free radical damage, or high levels of free fatty acids. In the only human study to assess markers of ROS and serum cTnT after a marathon race no link was observed (Whyte et al. 2005). The obvious problem with this previous paper is that ROS markers were assayed in the systemic circulation and therefore could not be localized to action or damage in the myocardium. The current data are the first to assess the temporal association of serum cTnT with markers of ROS damage actually in the myocardium. Serum cTnT was elevated in all animals 0HR and 2HR post-prolonged exercise and the same temporal pattern occurred for MDA a marker of lipid peroxidation in cell membranes subsequent to reactions with ROS. The increases in MDA post-exercise are similar to findings of other a study, which used a similar prolonged swimming model in rats but no blood cTnT data (Venditti and Di Meo 1996). It is important to note that the two concomitant phenomena are not necessarily dependent on each other and, therefore, we would be the first to admit that temporal association does not equal causality; the nature of the data suggests that further research in this field may be warranted.

Likely, as a consequence of the oxidant challenge of the exercise, a major GSH content was depressed at 2HR and 24HR in the present study. The decreases in myocardial GSH content post-exercise are similar to findings of other a study, which used model of Swiss-Webster mice performing swim exercise to exhaustion (Leeuwenburgh et al. 1996). In male rats that swam for 210 min, Venditti et al. (1996) reported a small decrease in anti-oxidant capacity of the myocardium. Data for T-AOC did not alter significantly in rats assessed at all time points of the current study. Our data suggests that myocardial anti-oxidant capacity is not elevated in the face of an exercise stress that is known to increase ROS. Whether this simply reflects inadequate activation or the hearts limited anti-oxidant capacity or some of the limitations of the estimation of total anti-oxidant capacity is not known. The lack of change in anti-oxidant capacity does promote the idea that exogenous anti-oxidant feeding, in future animals and/or human studies of prolonged exercise, may be insightful.

Irrespective of whether the rise in serum cTnT after prolonged exercise is due partially or wholly to elevated ROS increasing myocyte membrane permeability, it is important to ask if this cTnT appearance is clinically relevant. Given the temporal nature of the rise in cTnT, it is unlikely that the serum cTnT observed in the current study reflects irreversible myocyte damage and appearance of previously bound cTnT (that normally occurs with cell necrosis). At 24HR post-exercise serum cTnT had returned to baseline/undetectable levels in all rats, and this rapid return to normal is not what would be observed in a classical clinical presentation of elevated cTnT after a myocardial infarction (Shave et al. 2007). Even though the appearance of cTnT in the serum of rats is noteworthy, and the association with MDA levels of interest mechanistically, there is no experimental evidence suggesting that this is a mal-adaptation to prolonged exercise. Indeed, it may reflect a "natural" or "normal" adaptation to the biochemical and mechanical stress of such exercise which is important in preparation for the next bout of prolonged exercise.

The current study has some major limitations that also provide some guidance for further research, beyond those already noted. This is a small initial study with only a limited number of markers of ROS damage and anti-oxidant activity. Future research may wish to employ a more sensitive marker of lipid peroxidation such as HPLC assessment of MDA or the measurement of F_2-isoprostanes. Moreover, T-AOC as assessed in the current study does not measure anti-oxidant enzymes and future work should determine changes in superoxide dismutase, glutathione peroxidase and catalase. It may also be pertinent to study the temporal responses of cTnI as well as to assess different exercise bouts where duration, intensity and volume of cardiac activity are carefully manipulated. Also we did not study other potential mechanisms, such as

ischemia-induced bleb formation (Hickman et al. 2010), which require further evaluation in an exercise setting.

In conclusion, the completion of 3 h of strenuous swimming exercise in male SD rats resulted in the appearance of detectable levels of cTnT, a biomarker of cardiomyocyte damage, in all animals. All cTnT had disappeared by 24 h post-exercise. The temporal change in serum cTnT was matched by an increase in myocardial tissue concentration of MDA, a marker of free-radical related lipid peroxidation. Further research should assess the role of ROS damage in the mediation of cTnT release commonly observed in humans after prolonged exercise.

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