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ORIGINAL ARTICLE

The influence of exogenous carbohydrate provision and pre-exercise alkalosis on the heat shock protein response to prolonged interval cycling

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Abstract The aim of this study was to observe the intracellular heat shock protein 72 (HSP72) and heme oxygenase-1 (HSP32) response to prolonged interval cycling following the ingestion of carbohydrates (CHO) and sodium bicarbonate ($NaHCO_3$). Six recreationally active males (mean ± SD; age 23.2 ± 2.9 years, height 179.5 ± 5.5 cm, body mass 76.5 ± 6.8 kg, and peak power output 315 ± 36 W) volunteered to complete a 90 min interval cycling exercise on four occasions. The trials were completed in a random and blinded manner following ingestion of either: placebo and an artificial sweetener (P–P), $NaHCO_3$ and sweetener (B–P), placebo and CHO (P–CHO), and $NaHCO_3$ and CHO (B–CHO). Both HSP72 and HSP32 were significantly increased in monocytes and lymphocytes from 45 min post-exercise ($p \leq 0.039$), with strong relationships between both cell types (HSP72, $r = 0.83$; HSP32, $r = 0.89$). Exogenous CHO had no influence on either HSP72 or HSP32, but the ingestion of $NaHCO_3$ significantly attenuated HSP32 in monocytes and lymphocytes ($p \leq 0.042$). In conclusion, the intracellular stress protein response to 90 min interval exercise is closely related in monocytes and lymphocytes, and HSP32 appears to be attenuated with a pre-exercise alkalosis.

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Introduction

The research surrounding the benefits of exogenous carbohydrate (CHO) provision for sport and exercise is vast, and it has been shown on numerous occasions to improve physical performance when taken before (Moore et al. 2010) and during exercise (Jeukendrup 2004), and to also improve recovery when taken post-exercise (Stevenson et al. 2004). A training technique that has attracted research interest is that of 'low carbohydrate' training (Drust and Morton 2009). This training method withholds the ingestion of CHO during exercise and has been shown to have the potential to enhance training adaptation via the up-regulation of oxidative enzyme activity (De Bock et al. 2008; Hansen et al. 2005; Morton et al. 2009; Yeo et al. 2008). However, the influence of training in the absence of CHO provision on the stress protein response to exercise is currently unclear. In particular there is conflicting research surrounding heat shock protein 72 (HSP72 or HSPA1A (Kampinga et al. 2009)). This protein is highly inducible under periods of physiological stress and its protective effects against cell degradation is suggested to have the potential to pre-condition for subsequent periods of exercise via its defensive properties (Madden et al. 2008; Taylor et al. 2012). Both the extracellular (Febbraio et al. 2004) and intramuscular HSP72 (Febbraio et al. 2002) responses to acute exercise have been shown to be attenuated with the ingestion of CHOs, but other authors have reported no effect of CHO intake on intramuscular HSP72 following a more chronic bout of training (Morton et al. 2009). It is currently

unknown what effect CHO consumption has on intracellular HSP72 in leukocytes, despite this source of the stress protein being considered highly sensitive in humans in response to exercise (Hillman et al. 2011; Peart et al. 2011; Shastry et al. 2002).

A nutritional practice that has been shown to attenuate intracellular HSP72 is inducing a pre-exercise alkalosis via the ingestion of sodium bicarbonate ($NaHCO_3$) (Peart et al. 2011). This enhances the natural bicarbonate (HCO_3^-) reserve, which acts to enhance H^+/lactate efflux from cells by accepting a proton to form carbonic acid (McNaughton et al. 2008). The mechanism by which $NaHCO_3$ attenuated stress in this study is currently unclear, although it may be assumed to be due to the maintenance of acid–base variables closer to homeostatic values. However, we have also proposed that the enhanced buffering capacity may attenuate an oxidative stress (Peart et al. 2011), a stimulus for the induction of HSP72 (Khassaf et al. 2003). It must be considered though that the only measure of oxidative stress observed in this study was lipid peroxidation via the thiobarbituric acid reactive substances (TBARS) assay. Examining more direct markers of exercise induced oxidative stress such as glutathione activity (Gohil et al. 1988; Hillman et al. 2011; Tanskanen et al. 2011), and the inclusion of a marker more specific to protein damage will allow greater clarity on this issue. One such marker is heme oxygenase-1, another heat shock protein otherwise known as HSP32. This inducible stress protein catabolises free heme produced following the breakdown of heme bound proteins following oxidative stress. This results in improved cytoprotection via the synthesis of the antioxidant bilirubin and stabilisation of labile iron (Fe^{2+}) (Gozzelino et al. 2010).

Any nutritional mediated modifications to the HSP72 and HSP32 responses to exercise may have implications for training as these proteins have a role in conferring cellular tolerance to future stressors. Therefore, the influence of ingesting $NaHCO_3$ prior to and CHO during exercise on their expression following an acute exercise bout warrants further investigation. The magnitude of changes in HSP72 (Fehrenbach et al. 2005), HSP32 (Fehrenbach et al. 2003) and glutathione (Fisher-Wellman and Bloomer 2009) following exercise in humans has been shown to be related to exercise duration. Furthermore, despite $NaHCO_3$ being primarily recognised for its potential to influence performance of a shorter duration, it has been reported to maintain acid–base variables closer to homeostatic values during longer exercise protocols (McNaughton et al. 1999; Price et al. 2003; Stephens et al. 2002). The aim of this study was to investigate the influence of CHO and $NaHCO_3$ on markers of stress following prolonged exercise.

Methods

Participants

Six recreationally active non-smoking males (mean ± SD; age 23.2 ± 2.9 years, height 179.5 ± 5.5 cm, body mass 76.5 ± 6.8 kg, and peak power output (PPO) 315 ± 36 W) volunteered for the study. All procedures were approved by the institutional ethics committee and written informed consent was given. Participants were treated in accordance with the Declaration of Helsinki. None of the participants were supplementing their diet with any ergogenic aids prior to testing. Participants were instructed not to exercise in the 24 h prior to testing, and to also abstain from foods and beverages high in alcohol, fat and caffeine (Sandstrom et al. 2009; Taylor et al. 2010a).

Exercise protocols

Prior to the experimental trials all participants performed a ramped PPO test on a cycle ergometer (Lode Sport Excalibur, Netherlands). The test started with a 5 min warm up at 50 W, and workload increased thereafter by 30 W min^{-1} until volitional exhaustion. The PPO from this test was used to prescribe an individual workload for the 90 min intermittent cycling protocol used during the experimental trials. The 90 min exercise was adapted from (Price et al. 2003) to consist of 10 × 9 min blocks of exercise, each with 306 s at 40 % PPO, 204 s at 60 % PPO, 8 s at 0 W, 14 s sprint at 120 % PPO and 8 s at 0 W (Fig. 1).

Experimental design

Participants reported to the laboratory on five occasions, with the first being the PPO test followed by a familiarisation to the 90 min protocol. The following four visits were the experimental trials completed in a blinded and randomised manner, namely placebo (NaCl) and an artificial sweetener (P–P), $NaHCO_3$ and sweetener (B–P), placebo and CHO (P–CHO), and $NaHCO_3$ and CHO (B–CHO). The $NaHCO_3$ and NaCl placebo were taken in gelatine capsules 60 min prior to exercise at a dosage of 0.3 g kg^{-1} body mass and 0.045 g kg^{-1} body mass, respectively, with flour added to the placebo to equal the same number of pills. Each CHO drink was made to a concentration of 6.4 % in 250 ml of water corresponding to (Febbraio et al. 2004), with a taste matched sweetener (aspartame) as the placebo. The drinks were consumed at the onset of exercise and every 18 min during the exercise bout to allow consumption to be uninterrupted by a sprint. Participants reported for the experimental trials following an over-night fast and did not eat until after the final blood

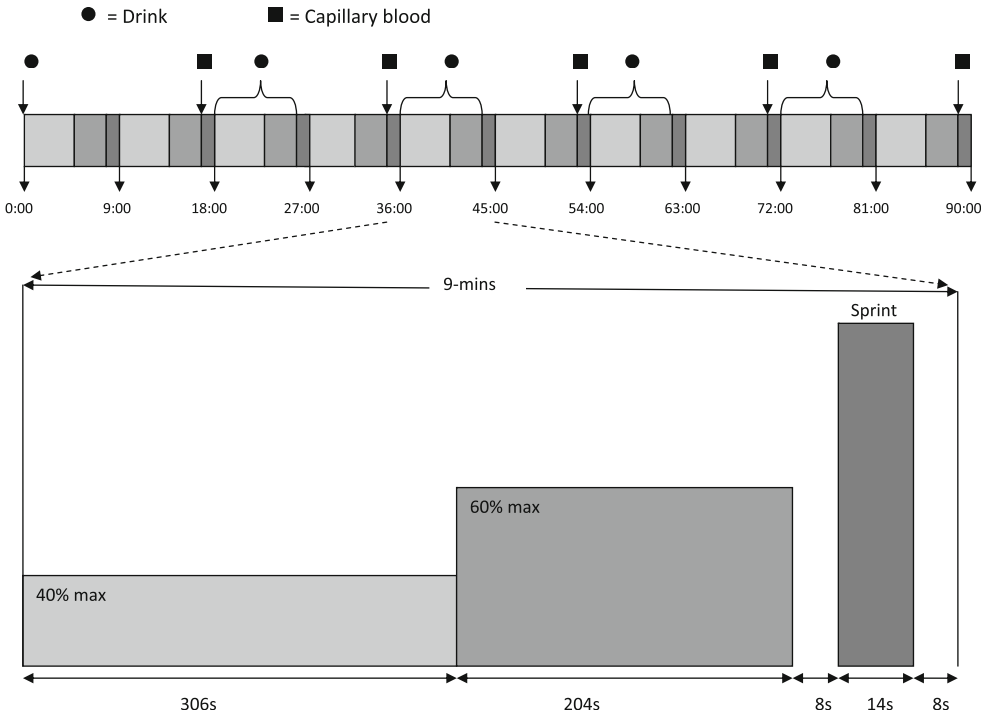

Fig. 1 Schematic of the 90 min interval exercise

draw. All testing took place at the same time of day (pill ingestion 8:00 am) to control for circadian variations in HSP72 expression (Sandstrom et al. 2009) and exercise performance (Drust et al. 2005).

Ingestion of $NaHCO_3$ has been associated with some negative gastro-intestinal symptoms, namely nausea, flatulence, stomach cramping, belching, stomach ache, bowel urgency, diarrhoea, vomiting and stomach bloating (Cameron et al. 2010). Participants were asked to mark their perception of each symptom on a 100 mm visual analogue scale (VAS) prior to and every 15 min during the 60 min post capsule ingestion period. Four of the participants reported no symptoms following the ingestion of either the $NaHCO_3$ or NaCl capsules, with marks on the VAS ranging from 1 to 5 mm. One participant reported some stomach bloating 15 min post NaCl ingestion (35 mm), and stomach ache (38 mm) with diarrhoea 45 min post $NaHCO_3$ ingestion. The final participant had a high rating of bowel urgency 60 min post ingestion of $NaHCO_3$ (80 mm) but reported no other symptoms.

Capillary blood samples for the measurement of acid–base variables (pH, HCO_3^-, base excess and lactate) were taken immediately before and 60 min after ingestion of the pills (both pre-exercise), and at 18 min intervals during exercise. All capillary blood samples were collected in 100 µl balanced heparin blood gas capillary tubes and analysed immediately (Radiometer, ABL800, Copenhagen, Denmark). Venous blood samples for the measurement of HSP72, HSP32, oxidised (GSSG) and total (TGSH) glutathione were drawn from the antecubital vein into potassium EDTA and tri-sodium citrate coagulation Vacuette tubes (Vacuette®, Greiner BIO-one, UK) pre-exercise, immediately post-exercise, and then 45, 90 and 180 min post-exercise. Glutathione measurement was excluded at 180 min as levels typically return to baseline within the hour post-exercise (Fisher-Wellman and Bloomer 2009).

Measurement of stress markers

The expression of HSP72 and HSP32 was measured via flow cytometry in monocytes (*m*HSP72, *m*HSP32) and lymphocytes (*l*HSP72, *l*HSP32) using an established assay method (Sandstrom et al. 2009; Vince et al. 2010). In brief, whole venous blood underwent red cell lysis, and the resultant cells were fixed and permeablised (AbD Serotec, UK) and a negative control:FITC (AbD Serotec, UK) or anti-HSP72:FITC/anti-HSP32:FITC antibody (Enzo Life Sciences, USA) was added. Following 30 min incubation in the dark samples were washed with phosphate buffering solution and analysed via flow cytometry on a BDFACSCalibur® (BD Biosciences, UK) running CELLQuest Software (BD Biosciences, UK). Monocytes and lymphocytes were gated by forward scatter/side scatter properties, with a total of 20,000 events counted. Results were calculated as the ratio of mean fluorescence intensity (MFI) gained with the anti-HSP72/anti-HSP32 antibody to that obtained with the isotype matched negative control. The concentration of GSSG and TGSH were analysed using a commercially available assay kit in accordance with manufacturer's instructions (Enzo Life Sciences, USA).

Statistical analysis

All statistical analyses were completed using IBM SPSS Statistics 18 (SPSS Inc., Chicago, IL). Central tendency and dispersion of the sample data are represented as the mean ± SD. The change in acid–base variables and biochemical markers across condition and time were analysed using linear mixed models. The expression of stress proteins were expressed as a percentage of the resting values to remain consistent with previous research (Morton et al. 2006; Peart et al. 2011; Taylor et al. 2010b). Quantile–quantile plots showed that residuals for the HSP72, HSP32 and GSSG/TGSH data were positively skewed, and were corrected using natural log transformations. Post hoc tests with Sidak-adjusted p values were used to locate significant paired differences, with two-tailed statistical significance accepted at $p < 0.05$. The relationship between monocyte and lymphocyte expression of HSP72 and HSP32 at individual time points irrespective of condition was analysed using Pearson Correlation Coefficients.

Results

Acid base status

There was a significant condition main effect for blood pH ($F = 22.374$, $p < 0.001$), HCO_3^- (21.667, $p < 0.001$), and base excess ($F = 18.890$, $p < 0.001$) where each were maintained closer to homeostatic values in B–P and B–CHO compared with P–P and P–CHO ($p \leq 0.033$) (Fig. 2a–c). Blood lactate, however, was similar for all trials ($F = 2.189$, $p = 0.111$) (Fig. 2d).

Hsp72

Monocyte and lymphocyte HSP72 data is presented in Fig. 3a, b. The expression of HSP72 in each cell type was closely related with a significant correlation observed ($r = 0.833$, $p < 0.001$, Fig. 3c). The exercise resulted in a significant increase in *m*HSP72 and *l*HSP72 ($F = 6.945$, $p < 0.001$ and $F = 5.888$, $p < 0.001$, respectively), whereby both were significantly higher than rest at 45, 90 and 180 min, with an approximate peak increase of 60 and 50 %, respectively ($p \leq 0.014$). In addition HSP72 was significantly increased at 90 min compared to immediately post-exercise (0 min) in both monocytes and lymphocytes ($p \leq 0.029$), and 180 min compared to 0 min post in monocytes ($p = 0.039$). There were no significant differences between conditions in either *m*HSP72 or *l*HSP72 ($F = 0.160$, $p = 0.923$ and $F = 0.190$, $p = 0.902$, respectively). These trends were evident in all participants.

Hsp32

There was a significant increase in *m*HSP32 from 45 min post-exercise regardless of condition ($F = 13.474$, $P < 0.001$) (Fig. 4a). There was also a significant main effect for condition ($F = 5.551$, $p = 0.003$) whereby *m*HSP32 was significantly attenuated in B–P and B–CHO compared to P–P (average peak increase of 65 and 55 versus 182 %, respectively; $p \leq 0.042$). The response of *l*HSP32 was similar as there was a main effect for time

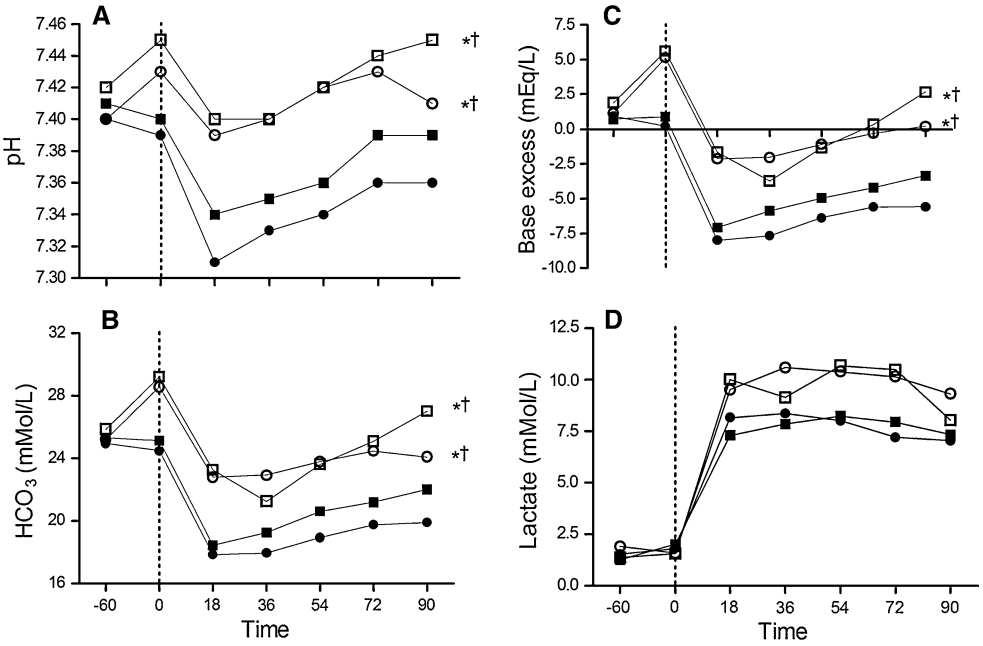

Fig. 2 Blood pH (**a**), HCO_3^- (**b**), base excess (**c**) and lactate (**d**) throughout the testing period in P–P (*filled square*), B–P (*open square*), P–CHO (*filled circle*) and B–CHO (*open circle*). *Significant difference to P–P ($p \leq 0.033$) and † significant difference to P–CHO ($p < 0.001$)

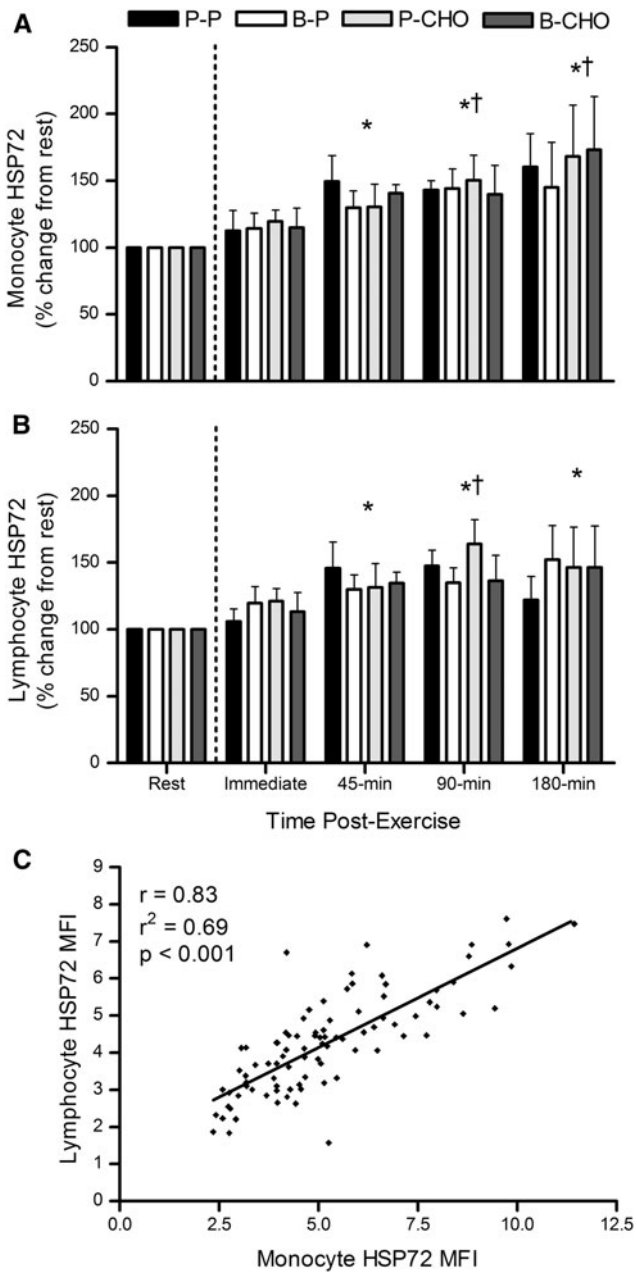

Fig. 3 Monocyte (**a**), lymphocyte (**b**) expressed HSP72 presented as mean ± SEM percentage change from rest and the correlation of monocyte to lymphocyte MFI (**c**). *Significant difference to rest ($p \leq 0.014$) and †significant difference to 0 min ($p \leq 0.039$)

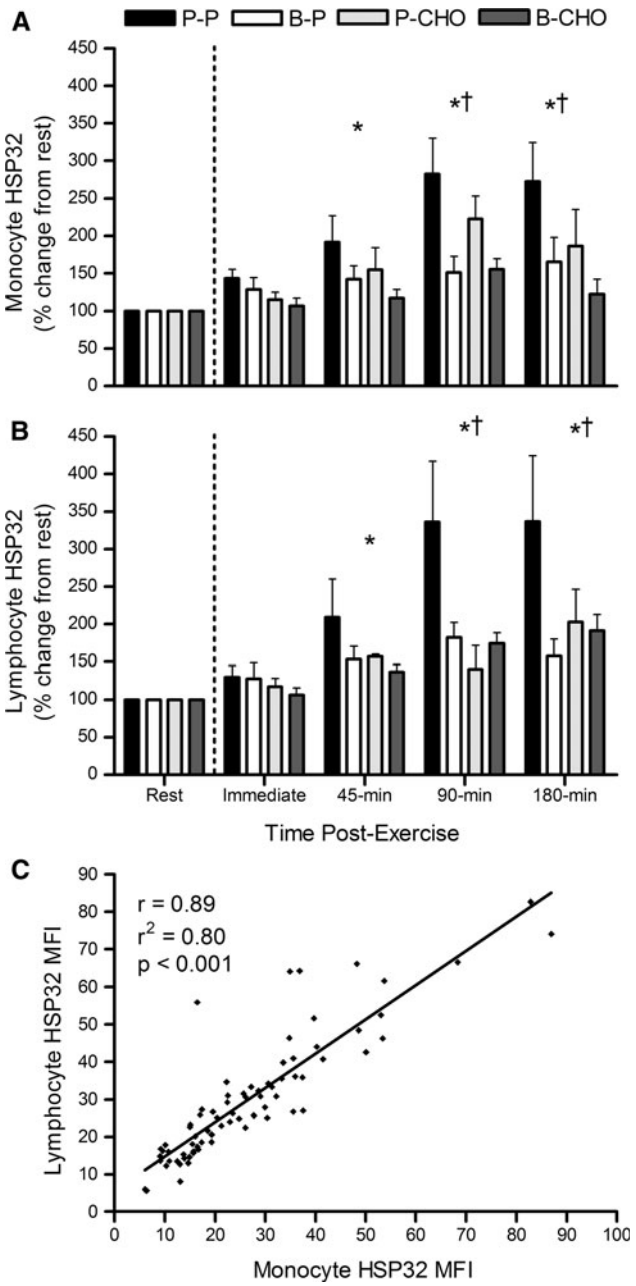

Fig. 4 Monocyte (**a**), lymphocyte (**b**) expressed HSP32 presented as mean ± SEM percentage change from rest and the correlation of monocyte to lymphocyte MFI (**c**). HSP32 is significantly lower in B–P and B–CHO compared to P–P ($p \leq 0.042$) in monocytes and lower in B–CHO compared to P–P ($p = 0.029$) in lymphocytes. *Significant difference to rest ($p = 0.001$) and †significant difference to 0 ($p = 0.039$)

($F = 15.240$, $p < 0.001$) with significant differences between rest and 45, 90 and 180 min post ($p < 0.001$), and between 0 min post to 90 and 180 min post ($p < 0.001$) (Fig. 4b). There was also a significant main effect for condition ($F = 3.633$, $p = 0.017$) whereby *l*HSP32 was significantly higher in P–P compared to B–CHO ($p = 0.029$). This attenuation was evident in all participants. There was a significant correlation between *m*HSP32 and *l*HSP32 post-exercise ($r = 0.89$, $p < 0.001$, Fig. 4c).

Glutathione

The percentage of GSSG/TGSH increased after the exercise ($F = 10.957$, $p < 0.001$) at 45 and 90 min compared to rest ($p \leq 0.001$) and 90 min compared to 0 min post ($p = 0.011$) (Table 1). There was no significant main

Table 1 Percentage of GSSG/TGSH following the 90 min protocol

		Pre	0	45	90
GSSG/TGSH (%)*	P–P	14.83 ± 1.02	15.55 ± 1.10	17.28 ± 2.49	18.34 ± 3.49
	B–P	15.46 ± 1.40	16.47 ± 1.55	17.64 ± 3.03	19.12 ± 3.07
	P–CHO	14.96 ± 0.42	17.10 ± 3.17	18.52 ± 2.32	18.73 ± 2.45
	B–CHO	15.37 ± 0.95	17.13 ± 2.08	18.59 ± 3.57	19.31 ± 2.23

* Indicates significant main effect for time ($p < 0.001$) where 45–90 min are significantly higher than pre-exercise

effect for condition ($F = 0.907$, $p = 0.442$) as all trials had a similar peak (~19 %) at 90 min post-exercise.

Discussion

The aim of the current study was to observe intracellular HSP72 and HSP32 expression following 90 min of interval cycling, and to examine if the response was influenced by the ingestion of CHO and/or $NaHCO_3$. The time course of each testing day in this study closely matched that of a study examining circadian changes in resting intracellular HSP72 by (Taylor et al. 2010b) (8:00–13:30 and 8:00–14:00, respectively). During this time period basal expression is proposed to follow a quadratic trend with a ~17 % decrease between 8:00 and 11:00, followed by a return to levels similar to the 8:00 expression by 14:00. The statistically significant post-exercise increases in both *m*HSP72 and *l*HSP72 in this study (observed peak increases of 50–70 and 20–50 %, respectively) were much higher than these reported changes at rest; therefore it can be reasonably assumed that the observed increases shown were due to the exercise bout. Furthermore, there was a strong relationship observed between the two cell types ($r = 0.833$, $p < 0.001$; Fig. 3c) despite it typically being reported that *l*HSP72 is less sensitive than *m*HSP72 following stress (Bachelet et al. 1998; Hillman et al. 2011; Lovell et al. 2007; Oehler et al. 2001; Peart et al. 2011; Sandstrom et al. 2009). The majority of this previous work was performed at rest, although one study from our own laboratory implemented a similar exercise to the current study (90 min cycling at 95 % lactate threshold) (Hillman et al. 2011). This exercise resulted in a significant ~20–30 % increase in *m*HSP72 immediately post-exercise, similar to the current study (~20 %), but no significant change in *l*HSP72. It must be considered though that *l*HSP72 was not significantly increased in the current study until after 45 min post-exercise, suggesting (Hillman et al. 2011) may have missed any subsequent increase in lymphocyte expression. It is proposed that there may be a threshold stress required before HSP72 is increased to the same extent in lymphocytes as in monocytes, peaking between 45 and 180 min post-exercise. Future work is needed to investigate any possible dose–response relationship following exercise in more detail.

A reduced availability of endogenous CHO has been shown to induce HSP72 in skeletal muscle following 4–5 h leg press exercise (Febbraio et al. 2002), and the same authors also reported that exogenous CHO ingestion can attenuate HSP72 in serum following 2 h of cycling at 65 % capacity (Febbraio et al. 2004). However, the results from our current study are more similar to that of a training study by (Morton et al. 2009) as CHO had no influence on either *l*HSP72 or *m*HSP72. It must be considered that the source of extracellular HSP72 following exercise is currently unclear, though (Febbraio et al. 2004) observed a reduced hepatosplanchnic release of HSP72 in conjunction with reduced HSP72 in the circulation following the ingestion of CHO. Therefore, it appears that CHO ingestion may primarily alter extracellular HSP72 via a blunted hepatosplanchnic release, unless the exercise stress is sufficient to deplete glycogen stores by targeting a specific muscle for an extended period of time (i.e. leg press for 4–5 h) (Febbraio et al. 2002). The other nutritional intervention examined in this study was inducing a pre-exercise alkalosis with $NaHCO_3$, as previous work from our laboratory reported a significant attenuation in *m*HSP72 with $NaHCO_3$ following a 4 min sprint exercise (~40 % observed peak increase in placebo vs. no change in $NaHCO_3$ trials) (Peart et al. 2011). The supplementation of $NaHCO_3$ maintained acid–base measures closer to homeostatic values during exercise (Fig. 2), similar to the original protocol that the 90 min exercise used in this study was adapted from (Price et al. 2003), but had no effect on the expression of HSP72. This may suggest that alterations to acid–base balance is one of the mechanisms by which $NaHCO_3$ attenuates HSP72, as although these variables were maintained significantly closer to basal levels during exercise their disruption was not to the same extent as observed in past research due primarily to differing exercise protocols (pH 7.16 observed in (Peart et al. 2011) versus pH 7.31 observed in the current study).

A secondary aim of this study was to examine the effect of $NaHCO_3$ on markers of oxidative stress. We have shown in previous work that $NaHCO_3$ can attenuate plasma TBARS following 4 min sprint exercise (Peart et al. 2011).

However, other authors have suggested that this method may not be the most appropriate for the quantification of exercise related oxidative stress due to its lack of specificity (Powers et al. 2010). Glutathione data demonstrated that an oxidative stress had occurred as the percentage of GSSG/TGSH significantly increased from 45 min post-exercise in all trials (Table 1), the same time at which the expression of intracellular HSP72 is increased in both cell types. Despite this both *m*HSP32 and *l*HSP32 were significantly attenuated in both $NaHCO_3$ trials compared to P–P (Fig. 4a, b). Previous in vitro research from (Christou et al. 2005) has demonstrated a relationship between the expression of HSP32 in smooth muscle cells and changes in pH. Therefore, when considered alongside the absence of an attenuation in glutathione activity, it may be assumed that the mechanism by which intracellular HSP32 is attenuated following a pre-exercise alkalosis is associated with acid–base balance as opposed to changes in the generation of reactive oxygen species. However, this is speculation as the extent of acidosis was to a much lesser extent in this study compared to (Christou et al. 2005) (7.31 vs. 6.8) and the source of HSP32 is different. Further work is needed to clarify the mechanism of HSP32 attenuation.

The main findings of this study were that the expression of HSP72 was closely related in monocytes and lymphocytes following 90 min of interval cycling, but neither was influenced by the ingestion of 6.4 % CHO during exercise or 0.3 g kg^{-1} body mass of $NaHCO_3$ prior to exercise. Although both monocyte and lymphocyte HSP72 are strongly correlated monocyte HSP72 still tended to be higher than in lymphocytes, confirming that determining the response in these cells would be most sensitive (Bachelet et al. 1998; Sandstrom et al. 2009). A further finding was that *m*HSP32 and *l*HSP32 were significantly attenuated following a pre-exercise alkalosis. The mechanism by which this occurred is currently unclear as glutathione activity, another marker of oxidative stress, was not altered by ingestion of $NaHCO_3$.

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