Title: Implications of a Pre-Exercise Alkalosis Mediated Attenuation of HSP72 on its Response to a Subsequent Bout of Exercise

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Abstract

The aim of this study was to investigate if a pre-exercise alkalosis mediated attenuation of HSP72 had any effect on the response of the same stress protein after a subsequent exercise. Seven physically active males (25.0 ± 6.5 years, 182.1 ± 6.0 cm, 74.0 ± 8.3 kg, peak aerobic power (PPO) 316 ± 46 W) performed a repeated sprint exercise (EXB1) following a dose of 0.3 g kg^{-1} body mass of sodium bicarbonate (BICARB), or a placebo of 0.045 g kg^{-1} body mass of sodium chloride (PLAC). Participants then completed a 90-min intermittent cycling protocol (EXB2). Monocyte expressed HSP72 was significantly attenuated after EXB1 in BICARB compared to PLAC, however there was no difference in the HSP72 response to the subsequent EXB2 between conditions. Furthermore there was no difference between conditions for measures of oxidative stress (protein carbonyl and HSP32). These findings confirm the sensitivity of the HSP72 response to exercise induced changes in acid-base status in vivo, but suggest that the attenuated response has little effect upon subsequent stress in the same day.

Key words

HSP72, bicarbonate, alkalosis, stress, monocyte

Introduction

Exposure to an extracellular acidosis has been demonstrated to increase HSP72 gene activity in monkey kidney cells (pH = 6.8) (Gapen and Moseley, 1995) and the expression of HSP72 protein in cultured rat astrocytes (pH = 5.2) (Narasimhan et al., 1996), with a potential mechanism being the initiation of protein degradation via the ATP dependent ubiquitin proteasome pathway (Mitch et al. 1994, Price et al. 1994). However, these *in vitro* models are difficult to apply to the whole organism due to the extreme pH limits employed. Recent research in human studies inducing a pre-exercise alkalosis using sodium bicarbonate ($NaHCO_3$) has demonstrated an attenuation of the stress protein following high-intensity anaerobic exercise (Peart et al. 2011, 2013a), but not following a longer sub-maximal 90-min cycling effort (Peart et al. 2013b). These findings therefore suggest that HSP72 is also sensitive to less extreme shifts in acid-base balance in vivo (pH = 7.15). However it was not investigated in this previous work whether the attenuated response of HSP72 would have any implications for the stress response from a subsequent exercise.

One of the primary purposes of HSP72 is to facilitate cross-tolerance to a subsequent stressor, and Madden et al. (2008) proposed that its expression could enhance cellular tolerance to the stress of exercise. Moreover authors have shown that increases in monocyte expressed HSP72 in particular can provide protection to oxidative stress in a subsequent exercise (Taylor et al, 2012). In light of this it is possible that attenuating the acute exercise induced HSP72 response to exercise via $NaHCO_3$ may inhibit this particular mechanism of defence to a second bout of exercise.

The aim of this study was to confirm previously published data and further expand whether this would have any implications for the subsequent HSP72 response and oxidative stress response to a second exercise in the same day.

Materials and Methods

Participants

Seven recreationally active non-smoking males (mean ± SD; age 25.0 ± 6.5 years, height 182.1 ± 6.0 cm, body mass 74.0 ± 8.3 kg, absolute peak power output (PPO) 316 ± 46 W and relative PPO 4.3 ± 1.0 $W.kg^{-1}$) volunteered for the study. All participants provided written informed consent in accordance with

the departmental and University ethical procedures and following the principles outlined in 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 both exercise bouts (EXB1 and EXB2 respectively). EXB1 was a high-intensity anaerobic exercise consisting of 10 x 15-s sprints against 120% PPO separated by 45-s active recovery and EXB2 was a 90-min interval cycling exercise (10 x 9-min blocks of exercise consisting of 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). Both protocols have been described in full in Peart et al (2013a) and Peart et al (2013b) respectively.

Experimental Design

Participants reported to the laboratory on three occasions, with the first visit consisting of the described PPO test followed by a familiarisation to the testing procedures. Visits two and three were the experimental trials completed in a blinded and randomised manner. These trials began with ingestion of either 0.3 $g.kg.BW^{-1}$ $NaHCO_3$ (BICARB) or an equimolar (sodium) dose of sodium chloride (NaCl - $0.045g.kg.BW^{-1}$) placebo (PLAC), followed by a 60-min rest period. The $NaHCO_3$ and NaCl were ingested in gelatine capsules as opposed to in a liquid solution as per previous research (Artioli et al 2007, Flinn et al 2014, Peart et al 2013b) as the capsules may facilitate participant blinding by removing the taste (Peart et al, 2012) and offer a more preferable mode of consumption (Carr et al, 2011). All of the gelatine capsules were opaque so participants could unknowingly ingest empty capsules to allow a matched number of capsules per condition.

A venous blood sample was taken 60-min post-ingestion, after which participants performed EXB1. After 90-min rest (determined from the peak HSP72 response observed in Peart et al., (2013b)) participants began EXB2. A venous blood sample was taken immediately post EXB2, and a final venous

blood sample was taken 60-min post EXB2. Fig 1 provides a visual representation of the experimental trials. 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), immediately post EXB1 and prior to EXB2. 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 were drawn from the antecubital vein into potassium EDTA Vacuette tubes (Vacuette®, Greiner BIO-one, UK) at the time points already described.

Oxidative stress was quantified by the measurement of protein carbonyl using a commercially available assay kit (Cayman, UK). The expression of HSP72 and HSP32 were measured via flow cytometry in monocytes using a well-established assay method (Sandstrom et al. 2009, Vince et al., 2010 Peart et al 2013b, Taylor et al, 2012). Whole blood (100 µl) was transferred from the potassium EDTA tubes into 2 ml of red blood cell lysis buffer (diluted 1/10 with distilled water; Erythrolyse, AbD Serotec, UK) and left to incubate for 10 minutes. Following incubation, samples were centrifuged for 5-min at 3000 rpm to pellet the white blood cells, and the resultant supernatant discarded. White blood cells were subsequently washed in 2 ml phosphate buffering solution (PBS) and centrifuged for 5-min at 3000 rpm, with the supernatant discarded. Following a second wash 100µl of Fix solution (Leucoperm Reagent A, AbD Serotec) was added to the samples and left to incubate for 15-min. After incubation, samples were washed as above and permeabilised by the addition of 100 µl of Perm solution (Leucoperm Reagent B, AbD Serotec). Each sample was then divided into two 50 µl aliquots. Added to these aliquots were 4 µl of either anti-HSP72:FITC (IgG1) / anti-HSP32:FITC (IgG2B) (Enzo Life Sciences, USA) or an isotype matched negative control:FITC (AbD Serotec). Following 30 min incubation in the dark, samples were washed with PBS and then re-suspended in 300 µl PBS ready for flow cytometry. Samples were analysed by flow cytometry on a BDFACSCalibur® (BD Biosciences, UK) running CELLQuest Software (BD Biosciences, UK), with monocytes and lymphocytes gated by forward scatter (FSC; cell size) and side scatter (SSC; cell granularity) properties, with a total of 20,000 events counted. Results were calculated as the ratio of mean fluorescence intensity (MFI) gained with the anti-HSP antibody to that obtained with the isotype matched negative control.

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 variables across condition and time were analysed using linear mixed models. The expression of HSP72 and HSP32 were expressed as the ratio of the mean fluorescence intensity (MFI) gained from the anti-HSP72/anti-HSP32 antibody to that obtained with the isotype matched negative control. Post hoc tests with Sidak-adjusted p values were used to locate significant paired differences, with two-tailed statistical significance accepted at $p < 0.05$.

Results

The $NaHCO_3$ ingestion protocol significantly elevated blood pH, HCO_3^- and base excess at rest. EXB1 significantly altered all acid-base variables in PLAC, with pH, HCO_3^-, and base excess all maintained closer to pre-exercise values in BICARB (Table 1).

HSP72 was significantly higher post EXB1 in PLAC compared to BICARB (Fig 2) (33 vs. 8% increase respectively; $F = 4.588$, $p = 0.050$), (Fig 1) a result apparent in all participants (Fig 3). The HSP72 response following EXB2 was similar in both conditions ($F = 2.633$, $p = 0.115$), with a significant main effect for time ($F = 13.484$, $p < 0.001$) whereby HSP72 significantly increased 60-min post exercise ($p < 0.001$) (Fig 2). HSP32 was unchanged following EXB1 (less than 10% change in both trials), but was significantly higher ($F = 11.085$, $p < 0.001$) 60-min post EXB2 compared to rest and pre EXB2 ($p < 0.001$) regardless of condition (Fig 4). Furthermore HSP32 was significantly higher immediately post EXB2 compared with immediately before ($p = 0.018$) regardless of condition. The concentration of protein carbonyl did not change significantly from rest throughout the testing period ($F = 1.920$ $p = 0.174$), and was comparable between conditions ($F = 1.476$, $p = 0.249$) (Table 2).

Discussion

The efficacy of the supplementation protocol was demonstrated by the observation of a significantly altered blood acid-base balance prior to EXB1 (Table 1), and a significantly attenuated HSP72 response post EXB1 in the BICARB trial as per previous research (Peart et al, 2013a). A lower basal level of HSP72 prior to EXB2 in BICARB though did not affect the magnitude of the response following the 90-

min protocol of EXB2. Hypoxia is another stimulus that can result in ubiquitin- proteasome mediated protein degradation, via induction by hypoxia-inducible factor (HIF) (Brahimi-Horn and Pouysségur, 2005). Taylor et al (2012) reported a significantly attenuated HSP72 response post-exercise (~50%) after basal HSP72 expression was elevated via a hypoxic exposure, therefore it could have been expected that the trial with the lower basal HSP72 (BICARB) prior to EXB2 would have resulted in increased HSP72 expression following the bout. However it must be noted that the average changes in basal levels between experimental conditions in the research from Taylor et al (2012) was higher than in the present study (67% and 31% respectively). Therefore it may be the case that the HSP72 response to EXB1 in this study was not of a high enough magnitude to influence the response to EXB2.

As other authors have shown that increasing the level of HSP72 prior to exercise can improve defences to the oxidative damage associated with exercise (Taylor et al., 2012), both HSP32 and protein carbonyl were measured prior to and after EXB2. Monocyte HSP32 was induced after EXB2 to the same extent in both conditions. This is in contrast to the HSP32 response to the same 90-min exercise bout in previous research, which was attenuated under BICARB conditions (Peart et al. 2013b). In the current study acid-base values had returned to homeostasis prior to EXB2 resulting in comparable acid-base results between conditions (Table 1), whereas in the previous study blood pH, base excess and HCO_3 were significantly different between conditions prior to and throughout the exercise. This finding supports our previous suggestions that increased oxidative damage is not necessarily the only stimulus for increased HSP32 and that this particular HSP is highly sensitive to acid-base changes *in vivo* (Peart et al. 2013b) as well as *in vitro* (Christou et al, 2004).

The protein carbonyl data adds further support for the notion that oxidative stress is not the sole stimulus for increases in HSP32. Protein carbonyl was measured in this study as a second marker more specific to protein oxidative damage after HSP32 was significantly attenuated in the previous research (Peart et al. 2013b). Although quantification of this marker of protein oxidative damage has previously produced conflicting responses following anaerobic exercise (Alessio et al., 2000, Bloomer et al., 2006, Bloomer et al., 2007b, Bloomer et al., 2005), it has been widely reported to increase following exercise typically aerobic in nature. Such exercises include cycling interventions lasting 15 to 120-min (Alessio et al., 2000, Bloomer et al., 2007a, Michailidis et al., 2007), a half marathon race (Sureda et al., 2013) and

ultra-distance cycling (Tauler et al., 2003) and running (Radak et al., 2000). Despite this trend seen in previous research, protein carbonyl did not increase at any time point throughout the testing period in this study nor was any difference observed between conditions. The only other study reporting no increase in protein carbonyl following aerobic exercise utilised a 90-min cycling protocol at 70% of maximum (Morillas-Ruiz et al., 2005). Fisher-Wellman and Bloomer (2009) have suggested that studies reporting null findings for protein carbonyl may be due to insufficient sampling times, exercise protocols that are too short or physical training status of the participants. Additionally, some authors have reported peaks in protein carbonyl occurring around 4-6 hours post-exercise (Bloomer et al., 2005, Michailidis et al., 2007), therefore it is possible that sample timings in this study may have contributed to no observed increases in protein carbonyl, as the final blood sample was 60-min post EXB2, and Morillas-Ruiz et al, (2005) only took a sample 20-min post exercise. However, other authors have demonstrated peaks in protein carbonyl immediately post-exercise of a similar duration to the current study (Bloomer 2007a, Sureda et al, 2013). Finally the training status is unlikely to have resulted in the absence of an increased protein carbonyl from rest in this study as the participants were less physically active compared with other studies, and lesser trained individuals have been shown to have greater protein carbonyl responses following exercise (Tauler et al., 2003). In the current study participants cycled predominantly at 40-60% of maximum during EXB2, whereas Bloomer et al, (2007a) used an intensity of 70% and Michailidis et al, (2007) 70-90% maximum suggesting the current protocol may not have been a high enough stimulus to elicit increases in protein carbonyl. However Morillas-Ruiz et al, (2005) also applied an intensity of 70% with null findings. Furthermore increases in oxygen consumption are unlikely to be the sole cause of protein carbonyl increases following exercise as authors have at times demonstrated increases in protein carbonyl following exercises of relatively low demands compared to sub-maximal exercise (Alessio et al., 2000, Bloomer et al., 2005). In summary we witnessed no increase in protein carbonyl concentration following either exercise bout, which is in contrast to previous work using similar exercise modalities/duration. The reason for this is unclear as this study also had similar sampling times following exercise to other studies, a factor which has been suggested to contribute to null findings (Fisher-Wellman and Bloomer, 2009).

The increase in HSP72 after EXB1 observed in this study is less than the results reported in previous research (~30% and ~80% respectively) (Peart et al, 2013a). So as acknowledged earlier in the

discussion, the magnitude of the response may have been inadequate to influence HSP72 expression following EXB2, and it is unclear whether an increase similar to the original study would have had more effect upon the subsequent response. This draws attention to whether documenting the stress response on a single day of training is necessarily fully reflective of the role of HSP72 in sport and exercise and future investigations should document intra-individual variance in the response to acute exercise. Other authors have shown that protein damage can progressively increase over several days training, as can the intracellular HSP72 response to exercise (Whitham et al., 2004). Therefore future work may also consider further examining the effect of pre-exercise mediated alterations in the response of HSP72 by documenting the implications of manipulating the HSP72 stress response over a longer period training, and any subsequent effect upon exercise performance and recovery times. This would allow further insight into whether chronic attenuation of HSP72 would have additional implications during a presumably more stressful period, and may inform future work investigating the hormetic nature of oxidative stress for training adaptation (Powers et al, 2010). From a practical viewpoint these findings suggest that individuals choosing to ingest $NaHCO_3$ acutely for its possible ergogenic effect (Peart et al. 2012) do not risk interfering with the physiological stress response to a subsequent exercise in the same day.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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Fig 1 Schematic of the experimental trials

Fig 2 Mean ± SD Monocyte expressed HSP72 pre and post EXB1 and EXB2 during the PLAC and BICARB trials. * significantly different to BICARB ($p = 0.05$), † significantly different to pre EXB2 ($p < 0.001$), ‡ significantly different to 0-min post EXB2 ($p < 0.001$).

Fig 3 Individual changes in monocyte expressed HSP72 after EXB1, expressed as percentage change from rest

Fig 4 Mean ± SD Monocyte expressed HSP32 during the PLAC and BICARB trials. * significantly different to pre EXB1 and pre EXB2 ($p \leq 0.041$).

Table 1 Acid-base characteristics pre- and post-$NaHCO_3$ ingestion, immediately post EXB1 and pre EXB2 (means ± SD)

		Pre-ingestion	Post-ingestion	Post EXB1	Pre EXB2
pH	PLAC	7.40 ± 0.01	7.40 ± 0.02	7.19 ± 0.04†	7.39 ± 0.02
	BICARB	7.39 ± 0.01	7.44 ± 0.02*‡	7.27 ± 0.05†‡	7.40 ± 0.02
HCO_3^- (mmol/L)	PLAC	24.56 ± 0.70	23.02 ± 3.49	14.85 ± 4.35†	24.22 ± 1.19
	BICARB	24.28 ± 1.15	27.5 ± 0.97*‡	15.45 ± 2.21†	25.9 ± 1.53
Base excess (mEq/L)	PLAC	0.35 ± 1.37	0.30 ± 1.00	-13.93 ± 2.05†	0.43 ± 1.21
	BICARB	0.44 ± 1.21	3.84 ± 1.18*‡	-10.82 ± 3.09†‡	2.47 ± 1.49‡
Lactate (mmol/L)	PLAC	1.40 ± 0.47	1.38 ± 0.28	13.65 ± 1.09†	2.16 ± 0.38†
	BICARB	1.28 ± 1.66	1.65 ± 0.23	14.30 ± 1.89†	2.50 ± 0.48†

*significantly different to pre-ingestion ($p<0.05$), †significantly different to post-ingestion ($p<0.05$), ‡significantly different to PLAC ($p<0.05$)

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Table 2 Protein carbonyl (nmol/ml) during the PLAC and BICARB trials (means ± SD)

		Pre EXB1	90-min post EXB1 / Pre EXB2	0-min post EXB2	60-min post EXB2
Protein carbonyl	PLAC	44.18 ± 8.07	42.23 ± 5.02	40.55 ± 5.55	36.73 ± 11.45
	BICARB	48.69 ± 12.27	49.18 ± 8.32	38.16 ± 12.04	46.05 ± 13.54

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