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Citation: Peart, Daniel, Kirk, Richard, Madden, Leigh and Vince, Rebecca (2016) Implications of a Pre-Exercise Alkalosis Mediated Attenuation of HSP72 on its Response to a Subsequent Bout of Exercise. *Amino Acids*, 48 (2). pp. 499-504. ISSN 0939-4451

Published by: Springer

URL: <http://dx.doi.org/10.1007/s00726-015-2103-1> <<http://dx.doi.org/10.1007/s00726-015-2103-1>>

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

3

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24 **Abstract**

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

36

37 **Key words**

38 HSP72, bicarbonate, alkalosis, stress, monocyte

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53 **Introduction**

54 Exposure to an extracellular acidosis has been demonstrated to increase HSP72 gene activity in monkey
55 kidney cells (pH = 6.8) (Gapen and Moseley, 1995) and the expression of HSP72 protein in cultured rat
56 astrocytes (pH = 5.2) (Narasimhan et al., 1996), with a potential mechanism being the initiation of protein
57 degradation via the ATP dependent ubiquitin proteasome pathway (Mitch et al. 1994, Price et al.
58 1994). However, these in vitro models are difficult to apply to the whole organism due to the extreme
59 pH limits employed. Recent research in human studies inducing a pre-exercise alkalosis using sodium
60 bicarbonate (NaHCO₃) has demonstrated an attenuation of the stress protein following high-intensity
61 anaerobic exercise (Peart et al. 2011, 2013a), but not following a longer sub-maximal 90-min cycling
62 effort (Peart et al. 2013b). These findings therefore suggest that HSP72 is also sensitive to less extreme
63 shifts in acid-base balance in vivo (pH = 7.15). However it was not investigated in this previous work
64 whether the attenuated response of HSP72 would have any implications for the stress response from a
65 subsequent exercise.

66

67 One of the primary purposes of HSP72 is to facilitate cross-tolerance to a subsequent stressor, and
68 Madden et al. (2008) proposed that its expression could enhance cellular tolerance to the stress of
69 exercise. Moreover authors have shown that increases in monocyte expressed HSP72 in particular can
70 provide protection to oxidative stress in a subsequent exercise (Taylor et al, 2012). In light of this it is
71 possible that attenuating the acute exercise induced HSP72 response to exercise via NaHCO₃ may inhibit
72 this particular mechanism of defence to a second bout of exercise.

73

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

77

78 **Materials and Methods**

79 **Participants**

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

83 the departmental and University ethical procedures and following the principles outlined in the
84 Declaration of Helsinki. None of the participants were supplementing their diet with any ergogenic aids
85 prior to testing. Participants were instructed not to exercise in the 24-h prior to testing, and to also abstain
86 from foods and beverages high in alcohol, fat and caffeine (Sandstrom et al., 2009, Taylor et al., 2010a)

87

88 **Exercise protocols**

89 Prior to the experimental trials all participants performed a ramped PPO test on a cycle ergometer (Lode
90 Sport Excalibur, Netherlands). The test started with a 5-min warm up at 50 W, and workload increased
91 thereafter by 30 W.min⁻¹ until volitional exhaustion. The PPO from this test was used to prescribe an
92 individual workload for both exercise bouts (EXB1 and EXB2 respectively). EXB1 was a high-intensity
93 anaerobic exercise consisting of 10 x 15-s sprints against 120% PPO separated by 45-s active recovery
94 and EXB2 was a 90-min interval cycling exercise (10 x 9-min blocks of exercise consisting of 306 s at
95 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
96 been described in full in Peart et al (2013a) and Peart et al (2013b) respectively.

97

98 **Experimental Design**

99 Participants reported to the laboratory on three occasions, with the first visit consisting of the described
100 PPO test followed by a familiarisation to the testing procedures. Visits two and three were the
101 experimental trials completed in a blinded and randomised manner. These trials began with ingestion of
102 either 0.3 g.kg.BW⁻¹ NaHCO₃ (BICARB) or an equimolar (sodium) dose of sodium chloride (NaCl -
103 0.045g.kg.BW⁻¹) placebo (PLAC), followed by a 60-min rest period. The NaHCO₃ and NaCl were
104 ingested in gelatine capsules as opposed to in a liquid solution as per previous research (Artioli et al
105 2007, Flinn et al 2014, Peart et al 2013b) as the capsules may facilitate participant blinding by removing
106 the taste (Peart et al, 2012) and offer a more preferable mode of consumption (Carr et al, 2011). All of
107 the gelatine capsules were opaque so participants could unknowingly ingest empty capsules to allow a
108 matched number of capsules per condition.

109

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

113 blood sample was taken 60-min post EXB2. Fig 1 provides a visual representation of the experimental
114 trials. Capillary blood samples for the measurement of acid-base variables (pH, HCO₃⁻, base excess and
115 lactate) were taken immediately before and 60-min after ingestion of the pills (both pre-exercise),
116 immediately post EXB1 and prior to EXB2. All capillary blood samples were collected in 100 µl
117 balanced heparin blood gas capillary tubes and analysed immediately (Radiometer, ABL800,
118 Copenhagen, Denmark). Venous blood samples for the measurement of HSP72 were drawn from the
119 antecubital vein into potassium EDTA Vacuette tubes (Vacuette[®], Greiner BIO-one, UK) at the time
120 points already described.

121

122 Oxidative stress was quantified by the measurement of protein carbonyl using a commercially available
123 assay kit (Cayman, UK). The expression of HSP72 and HSP32 were measured via flow cytometry in
124 monocytes using a well-established assay method (Sandstrom et al. 2009, Vince et al., 2010 Peart et al
125 2013b, Taylor et al, 2012). Whole blood (100 µl) was transferred from the potassium EDTA tubes into
126 2 ml of red blood cell lysis buffer (diluted 1/10 with distilled water; Erythrolyse, AbD Serotec, UK) and
127 left to incubate for 10 minutes. Following incubation, samples were centrifuged for 5-min at 3000 rpm
128 to pellet the white blood cells, and the resultant supernatant discarded. White blood cells were
129 subsequently washed in 2 ml phosphate buffering solution (PBS) and centrifuged for 5-min at 3000 rpm,
130 with the supernatant discarded. Following a second wash 100µl of Fix solution (Leucoperm Reagent A,
131 AbD Serotec) was added to the samples and left to incubate for 15-min. After incubation, samples were
132 washed as above and permeabilised by the addition of 100 µl of Perm solution (Leucoperm Reagent B,
133 AbD Serotec). Each sample was then divided into two 50 µl aliquots. Added to these aliquots were 4 µl
134 of either anti-HSP72:FITC (IgG1) / anti-HSP32:FITC (IgG2B) (Enzo Life Sciences, USA) or an isotype
135 matched negative control:FITC (AbD Serotec). Following 30 min incubation in the dark, samples were
136 washed with PBS and then re-suspended in 300 µl PBS ready for flow cytometry. Samples were analysed
137 by flow cytometry on a BDFACSCalibur[®] (BD Biosciences, UK) running CELLQuest Software (BD
138 Biosciences, UK), with monocytes and lymphocytes gated by forward scatter (FSC; cell size) and side
139 scatter (SSC; cell granularity) properties, with a total of 20,000 events counted. Results were calculated
140 as the ratio of mean fluorescence intensity (MFI) gained with the anti-HSP antibody to that obtained with
141 the isotype matched negative control.

142 **Statistical Analysis**

143 All statistical analyses were completed using IBM SPSS Statistics 18 (SPSS Inc. Chicago, IL). Central
144 tendency and dispersion of the sample data are represented as the mean \pm SD. The change in acid-base
145 variables and biochemical variables across condition and time were analysed using linear mixed models.
146 The expression of HSP72 and HSP32 were expressed as the ratio of the mean fluorescence intensity
147 (MFI) gained from the anti-HSP72/anti-HSP32 antibody to that obtained with the isotype matched
148 negative control. Post hoc tests with Sidak-adjusted p values were used to locate significant paired
149 differences, with two-tailed statistical significance accepted at $p < 0.05$.

150

151 **Results**

152 The NaHCO₃ ingestion protocol significantly elevated blood pH, HCO₃⁻ and base excess at rest. EXB1
153 significantly altered all acid-base variables in PLAC, with pH, HCO₃⁻, and base excess all maintained
154 closer to pre-exercise values in BICARB (Table 1).

155

156 HSP72 was significantly higher post EXB1 in PLAC compared to BICARB (Fig 2) (33 vs. 8% increase
157 respectively; $F = 4.588$, $p = 0.050$), (Fig 1) a result apparent in all participants (Fig 3). The HSP72
158 response following EXB2 was similar in both conditions ($F = 2.633$, $p = 0.115$), with a significant main
159 effect for time ($F = 13.484$, $p < 0.001$) whereby HSP72 significantly increased 60-min post exercise (p
160 < 0.001) (Fig 2). HSP32 was unchanged following EXB1 (less than 10% change in both trials), but was
161 significantly higher ($F = 11.085$, $p < 0.001$) 60-min post EXB2 compared to rest and pre EXB2 ($p <$
162 0.001) regardless of condition (Fig 4). Furthermore HSP32 was significantly higher immediately post
163 EXB2 compared with immediately before ($p = 0.018$) regardless of condition. The concentration of
164 protein carbonyl did not change significantly from rest throughout the testing period ($F = 1.920$ $p =$
165 0.174), and was comparable between conditions ($F = 1.476$, $p = 0.249$) (Table 2).

166

167 **Discussion**

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

172 min protocol of EXB2. Hypoxia is another stimulus that can result in ubiquitin- proteasome mediated
173 protein degradation, via induction by hypoxia-inducible factor (HIF) (Brahimi-Horn and Pouyssegur,
174 2005). Taylor et al (2012) reported a significantly attenuated HSP72 response post-exercise (~50%) after
175 basal HSP72 expression was elevated via a hypoxic exposure, therefore it could have been expected that
176 the trial with the lower basal HSP72 (BICARB) prior to EXB2 would have resulted in increased HSP72
177 expression following the bout. However it must be noted that the average changes in basal levels between
178 experimental conditions in the research from Taylor et al (2012) was higher than in the present study
179 (67% and 31% respectively). Therefore it may be the case that the HSP72 response to EXB1 in this study
180 was not of a high enough magnitude to influence the response to EXB2.

181

182 As other authors have shown that increasing the level of HSP72 prior to exercise can improve defences
183 to the oxidative damage associated with exercise (Taylor et al., 2012), both HSP32 and protein carbonyl
184 were measured prior to and after EXB2. Monocyte HSP32 was induced after EXB2 to the same extent
185 in both conditions. This is in contrast to the HSP32 response to the same 90-min exercise bout in previous
186 research, which was attenuated under BICARB conditions (Peart et al. 2013b). In the current study acid-
187 base values had returned to homeostasis prior to EXB2 resulting in comparable acid-base results between
188 conditions (Table 1), whereas in the previous study blood pH, base excess and HCO₃ were significantly
189 different between conditions prior to and throughout the exercise. This finding supports our previous
190 suggestions that increased oxidative damage is not necessarily the only stimulus for increased HSP32
191 and that this particular HSP is highly sensitive to acid-base changes in vivo (Peart et al. 2013b) as well
192 as in vitro (Christou et al, 2004).

193

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

202 ultra-distance cycling (Tauler et al., 2003) and running (Radak et al., 2000). Despite this trend seen in
203 previous research, protein carbonyl did not increase at any time point throughout the testing period in
204 this study nor was any difference observed between conditions. The only other study reporting no
205 increase in protein carbonyl following aerobic exercise utilised a 90-min cycling protocol at 70% of
206 maximum (Morillas-Ruiz et al., 2005). Fisher-Wellman and Bloomer (2009) have suggested that studies
207 reporting null findings for protein carbonyl may be due to insufficient sampling times, exercise protocols
208 that are too short or physical training status of the participants. Additionally, some authors have reported
209 peaks in protein carbonyl occurring around 4-6 hours post-exercise (Bloomer et al., 2005, Michailidis et
210 al., 2007), therefore it is possible that sample timings in this study may have contributed to no observed
211 increases in protein carbonyl, as the final blood sample was 60-min post EXB2, and Morillas-Ruiz et al,
212 (2005) only took a sample 20-min post exercise. However, other authors have demonstrated peaks in
213 protein carbonyl immediately post-exercise of a similar duration to the current study (Bloomer 2007a,
214 Sureda et al, 2013). Finally the training status is unlikely to have resulted in the absence of an increased
215 protein carbonyl from rest in this study as the participants were less physically active compared with
216 other studies, and lesser trained individuals have been shown to have greater protein carbonyl responses
217 following exercise (Tauler et al., 2003). In the current study participants cycled predominantly at 40-
218 60% of maximum during EXB2, whereas Bloomer et al, (2007a) used an intensity of 70% and
219 Michailidis et al, (2007) 70-90% maximum suggesting the current protocol may not have been a high
220 enough stimulus to elicit increases in protein carbonyl. However Morillas-Ruiz et al, (2005) also applied
221 an intensity of 70% with null findings. Furthermore increases in oxygen consumption are unlikely to be
222 the sole cause of protein carbonyl increases following exercise as authors have at times demonstrated
223 increases in protein carbonyl following exercises of relatively low demands compared to sub-maximal
224 exercise (Alessio et al., 2000, Bloomer et al., 2005). In summary we witnessed no increase in protein
225 carbonyl concentration following either exercise bout, which is in contrast to previous work using similar
226 exercise modalities/duration. The reason for this is unclear as this study also had similar sampling times
227 following exercise to other studies, a factor which has been suggested to contribute to null findings
228 (Fisher-Wellman and Bloomer, 2009).

229

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

232 discussion, the magnitude of the response may have been inadequate to influence HSP72 expression
233 following EXB2, and it is unclear whether an increase similar to the original study would have had more
234 effect upon the subsequent response. This draws attention to whether documenting the stress response
235 on a single day of training is necessarily fully reflective of the role of HSP72 in sport and exercise and
236 future investigations should document intra-individual variance in the response to acute exercise. Other
237 authors have shown that protein damage can progressively increase over several days training, as can the
238 intracellular HSP72 response to exercise (Whitham et al., 2004). Therefore future work may also
239 consider further examining the effect of pre-exercise mediated alterations in the response of HSP72 by
240 documenting the implications of manipulating the HSP72 stress response over a longer period training,
241 and any subsequent effect upon exercise performance and recovery times. This would allow further
242 insight into whether chronic attenuation of HSP72 would have additional implications during a
243 presumably more stressful period, and may inform future work investigating the hormetic nature of
244 oxidative stress for training adaptation (Powers et al, 2010). From a practical viewpoint these findings
245 suggest that individuals choosing to ingest NaHCO₃ acutely for its possible ergogenic effect (Peart et al.
246 2012) do not risk interfering with the physiological stress response to a subsequent exercise in the same
247 day.

248

249 **Acknowledgments**

250 The authors wish to thank those who volunteered for the study, and declare that the research project was
251 internally funded.

252

253 **Ethical approval**

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

257

258 **Compliance with Ethical Standards**

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

260

261

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380 **Fig 1** Schematic of the experimental trials

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

385

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

388

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

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Table 1 Acid-base characteristics pre- and post-NaHCO₃ 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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