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DOI:
10.3109/10715762.2015.1131824

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Document Version
Peer reviewed version

Citation for published version (Harvard):
Aldred, S, Turner, J & Wadley, A 2016, 'Factors influencing post-exercise plasma protein carbonyl concentration', *Free Radical Research*, vol. 50, no. 4, pp. 375-384.
https://doi.org/10.3109/10715762.2015.1131824

Factors influencing post-exercise plasma protein carbonyl concentration

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Running Head: Protein carbonyl groups and exercise

Word Count: 2967

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Abstract

Exercise of sufficient intensity and duration can cause acute oxidative stress. Plasma protein carbonyl (PC) moieties are abundant, chemically stable and easily detectable markers of oxidative stress that are widely used for the interpretation of exercise-induced changes in redox balance. Despite many studies reporting acute increases in plasma PC concentration in response to exercise, some studies, including those from our own laboratory have shown decreases. This review will discuss the differences between studies reporting increases, decreases and no change in plasma PC concentration following exercise in humans; highlighting participant physiology (i.e. training status) and study design (i.e. intensity, duration and novelty of the exercise bout) as the main factors driving the direction of the PC response to exercise. The role of the 20S proteasome system is proposed as a possible mechanism mediating the clearance of plasma PC following exercise. Resting and exercise-induced differences in plasma protein composition and balance between tissues are also discussed. We suggest that exercise may stimulate the clearance of plasma PC present at baseline, while simultaneously increasing reactive oxygen species production that facilitates the formation of new PC groups. The balance between these two processes likely explains why some studies have reported no change or even decreases in plasma PC level post-exercise when other biomarkers of oxidative stress (e.g., markers of lipid peroxidation) were elevated. Future studies should determine factors that influence the balance between PC clearance and formation following acute exercise.

Keywords: Protein oxidation, exercise, proteasome, protein degradation, reactive oxygen species

Introduction

Exercise can induce a wide range of whole body physiological adaptations that improve metabolic health and lower oxidative stress [1–3]. Oxidative stress is a biological state whereby reactive oxygen species (ROS) overwhelm antioxidant defences, increasing the oxidation of proteins, lipids and DNA. It is widely accepted that transient increases in exercise-induced ROS can initiate a diverse range of signalling pathways that lead to adaptation [4–6]. Indirect biomarkers of exercise-induced oxidative stress, such as protein oxidation [7–9], lipid peroxidation [8–10] and antioxidant capacity [8,11,12] are routinely measured to give an indication of altered redox balance. One of the most frequently examined biomarkers of protein oxidation is plasma protein carbonyl (PC) concentration. Carbonylation is a stable and quantifiable post-translational protein modification which is ten times more abundant than other protein adducts, such as 4-Hydroxynonenal and glycooxidation end-products [13,14]. Most biomarkers of oxidative stress increase in plasma in response to exercise [11,12,15], as would be expected following an acute bout of increased metabolic activity. However some studies have reported decreases in plasma PC concentration post-exercise alongside increases in other biomarkers of oxidative stress [8,16,17]. The focus of this review is to explore key physiological factors that might explain these different responses, with a primary emphasis on aerobic, steady state exercise bouts where PC groups have been measured in blood plasma or serum of human participants exercising under fasted conditions. It is beyond the scope of this review to include the results of studies that have examined the effect of habitual diet or dietary supplementation (e.g., high dose antioxidants) on resting or exercise-induced changes in PC levels.

Protein carbonyl formation

PC groups are present in all proteins (carboxylic acid (-COOH) groups), form the basis of their structural integrity, and influence their capacity to function and interact with other molecules. Formation of additional, non-native PC groups can be a result of a variety of irreversible, non-enzymatic oxidative pathways (*Figure 1)* that are a normal part of metabolic processes [18]. These include direct oxidation of amino acids (in conjunction with oxidising agents such as transition metal clusters in protein structures) and secondary oxidation reactions with lipid peroxidation or glucose-protein oxidation products [19]. PC groups are highly polar, thus increasing the proteins susceptibility to further oxidation and/or formation of cross-linkages and protein aggregates (via ketone or aldehyde links) [20]. Excessive introduction of new PC groups can result in altered or disrupted protein function and exposure of previously embedded hydrophobic groups in the protein core, resulting in targeted proteolytic degradation by 20S and 11S proteasome systems [20,21]. The degree of PC formation is dependent on the presence of conjugated metals (e.g., iron [19]), the orientation of specific amino acids that are more susceptible to PC formation (i.e., Proline, Arginine, Lysine, and Threonine) and importantly, the magnitude and origin of ROS production in relation to the protein [22].

[Insert Figure 1 here]

Exercise-induced changes in protein carbonylation

A variety of cells can produce ROS in response to exercise, most notably myocytes [23], leukocytes [24] and endothelial cells [25]. Superoxide ($O_2^{-\cdot}$) is produced from a range enzymatic sources within these cell types during exercise, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase and nitric oxide synthases [26]. These cells can also release $O_2^{-\cdot}$ into the extracellular space via enzymes expressed on the

plasma membrane such as NADPH oxidase [26–28] or via passive diffusion of uncharged ROS, such as hydrogen peroxide [29]. Consequently, plasma proteins are susceptible to the formation of PC groups during exercise [7,9,30,31], with evidence that new PC moieties are stable in plasma for up to 4 hours, before selective degradation [32,33]. Increased formation of plasma PC groups following exercise is considered to be a non-specific reflection of increased systemic oxidative stress (i.e. the origin of ROS and biological impact is unknown). As a result, many studies over the last 15 years have investigated associations between changes in plasma PC concentration and aspects of the acute physiological stress caused by exercise, in a variety of populations [7,9].

Factors influencing post-exercise changes in protein carbonylation

Exercise intensity

Aside from the potential for greater production of ROS from the cellular sources discussed above, specific hypoxic mechanisms can also contribute during high intensity exercise. Repetitive cycles of temporary occlusion (hypoxia) and re-oxygenation of the blood vessels surrounding actively contracting muscle can produce large quantities of ROS via the enzyme xanthine oxidase [34], that may increase PC formation. Previous studies have reported increases in plasma PC concentration following high-intensity exercise to exhaustion relative to baseline values [12,31,35–37]. Lamprecht et al [30] assessed the impact of exercise intensity on plasma PC formation by examining three 40-minute cycling bouts of different intensities (70, 75 and 80% $\dot{V}O_{2max}$) in three independent groups of moderately active participants (*Table 1).* Plasma PC concentration increased in response to cycling at 80% $\dot{V}O_{2max}$ only, suggesting that exercise intensity is a determinant of the formation of new non-native carbonyl moieties in the bloodstream. However it is likely that exercise duration and other physiological factors also have an impact upon these processes.

Exercise duration

There is some evidence to suggest that exercise duration is also a key factor in post-exercise plasma PC formation. Bloomer et al [7] reported that 120 minutes of cycling at 70% $\dot{V}O_{2max}$ caused a greater increase in post-exercise plasma PC concentrations than 30 and 60-minute bouts of the same exercise intensity in male and female participants. Moreover, an exercise bout of moderate intensity, but long duration (ultra-endurance running: 174 km, 30-44 hours) has been reported to elicit immediate and prolonged (7 days) post-exercise increases in plasma PC concentration [38]. This increased protein oxidation occurred simultaneously with a decline of exogenous and/or endogenous antioxidants, which may have reduced the capacity to clear PC groups within this seven day period. The roles of exercise intensity and duration on post-exercise PC concentrations are inevitably linked; however there is evidence that other physiological factors (e.g., training status) contribute [7,15].

Training status and habituation to exercise

Exercise-induced ROS production can initiate a cascade of cellular signals which result in various post-translational modifications (i.e. phosphorylation, acetylation and thiol modifications), and up-regulate the expression of antioxidant and stress proteins following exercise [4,39,40]. Differences in the resting expression of antioxidant proteins between participants (i.e. due to training status and/or habituation to exercise) will no doubt have consequences for changes in exercise-induced oxidative stress. Indeed, it has been demonstrated that exercise training can stimulate an increased expression of endogenous antioxidant proteins [5,41], with some direct evidence of this in humans [42]. However, it is largely unclear how much resting antioxidant capacity impacts upon the acute oxidative stress response to exercise. Elevated endogenous antioxidant capacity may enable a buffering

of ROS production subsequently reducing the magnitude of oxidative stress biomarker formation during exercise in trained individuals [43]. Bloomer et al [7] reported increases in plasma PC concentration immediately following cycling exercise (30 minutes at 70% $\dot{V}O_{2max}$) (*Table 1)*, but in a separate study, with different research participants, reported no change in plasma PC level following an identical cycling protocol [15]. Differences in the physiology and exercise training habits of the participants featured in these studies, rather than the intensity and duration of the exercise bout are likely to have influenced the differential net changes in PC post-exercise. For example, there were clear differences in both the aerobic fitness ($\dot{V}O_{2max}$: 57 ± 5 ml/kg/min [7] vs. 45 ± 8 ml/kg/min [15]) and time engaged in aerobic exercise prior to the study (10.4 ± 1.6 hours/week [7] vs. 2.8 ± 2.2 hours/week [15]). In addition, the participants in the study published by Bloomer et al, 2005 [15] were more resistance (3.8 ± 1.8 hours/week), than aerobically trained. This suggests that the exercise stimulus implemented in both studies was more novel for the participants in the 2005 study [15]. This supports some previous work reporting a greater magnitude of oxidative stress biomarker formation following unaccustomed exercise [44,45]. Thus, a combination of factors such as the novelty of exercise, and aspects of study design (i.e., exercise intensity and duration) are likely to interact to govern the magnitude of increase in plasma PC formation following exercise. However, it is perhaps more challenging to explain why *decreases* in plasma PC level have been observed.

Studies reporting *decreases* in plasma PC concentration after exercise

The available evidence suggests exercise intensity [30] and duration [7] influence the magnitude of ROS production and the associated increase in biomarkers of oxidative stress. It is therefore not surprising that many studies have reported no change in plasma PC concentration following sub-maximal exercise [15,46–50]. However, counter-intuitively,

many studies report a *decrease* in plasma PC concentration following both sub-maximal and maximal exercise [8,16,17,51] (*Table 1).* Importantly, these changes have sometimes been reported alongside increases in other biomarkers of oxidative stress [8,17]. This is unexpected and implies that exercise stimulates clearance processes alongside exercise-induced ROS production.

We have shown that steady state submaximal cycling (60% and 80% $\dot{V}O_{2max}$ for 27 [moderate intensity] and 20 minutes [high intensity] respectively) undertaken by untrained males ($\dot{V}O_{2max}$; 42.7 ± 5.0 ml/kg/min) elicits a 9% (moderate intensity, p<.0001) and 4% (high intensity, p<.0001) mean decrease in plasma PC concentration (see figure 2: A and B) [8]. Importantly, these changes occurred in parallel with increases in plasma lipid hydroperoxides (LOOH), total antioxidant capacity (TAC) and cellular markers of oxidative stress [52]. Interestingly, the participants in this study engaged in less than 3 hours of generic aerobic exercise per week, indicating that these bouts were relatively unaccustomed. These findings are not limited to just moderately trained individuals. We have also shown a 7% decrease (p=.016) in plasma PC concentration immediately after a bout of cycling exercise (70% $\dot{V}O_{2max}$, 75 min) in highly trained cyclists ($\dot{V}O_{2max}$; 63.7 ± 5.3 ml/kg/min) (Wadley et al, 2015; In Preparation; see figure 2D). Finally, our findings are not limited to sub-maximal exercise: we have also shown a 10% mean decrease (p=.002) in plasma PC concentration immediately after a graded exercise test to volitional exhaustion (i.e., 100% $\dot{V}O_{2max}$, approximately 15-minutes) in very active young men ($\dot{V}O_{2max}$; 61.9 ± 4.7 ml/kg/min; see Figure 2C) (Turner *et al;* unpublished data) and a 13% mean decrease (p<.0001) following a bout of low volume high intensity interval exercise (10 × 1 minute stages at 90%$\dot{V}O_{2max}$, with 9 × 1 minute rest intervals at 40% $\dot{V}O_{2max}$) in moderately active participants ($\dot{V}O_{2max}$; 42.7 ± 5.0 ml/kg/min) [8].

There are a number of other studies that report (but do not always comment upon) decreases in plasma PC levels following exercise (Table 1). For example, Chevion et al, [16] reported decreases in PC concentration following a study involving two walks (50km and 80km) of unreported intensity. The magnitude of this decrease in PC concentration was greater following the first walk compared to the second, highlighting again that the novelty of the exercise stimulus may be a key factor modulating post-exercise PC concentration. Furthermore, reductions in plasma PC level have been reported immediately [17,51] and up to four hours [17] following submaximal cycling exercise in moderately trained participants.

[Insert Figure 2 here]

Possible mechanisms mediating protein carbonyl clearance in response to exercise

The 20S proteasome system

The proteasome system is an organised assembly of proteins present in all cell types that functions to degrade irreversibly modified proteins, such as those containing carbonyl groups. The ubiquitin-independent 20S proteasome is the primary system in place to degrade oxidatively damaged proteins [21,32,53], with recent evidence also suggesting the 11S proteasome facilitates this process under conditions of heightened oxidative stress [20]. Increased exposure of carbonyl-mediated hydrophobic groups within the protein core can increase targeted degradation by these proteasome systems within cells. The 20S proteasome is also excreted into extracellular fluids such as plasma [54–56] and is known to be enzymatically functional [57], suggesting that it could cleave PC groups in plasma directly. Studies in humans have found that exercise can acutely increase ubiquitin-dependent proteasome gene expression in response to resistance [58–60] and ultra-endurance exercise [61]. However, the impact of acute shorter-duration aerobic-type exercise on the ubiquitin-

independent 20S and 11S proteasome subunits (either intra- or extra-cellular; expression or activity) and the relationship with plasma PC has not been investigated in humans. Data from rats has indicated that chymotrypsin-like activity of the ubiquitin-independent 20S proteasome subunit increases in brain tissue following 8 weeks of exercise overload (1 hour swimming/day, 5 days a week for 6 weeks) [62].

Exercise bouts below a certain intensity and/or duration, in combination with the other physiological parameters discussed above may activate intracellular and extracellular proteasome pathways to clear modified proteins and thus lower the concentration of PC groups in plasma (*Figure 3*). The net increase in PC level observed in response to high intensity or prolonged duration exercise may result from the formation of new PC groups outnumbering proteolytic clearance of those present at baseline. Furthermore, inferences from cell culture data [53] led Radak et al [63] to propose that proteasome activity may follow a hormetic-type response with increasing exercise-induced oxidative stress. Proteasome activity may be reduced at higher exercise intensities or after prolonged duration exercise, due to ROS-induced inactivation of the functional 20S or 11S proteasome. Future work is needed to explore this mechanism.

<u>Resting or exercise-induced differences in total plasma protein composition and balance between tissues</u>

Studies examining plasma PC concentration typically express results relative to the total protein concentration of plasma (i.e., nM of carbonyls per mg of protein) [64]. Measuring protein carbonylation in this way does not account for possible exercise-induced shifts in protein composition. This is relevant because certain plasma proteins, such as serotransferrin and fibrinogen (approximately 2-4% of total plasma proteins) are more susceptible to exercise-induced oxidation than other plasma proteins [18]. Therefore,

individual differences in baseline plasma protein composition may influence post-exercise changes in PC. Furthermore, exercise could induce shifts in the proportion of certain proteins in plasma, which could result in exaggerated or suppressed protein carbonylation.

Although not as susceptible to oxidation as fibrinogen, albumin, the most abundant plasma protein, has also been shown to exhibit a high concentration of PC groups [65]. The extent to which albumin becomes oxidised in response to exercise is dependent on intensity, and albumin carbonylation is marked at intensities of 80% $\dot{V}O_{2max}$ or more [30]. Inter-individual variation in exercise-induced proteinurea or albuminuria has been documented [66,67], and might affect the amount and type of plasma proteins that can be oxidised during exercise. Furthermore, it has been shown that during and following both aerobic and resistance exercise, there is an increase in the uptake and turnover of proteins such as albumin and fibrinogen by muscle [68,69]. Thus, differences in PC excretion and balance between tissues might explain to some degree, alterations in the composition of plasma proteins, which may increase or decrease plasma PC level.

Experimental approaches for monitoring changes in plasma protein carbonyl level

The evidence presented in this review highlights the uncertainty with regards to the mechanistic underpinnings of post-exercise decreases in plasma protein carbonylation. Despite the factors discussed, experimental design and the analytical techniques used to quantify protein carbonyl concentration in the cited studies warrants discussion.

The majority of studies included in *Table one* controlled for factors such as age, training status, dietary habits (i.e. fasted exercise trials), and the time of day that exercise was undertaken. All of these variables have been shown to alter protein balance through changes in protein uptake to skeletal muscle during exercise [68,69], highlighting the rigorous experimental approach taken in these studies. However, an important consideration is the

timing of blood sampling. Many of the studies presented assessed protein carbonyl concentration immediately following cessation of the exercise bout. It is conceivable that changes within the minutes and hours after exercise might give more insight into the 'conundrum' of post-exercise changes in protein carbonyl level. Indeed, independent studies have shown increases [70] and decreases [17] in protein carbonyl concentration up to 4 hours post-exercise. In this regard, it is clear that extensive timecourse analysis of protein carbonyl concentration is needed to validate these findings and importantly, to elucidate the mechanisms influencing decreases following exercise.

It is important to note that a variety of analytical techniques are used to quantify protein carbonylation in the literature. These have primarily included spectrophotometric and ELISA-based methods, which have recently come under scrutiny with regards to their sensitivity [71] and reproducibility [72]. However, while these studies provide an example of an important limitation of quantifying protein carbonyl concentration, they do not explain why protein carbonyl concentration might decrease following exercise. Furthermore, with independent laboratories now reporting decreases in protein carbonyl level following exercise [8,16,17,51], this reduces the chances of experimental variability causing this effect.

[Insert Figure 3 here]

Conclusions

Plasma protein carbonyl concentration is a marker of oxidative stress routinely used to assess exercise-induced redox regulation. The evidence presented in this review suggests that certain exercise conditions can result in a net *decrease* in plasma PC concentration following exercise, which occurs in parallel with increases in other biomarkers of oxidative stress. Exercise intensity (>70% $\dot{V}O_{2max}$) and prolonged duration (>60 minutes) appear to be

the main contributing factors in the observed post-exercise increases in PC concentration. The factors influencing *decreases* in protein carbonyl level are more difficult to interpret, but likely involve the clearance of oxidised proteins from plasma, potentially by plasma proteasomes, excretion, or uptake into active tissues. Studies wishing to assess markers of oxidative stress in response to exercise should assess PC concentration together with other biomarkers over an extended time course (immediately after and up to 4 hours post-exercise) for a true representative assessment [31]).

Acknowledgments

All authors contributed to the following aspects of this study: conception and outline of the brief review, collection and analysis of the unpublished data presented, and drafting the article and revising it critically for important intellectual content.

Declaration of Interest

None of the authors declare a conflict of interest and have no financial interest in the study.

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Figure Legends

Figure 1: Formation of non-native carbonyl groups in proteins. Non-native carbonyl groups can be introduced into proteins by; (1) direct oxidation of amino acids, (2) via secondary oxidation products of lipid peroxidation, and (3) the oxidation products of reducing sugars. An example carbonyl modification is included for each pathway; (A) (2-amino-3-ketobutyric acid, (B) malondialdehyde-Lysine adduct, and (C) carboxymethyl lysine (3)). The non-native carbonyl group in each example is indicated by a dashed circle around the C=O bond of the amino acid or amino acid side chain (adduct).

Figure 2: Published and unpublished data indicating decreases in protein carbonyl concentration (nM/mg protein) in response to exercise. PC level decreases during the last minute (End-Exercise) (A) 27 minutes of cycling at 60% $\dot{V}O_{2max}$ and (B) 20 minutes of cycling at 80% VO_{2max} in untrained young men (n=10; mean ± SD: age 22 ± 3 yrs; $\dot{V}O_{2max}$ 42.7 ± 5.0 ml/kg/min) [8]; C) PC level decreases immediately following a $\dot{V}O_{2max}$ test to exhaustion in trained young men (n=10; mean ± SD: age 23 ± 3 yrs; $\dot{V}O_{2max}$ 61.9 ± 4.7 ml/kg/min) (Turner & Aldred; unpublished work); D) PC level decreases immediately following 75 minutes of cycling at 70% $\dot{V}O_{2max}$, and is elevated above baseline levels 2 hours post-exercise in trained young men (n=12; mean ± SD: age: 28 ± 4 years, $\dot{V}O_{2max}$ 63.7 ± 5.3 ml/kg/min) (Wadley et al, 2015; *in preparation*). Individual (grey bars) and mean (black line) PC concentration changes are reported. Mean percentage change and statistical significance are indicated above each graph. Paired samples T-tests (A-C) and repeated measures ANOVA (D) were performed using SPSS (PASW Statistics, 22.0).

Figure 3: Proposed formation/ clearance of protein carbonyl groups in response to exercise in humans. Exercise can cause an increase in plasma protein carbonylation when the exercise bout is of sufficient intensity and/or duration. Activation of clearance mechanisms may drive a decrease in PC groups present at baseline when certain exercise conditions are met.