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Passive smoking reduces and vitamin C increases exercise-induced oxidative stress: Does this make passive smoking an anti-oxidant and vitamin C a pro-oxidant stimulus?

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ABSTRACT

The current interpretative framework states that, for a certain experimental treatment (usually a chemical substance) to be classified as "anti-oxidant", it must possess the property of reducing (or even nullifying) exercise-induced oxidative stress. The aim of the study was to compare side by side, in the same experimental setup, redox biomarkers responses to an identical acute eccentric exercise session, before and after chronic passive smoking (considered a pro-oxidant stimulus) or vitamin C supplementation (considered an anti-oxidant stimulus). Twenty men were randomly assigned into either passive smoking or vitamin C group. All participants performed two acute eccentric exercise sessions, one before and one after either exposure to passive smoking or vitamin C supplementation for 12 days. Vitamin C, oxidant biomarkers (F_2-isoprostanes and protein carbonyls) and the non-enzymatic antioxidant (glutathione) were measured, before and after passive smoking, vitamin C supplementation or exercise. It was found that chronic exposure to passive smoking increased the level of F_2-isoprostanes and decreased the level of glutathione at rest, resulting in minimal increase or absence of oxidative stress after exercise. Conversely, chronic supplementation with vitamin C decreased the level of F_2-isoprostanes and increased the level of glutathione at rest, resulting in marked exercise-induced oxidative stress. Contrary to the current scientific consensus, our results show that, when a pro-oxidant stimulus is chronically delivered, it is more likely that oxidative stress induced by subsequent exercise is decreased and not increased. Reversely, it is more likely to find greater exercise-induced oxidative stress after previous exposure to an anti-oxidant stimulus. We believe that the proposed framework will be a useful tool to reach more pragmatic explanations of redox biology phenomena.

1. Introduction

In chemistry, oxidants are defined as elements or compounds in an oxidation–reduction (redox) reaction that accept electrons from another species, whereas reductants are defined as elements or compounds that donate electrons to another species. For their property to act as electron donors, reductants in biology are referred to as "anti-oxidants", whereas oxidants retain their "chemical" name, unless the substance in question is not a free radical per se (albeit it can promote oxidation), in that case referred to as "pro-oxidants". Considering the very high rate of redox reactions and the inherent complexity of living biological organisms, monitoring redox reactions in vivo is essentially infeasible. To overcome this obstacle, redox biologists invented the term "oxidative stress" to follow the effects of pro-oxidants and anti-oxidants in living biological organisms. Due to the elusive nature of free radicals and their difficulty to be directly assessed, a common practice in redox biology is to measure the levels of redox biomarkers (both oxidant and anti-oxidant). Most researchers use the term "oxidative stress" to indicate "an increase in the level of reactive species and/or oxidant biomarkers" [1]. In this context, relevant review and opinion papers have been published, providing methodological guidelines for redox biomarkers assessment and recommendations on data interpretation on the basis of which alterations in redox biomarkers indicate oxidative stress (e.g.,

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[1]). These theory-based interpretative frameworks assume a stimulus (ranging from a chemical substance to physical activity) as a pro-oxidant, if it increases the levels of oxidant biomarkers and/or decreases the levels of anti-oxidant biomarkers, or as an anti-oxidant, if it does the opposite.

Recently, a central concept has emerged supporting that, to define the mechanisms regulating health and organismal performance, monitoring the responses to a homeostatic challenge is more informative than static homeostatic measures (e.g., [2]). Many studies have used either pro-oxidant (e.g., [3]) or anti-oxidant (e.g., [4]) stimuli to test the robustness and elasticity of functions involved in maintaining redox homeostasis. Acute exercise is probably the most commonly used physiological stimulus generating an oxidative stress response [1]. It is very common that an acute exercise session is performed before and after a redox treatment (e.g., an anti-oxidant supplement) to facilitate the disclosure of a redox effect in vivo (e.g., [5]). The current framework states that, for a certain experimental treatment (usually a chemical substance) to be classified as "anti-oxidant", it must possess the property of reducing (or even nullifying) exercise-induced oxidative stress, compared to that appeared before the treatment (e.g., [6]).

Noteworthily, the appropriateness of the current framework in redox biology is mainly based on intuition and theoretical considerations. To our knowledge, original data on a direct in vivo comparison between a supposed pro-oxidant and/or anti-oxidant stimulus are lacking. In human research, the most frequently used anti-oxidant stimulus is probably vitamin C. This is mainly because consumption of vitamin C above the recommended values is considered safe and its antioxidant capacity in vitro is undisputed, although pro-oxidant effects of vitamin C in vivo have also been described [7]. On the other hand, smoking is probably the most well investigated pro-oxidant stimulus in humans [8,9]. However, for ethical reasons, the use of smoking as an oxidant stimulus is restricted to smokers, thus limiting its applicability in research. Passive smoking may be an appropriate replacement for smoking but, to our knowledge, no study has yet investigated the effects of passive smoking on oxidative stress in comparison to an anti-oxidant stimulus. Thus, it becomes apparent that the lack of direct and well controlled in vivo comparisons between pro-oxidants and anti-oxidants before and after an exercise stimulus is raising serious doubts whether the current framework is appropriate for studying human redox responses, either at rest or after exercise. Therefore, the aim of the present study was to compare side by side, in the same experimental setup, redox biomarkers responses to an identical acute eccentric exercise session (considered a potent pro-oxidant stimulus; [10]), before and after chronic passive smoking or vitamin C supplementation. A main objective of this paper is to propose another interpretative framework within which researchers can assess more realistically the alterations in redox biomarkers after exercise.

2. Materials and methods

2.1. Participants

Twenty untrained men were randomly assigned into either passive smoking (22.6 ± 0.9 years; 73.1 ± 1.6 kg; mean ± SEM) or vitamin C group (22.8 ± 1.1 years; 72.2 ± 1.4 kg). The participants were asked to recall whether they had participated in regular exercise or in unaccustomed and/or heavy exercise in the 3 months before the study entry. Individuals who reported participation in such activities were precluded from the study. Smoking and consumption of nutritional supplementation the last 3 months before the study initiation were also exclusion criteria to participate in the present investigation. Volunteers were instructed to abstain from any strenuous exercise during their participation in the study as well as for 5 days prior and 2 days following the exercise session. A written consent was obtained from all participants. The procedures were in accordance with the Helsinki declaration of 1975, as revised in 2000, and approval was received from the institutional review board (016/12-05-2013).

2.2. Study design

An overview of the study design is shown in Fig. 1. All participants performed the first acute isokinetic eccentric exercise bout with the knee extensors of one leg. Plasma, erythrocytes and urine were collected at immediately before (day 0) and 48 h post-exercise (day 2), since previous studies have shown that oxidative stress peaks 1–3 days after eccentric exercise [1]. Fourteen days after, participants in the pro-oxidant group were exposed to passive smoking for 1 h, while the participants in the anti-oxidant group received oral supplementation of 1 g of vitamin C in a single dose at the time point "pre" (ascorbic acid; Lamberts Health Care Ltd., Kent, United Kingdom). Before and 1 h after exposure to passive smoking, or before and 1 h after supplement consumption plasma, erythrocytes and urine were collected. During the next 12 days, participants in the pro-oxidant group were exposed to passive smoking for 1 h daily, while the participants in the anti-oxidant group received oral supplementation of 1 g vitamin C daily. Supplements were taken every 8 h in capsules containing 333 mg of vitamin C, in order to achieve high and sustained blood concentration throughout the day [11]. After the 12 days of exposure to passive smoking or vitamin C supplementation, all participants performed the second acute isokinetic eccentric exercise bout with the knee extensors of the other leg. Plasma, erythrocytes and urine were collected at immediately before (day 14) and 48 h post-exercise (day 16). Evaluation of oxidant biomarkers (F_2-isoprostanes and protein carbonyls), the non-enzymatic antioxidant (glutathione) and vitamin C was performed at all sample collection

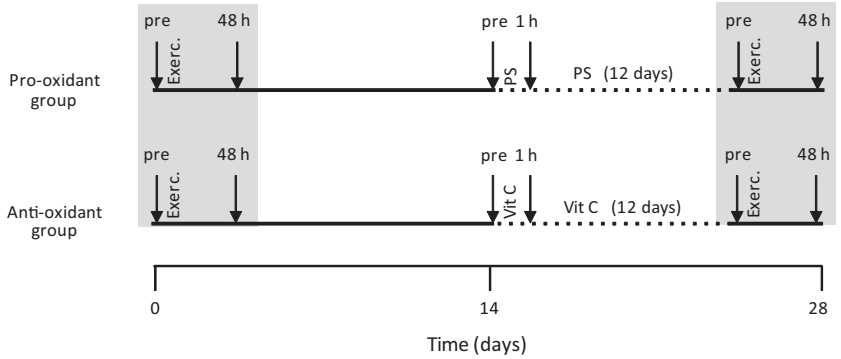

Fig. 1. Study design. Arrows indicate the time of body fluids collection. Exerc., exercise; PS, passive smoking; Vit C, vitamin C.

time points. Evaluation of muscle micro-damage (isometric torque and creatine kinase) was performed pre- and 48 h post-exercise only. Each volunteer was provided with a written set of instructions for monitoring dietary consumption, and with a record sheet for recording food intake.

2.3. Passive smoking exposure

During the exposure visits, subjects were instructed to remain seated at rest for 1 h inside a room (air temperature: 24 °C; humidity: 47%). The passive smoking exposure was adjusted at a carbon monoxide (CO) concentration of 23 ± 1 ppm to meet levels previously reported for bar/restaurant environments [12]. Gradients of gas concentrations and particle density were checked by continuous measurement of different areas inside the room by a CO90 (Martindale Electric Ltd., Watford, UK) CO–CO_2 analyzer. The desired CO concentration of the gas mixture was achieved by combustion of cigarettes from various popular brands as previously described [12].

2.4. Eccentric exercise protocol

The eccentric exercise session was performed on an isokinetic dynamometer (Cybex Norm, Ronkonkoma, NY). The exercise protocols were undertaken from the seated position (120° hip angle), after the participants were stabilized, according to the manufacturer's instructions. Participants completed 5 sets of 15 eccentric maximal voluntary contractions [knee range, 0° (full extension) to 90° flexion] at an angular velocity of 60°/s. A 2-min rest interval was used between sets.

2.5. Muscle micro-damage

The isokinetic dynamometer was used for the measurement of isometric knee extensor peak torque at 90° knee flexion. The average of the 3 maximal voluntary contractions with the exercised leg was recorded. To ensure that the subjects provided their maximal effort, the measurements were repeated, if the difference between the lower and the higher torque values exceeded 10%. There was a 2-min rest between isometric efforts. Creatine kinase was assayed in plasma in a Cobas Integra Plus 400 chemistry analyzer (Roche Diagnostics, Mannheim, Germany).

2.6. Collection and handling of body fluids

A blood sample was drawn from a forearm vein and collected in EDTA tubes. The blood was centrifuged immediately at 1370*g* for 10 min at 4 °C and the plasma was collected. The packed erythrocytes were lysed with 1:1 (v/v) distilled water, inverted vigorously and centrifuged at 4000*g* for 15 min at 4 °C. For urine sampling, spot samples were collected in a container. For standardizing urine dilution, creatinine levels were measured using a kit (Fisher Diagnostics, Middletown, USA). Body fluid samples were stored at −80 °C and thawed only once before analysis.

2.7. Redox biomarkers

A competitive immunoassay was used for the determination of F_2-isoprostanes in urine (Cayman Chemical, Charlotte, USA). Urine was purified using solid phase extraction cartridges. The purification and the subsequent ELISA assay were performed following the manufacturer's recommendations. There are some reservations regarding the validity and reliability of F_2-isoprostane ELISA kits [13]. Nevertheless, it has been shown that the kit employed in the present study for F_2-isoprostanes resulted in less variability among the ELISAs and reduced the positive analytical bias between the ELISA and LC/LC–MS/MS results in urine samples [14]. Plasma protein carbonyls and erythrocyte glutathione were determined spectrophotometrically, as previously described [10]. Oxidation of GSH was prevented using *N*-ethylmaleimide, which is widely considered the most appropriate blocking agent for preventing glutathione ex vivo oxidation. Vitamin C was measured spectrophotometrically in plasma by using a ferric reducing ascorbate assay kit (K671-100) from BioVision (Mountain View, CA) with the use of meta-phosphoric acid as stabilizer.

2.8. Statistical analysis

Differences on physical characteristics between the groups at baseline were examined by unpaired Student's *t*-test. A two-way ANOVA (group × time) with repeated measurements on time was used to examine the effect of passive smoking exposure for 1 h or acute vitamin C supplementation (0 and 1 h post). Two separate two-way ANOVAs (group × time) with repeated measurements on time were used to evaluate the effect of the first and second exercise session (pre- and 48 h post-exercise) in the pro-oxidant and the anti-oxidant group. If a significant interaction was obtained, pairwise comparisons were performed through the Sidak test. Data are presented as mean ± SEM. The level of significance was set at $\alpha = 0.05$. The SPSS version 18.0 was used for all analyses (SPSS Inc., Chicago, Illinois).

3. Results

3.1. Physical characteristics and dietary intake

No differences in physical characteristics at baseline between the 2 groups were observed. No significant differences were found in daily energy and macronutrient intakes between the 2 groups at baseline, pre- and post-exercise (data not shown).

3.2. Muscle micro-damage

For muscle torque and creatine kinase activity, a main effect of time ($P < 0.001$) appeared after both the first and the second exercise session (data not presented). In both exercise sessions, muscle torque was decreased and creatine kinase activity increased 48 h post-exercise, compared to the pre-exercise values in both the pro-oxidant and the anti-oxidant group.

3.3. Vitamin C

A significant group × time interaction ($P < 0.05$) and main effect of time ($P < 0.05$) concerning vitamin C concentrations were observed 1 h after the exposure to passive smoking for 1 h or vitamin intake. Vitamin C concentration was significantly higher in the anti-oxidant group at the pre-exercise time point compared to the pro-oxidant group (Fig. 2). Acute exercise did not significantly affect vitamin C concentration in either exercise sessions in both the pro-oxidant and the anti-oxidant group.

3.4. Redox biomarkers

Regarding F_2-isoprostanes (Fig. 3A), a significant group × time interaction ($P < 0.001$) appeared after the exposure to passive smoking for 1 h or vitamin intake. F_2-isoprostanes levels increased 1 h post in the pro-oxidant group, whereas F_2-isoprostanes decreased in the anti-oxidant group. After the first exercise session, a significant main effect of time ($P < 0.001$) in F_2-isoprostanes were observed. Levels of F_2-isoprostanes increased similarly in both the pro-oxidant and the anti-oxidant group. After the second exercise

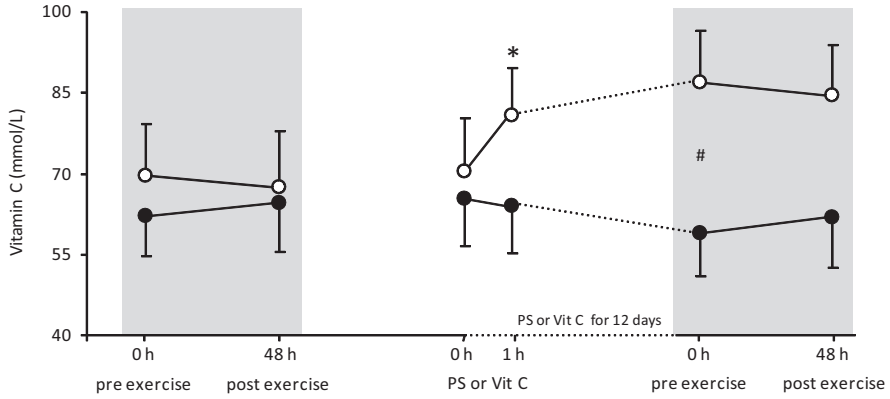

Fig. 2. Plasma vitamin C concentration in the passive smoking (closed circles) and vitamin C (open circles) groups (mean ± SEM). #Significantly different from the vitamin C group at the same time point. *Significantly different from the baseline (0 h) value in the same group.

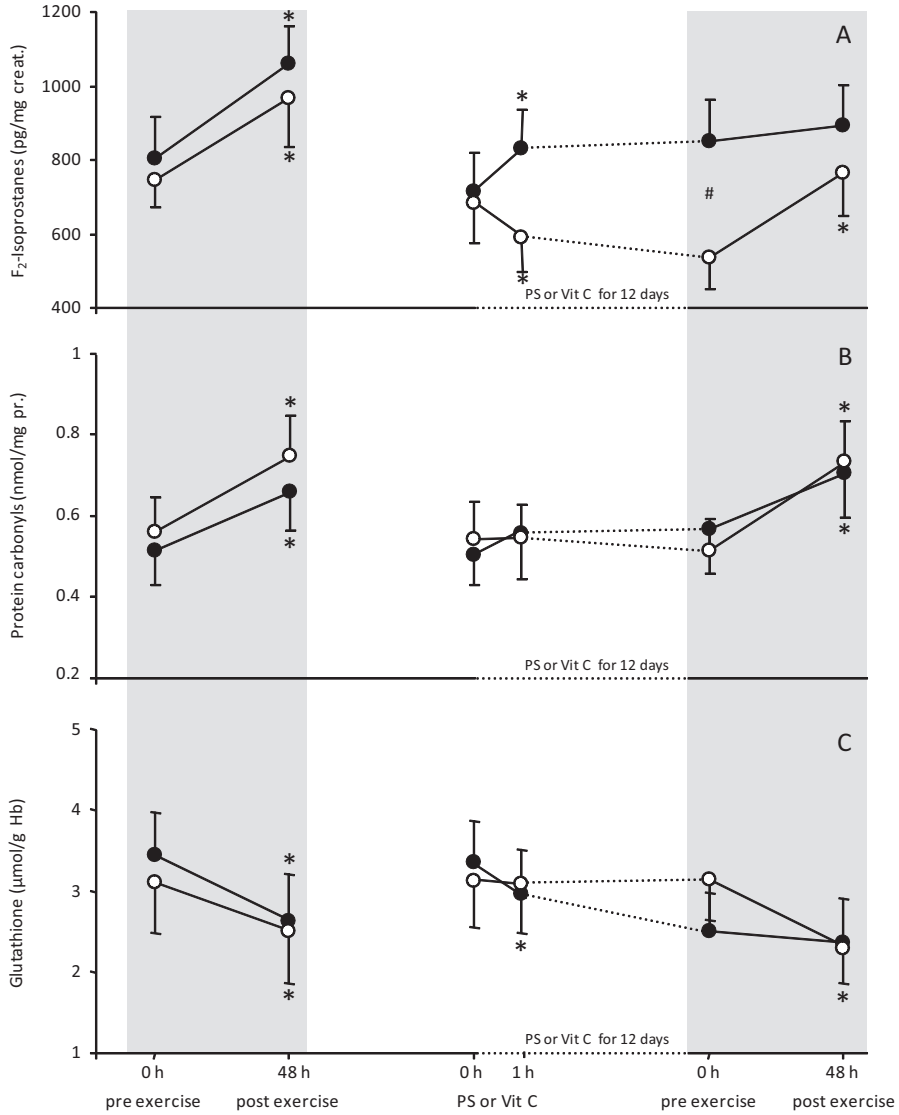

Fig. 3. Urine F_2-isoprostanes (A), plasma protein carbonyls (B) and erythrocyte glutathione (C) concentration in the passive smoking (closed circles) and vitamin C (open circles) groups (mean ± SEM). #Significantly different from the vitamin C group at the same time point. *Significantly different from the baseline (0 h) value in the same group.

session, a significant main effect of time ($P < 0.05$) and group - × time interaction ($P < 0.05$) in F_2-isoprostanes were observed. Pre-exercise F_2-isoprostanes levels were lower in the anti-oxidant group compared to the pro-oxidant group ($P < 0.05$). After the second exercise session, F_2-isoprostanes levels significantly increased only in the anti-oxidant group. For protein carbonyls (Fig. 3B), a

main effect of time ($P < 0.05$) appeared after both the first and the second exercise session. In both groups, protein carbonyls levels increased 48 h post exercise, compared to the pre-exercise values. Regarding glutathione (Fig. 3C), after exposure to passive smoking for 1 h or vitamin C intake, a main effect of time ($P < 0.05$) was observed. More specifically, glutathione levels decreased only after passive smoking compared to the pre-exposure value. After the first exercise session, a significant main effect of time ($P < 0.05$) was observed. Levels of glutathione decreased similarly in both the pro-oxidant and the anti-oxidant group. After the second exercise session, a significant main effect of time ($P < 0.05$) and group $\times$ time interaction ($P < 0.05$) were observed. Glutathione decreased 48 h post-exercise compared to the pre-exercise value only in the anti-oxidant group.

4. Discussion

To our knowledge, this is the first attempt to examine whether the current theory-based framework to interpret alterations in redox biomarkers after exercise is experimentally valid. To this end, we directly examined the effects of acute exercise-induced oxidative stress on participants that have been previously exposed to either a pro-oxidant stimulus (i.e., passive smoking) or an anti-oxidant stimulus (i.e., vitamin C supplementation) for 12 days. We found that chronic exposure to passive smoking (and consequently, higher level of F_2-isoprostanes and lower level of glutathione at rest) resulted in absence of oxidative stress after exercise, contrary to the current scientific consensus. Conversely, chronic supplementation with vitamin C (and consequently, lower level of F_2-isoprostanes and higher level of glutathione at rest) resulted in marked exercise-induced oxidative stress. If these findings are explained within the current framework, a rather confusing conclusion is reached that passive smoking acted as an anti-oxidant stimulus, by reducing exercise-induced oxidative stress, and vitamin C as a pro-oxidant stimulus, by increasing exercise-induced oxidative stress. To our opinion, this is a clear case of the limitations of the current interpretative approach, and below, we attempt to provide a novel framework for more realistically explaining alterations in redox biomarkers after exercise.

4.1. Passive smoking increased oxidative stress at rest

Cigarette smoke contains free radicals and free radical-derivatives that can lead to oxidative stress [8]. Today, numerous human studies have investigated the effects of either active smoking (e.g., [9]) or passive smoking (e.g., [15–17]) on redox status. In the present investigation, we report increased oxidative stress at rest after acute and chronic exposure to passive smoking, for 1 h and after 12 days of daily exposure, respectively, as indicated by the changes observed in F_2-isoprostanes and glutathione. Our results are in agreement with most of the studies in the literature, reporting increased oxidative stress after active or passive smoking (e.g., [9,15–17]).

4.2. Vitamin C decreased oxidative stress at rest

Vitamin C is probably the most commonly used anti-oxidant supplement. Despite some discrepancy, most of the studies have reported that vitamin C supplementation, either acutely or chronically, decreases oxidative stress [18]. In agreement with this literature, our data showed that vitamin C supplementation decreased the level of F_2-isoprostanes. However, no changes were detected in protein carbonyls or glutathione levels after vitamin C supplementation. In agreement with our findings, Reznick et al. [19] reported that vitamin C prevented oxidative damage to lipids but not to proteins after exposure of human plasma to gas-phase cigarette. We are not able to provide a satisfactory explanation why only F_2-isoprostanes responded in vitamin C supplementation. Nevertheless, judging from the decrease of F_2-isoprostanes (both acutely and chronically), we can conclude that vitamin C reduced lipid peroxidation at rest.

4.3. Passive smoking decreased and vitamin C increased exercise-induced oxidative stress

Our data reveal that 12 days exposure to a pro-oxidant environment generated redox adaptations that limited exercise-induced oxidative stress. Hormesis theory predicts that low levels of toxicants can actually have beneficial effects in the long term [1]. Several studies have clearly shown that chronic exercise actually leads to less oxidative stress than that observed after acute exercise [20]. We hypothesize that participants exposed to passive smoking developed adaptations allowing them to limit alterations in redox homeostasis (i.e., "tight" redox control). On the other hand, vitamin C supplementation exacerbated oxidative stress appeared after exercise (i.e., "loose" redox control). The essence of this observation is that a pro-oxidant stimulus delivered chronically has limited the alterations induced by a potent redox homeostatic challenge (i.e., exercise), whereas the anti-oxidant stimulus of vitamin C had the opposite effects, which cannot be appropriately explained within the current framework. Considering that exercise-induced alterations in free radical metabolism are now seen as signals activating redox signaling pathways leading to favorable exercise adaptations [21,22], it is tempting to speculate that passive smoking may hamper some of the redox-mediated exercise adaptations.

4.4. Rest values of redox biomarkers determine the redox responses to exercise

In a recent study from our group, we reported that the concentration of oxidant biomarkers is unlikely to be increased after an oxidant stimulus, if oxidant biomarkers are already high, and vice versa [23]. In the present study, the individuals exposed to passive smoking had increased systemic levels of lipid peroxidation and decreased antioxidant capacity at rest compared to the vitamin C group. Noteworthy, no significant changes were observed after exercise in the passive smoking group, whereas significant alterations appeared in the vitamin C group. Probably, the high initial values of F_2-isoprostanes and the low values of glutathione in the passive smoking group did not allow individuals to exhibit any further oxidative stress after exercise. Reversely, in the vitamin C group, the low initial values of F_2-isoprostanes increased and the high values of glutathione decreased markedly after exercise. The data presented herein emphasize that the initial values of redox biomarkers are important predictors of the responses to exercise.

4.5. A novel framework for characterizing a stimulus as pro-oxidant or anti-oxidant in vivo

We found that passive smoking induced oxidative stress at rest but reduced oxidative stress after exercise. Contrary, vitamin C reduced oxidative stress at rest but increased oxidative stress after exercise. Do these effects make passive smoking an anti-oxidant and vitamin C a pro-oxidant stimulus? We believe not. Based on our findings, it is more likely that the greater oxidative stress response appeared after vitamin C supplementation indicates that the anti-oxidant stimulus was effective to sufficiently decrease the oxidant biomarkers values, to such an extent, that allowed them for greater increases after a pro-oxidant stimulus. Consequently, what might be frequently interpreted as a pro-oxidant effect of a

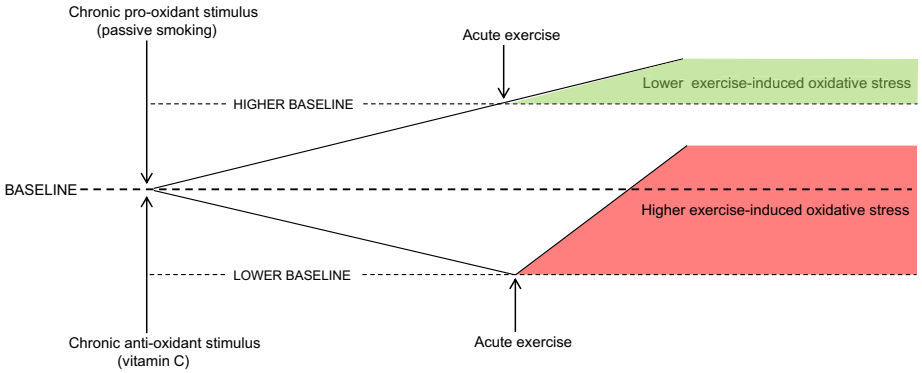

Fig. 4. A conceptual model, based on the study findings, showing how a chronic pro-oxidant stimulus preceding an exercise session can decrease the degree of exercise-induced oxidative stress, and conversely, how a chronic anti-oxidant stimulus preceding an exercise session can increase the degree of exercise-induced oxidative stress. The present experimental evidence supports the view that the degree of exercise-induced oxidative stress is to a great extent deterministically dependent on the initial levels of redox biomarkers before exercise; yet it is a highly plastic property, since a chronic either pro-oxidant or anti-oxidant stimulus can alter the initial levels of redox biomarkers.

repeatedly delivered anti-oxidant stimulus is more likely an anti-oxidant effect. As a result, a repeatedly delivered anti-oxidant stimulus is more likely to increase exercise-induced oxidative stress and not to decrease it. The opposite may be true for a pro-oxidant stimulus: chronic exposure to a pro-oxidant stimulus seems to accumulate oxidative burden, leading oxidant biomarkers to high enough levels that cannot be further increased by the exercise challenge. The proposed framework of alterations in redox homeostasis is schematically provided in Fig. 4.

4.6. Conclusion

Although it is straightforward to define oxidants and reductants chemically, characterizing the effects of an "oxidant" or an "anti-oxidant" in a biological environment has proven to be a very difficult task. We provide for the first time empirical evidence that the current interpretative framework of exercise-induced oxidative stress can be misleading and we propose a novel one. Contrary to the current scientific consensus, our results show that when a pro-oxidant stimulus is chronically delivered, it is more likely that oxidative stress induced by subsequent exercise is decreased, and not increased. Reversely, it is more likely to find greater exercise-induced oxidative stress after previous exposure to an anti-oxidant stimulus. We believe that the proposed framework will be a useful tool to reach more pragmatic explanations of redox biology phenomena.

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