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PII: S0891-5849(22)00071-5

DOI: https://doi.org/10.1016/j.freeradbiomed.2022.02.019

Reference: FRB 15552

To appear in: *Free Radical Biology and Medicine*

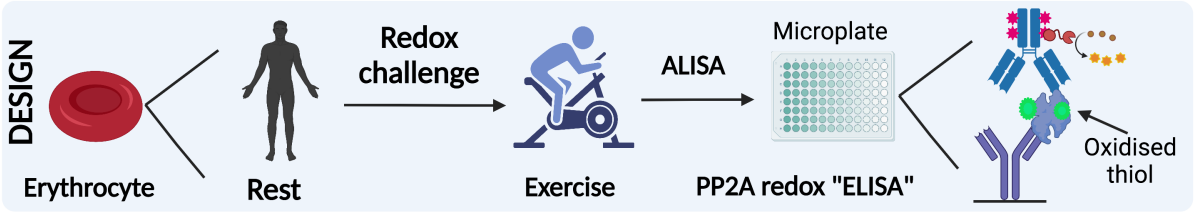
Received Date: 16 December 2021

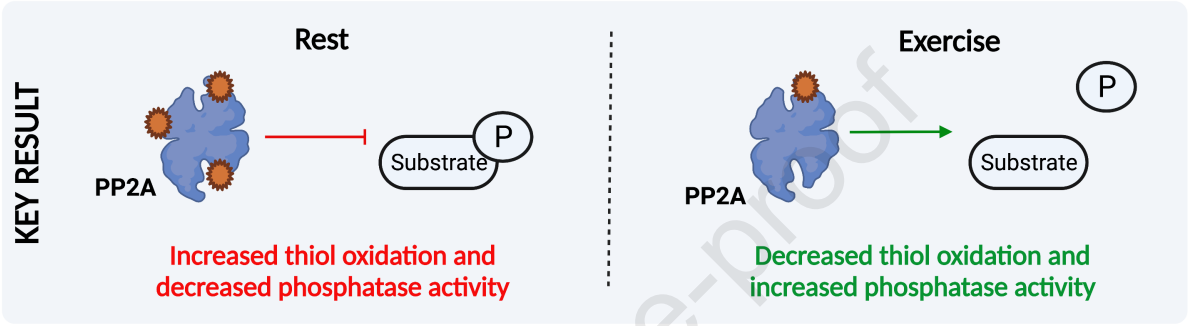
Revised Date: 7 February 2022

Accepted Date: 18 February 2022

Please cite this article as: D.J. Muggeridge, D.R. Crabtree, A. Tuncay, I.L. Megson, G. Davison, J.N. Cobley, Exercise decreases PP2A-specific reversible thiol oxidation in human erythrocytes: Implications for redox biomarkers, *Free Radical Biology and Medicine* (2022), doi: https://doi.org/10.1016/j.freeradbiomed.2022.02.019.

PP2A-specific reversible thiol oxidation is a new systemic redox biomarker





PP2A is a first-in-class readily accessible and easily measurable systemic redox biomarker.

Original research

Exercise decreases PP2A-specific reversible thiol oxidation in human erythrocytes: Implications for redox biomarkers

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Abstract

New readily accessible systemic redox biomarkers are needed to understand the biological roles reactive oxygen species (ROS) play in humans because overtly flawed, technically fraught, and unspecific assays severely hamper translational progress. The antibody-linked oxi-state assay (ALISA) makes it possible to develop valid ROS-sensitive target-specific protein thiol redox state biomarkers in a readily accessible microplate format. Here, we used a maximal exercise bout to disrupt redox homeostasis in a physiologically meaningful way to determine whether the catalytic core of the serine/threonine protein phosphatase PP2A is a candidate systemic redox biomarker in human erythrocytes. We reasoned that: constitutive oxidative stress (e.g., haemoglobin autoxidation) would sensitise erythrocytes to disrupted ion homeostasis as manifested by increased oxidation of the ion regulatory phosphatase PP2A. Unexpectedly, an acute bout of maximal exercise lasting ˜16 min decreased PP2A-specific reversible thiol oxidation (redox ratio, rest: 0.46; exercise: 0.33) without changing PP2A content (rest: 193 pg/ml; exercise: 191 pg/ml). The need for only 3-4 µl of sample to perform ALISA means PP2A-specific reversible thiol oxidation is a capillary—fingertip blood—compatible candidate redox biomarker. Consistent with biologically meaningful redox regulation, thiol reductant-inducible PP2A activity was significantly greater (+10%) at rest compared to exercise. We establish a route to developing new readily measurable protein thiol redox biomarkers for understanding the biological roles ROS play in humans.

Key words: ALISA, Protein thiol, Biomarker, Redox Signalling, Oxidative stress, Exercise, PP2A, Human.

Introduction

Unravelling regulatory roles for reactive oxygen species (ROS), a global term subsuming chemically heterogenous free radical and non-radical molecules [1], in humans relies on using biomarkers to understand redox functionality: what ROS do and how they do it [2]. For example, redox biomarkers (e.g., lipid peroxidation products) together with several molecular surrogates (e.g., mRNA transcripts) show that manipulating ROS with small-molecule antioxidants (e.g., vitamin C) impairs exercise adaptations [3–6]. ROS, therefore, regulate exercise adaptations (i.e., what) by activating beneficial cell signalling cascades (i.e., how) [7–9]. Discovering regulatory roles for ROS in humans is intrinsically linked to leveraging technical advances to develop new redox biomarkers [10].

The antibody-linked oxi-state assay (ALISA) is a new technical advance for measuring target-specific protein thiol redox state in a microplate [11]. A protein thiol (i.e., cysteine residue) can be reduced or oxidised (oxidation can be reversible or irreversible) [12,13]. The vast majority of the >200,000 cysteine residues in the human proteome are highly reduced (>85%) [14]. Much redox signalling is mediated by direct and/or indirect electron exchange between ROS and protein thiols [15–20]. Changing protein thiol redox state can control their activity, phase, lifetime, location, and interactome [21–24]. Redox signals can be catalysed and reversed by the glutathione and thioredoxin dependent systems. Measuring target-specific protein thiol redox state has great potential for understanding the pleiotropic roles ROS play in humans [25]. Reasons to measure target-specific protein thiol redox state are threefold:

1. **Redox signalling**. Target-specific protein thiol redox state changes can be used to infer context- and process-specific roles for ROS dependent redox signalling [26–28]. Measuring exercise-induced redox signalling is important because mechanistic links tying antioxidants to the target-specific protein thiol redox state changes responsible for regulating exercise adaptations are lacking [29].

2. **Biomarkers**. A target-specific protein thiol redox state change delivers an accessible and easily measurable process-specific or general candidate biomarker. For example, it might be possible to find new biomarkers of systemic exercise-induced oxidative stress: as chemically defined by a target-specific protein thiol redox state change. Beyond expanding the available analytical arsenal [30], clearly defining oxidative stress by specifying the change is essential because chemical ambiguity fosters misunderstanding [31–33]. Interpretational clarity stems from target-specific protein thiol redox state changes reflecting a difference in reversible thiol oxidation formation and/or reduction [34,35].

3. **Validity & specificity**. Unlike many overtly flawed or severely limited redox biomarker assays [36], ALISA is valid and target-specific. In considering specificity, global protein carbonylation, for example, is a general metric: it reports mean proteome-wide carbonylation. Consequently, relating altered protein carbonylation to function is challenging without targeted analysis. ALISA can relate functionally annotated redox switches to target-specific protein thiol redox state changes. For example, reversible thiol oxidation inhibits the tyrosine protein phosphatase PTP1B by disabling

nucleophilic catalysis [37–39]. One could infer an increase in PTP1B-specific reversible thiol oxidation as inhibitory, especially when combined with a protein activity proxy [35]. Understanding change functionality is key to interpreting oxidative stress (i.e., the change may be good, bad, or neutral).

To advance translational research, we used exercise to disrupt redox homeostasis in a biologically meaningful way to unearth target-specific protein thiol redox responses in human erythrocytes. Chronic oxidative stress sensitises erythrocytes to additional exercise-induced mechanical, thermal, chemical, and ionic stress [40]. We reasoned that: erythrocytes might struggle to defend ion homeostasis when exercise-induced oxidative stress is imposed, which may, in turn, increase the oxidation of the catalytic core subunit (PPP2CA, UniProt: #P67775) of the ion regulating and redox-sensitive serine/threonine protein phosphatase PP2A [41,42]. PP2A was selected, therefore, because it plays a key homeostatic role in the erythrocyte and it is a redox-sensitive target protein. Here, we used ALISA to measure PP2A-specific redox state at rest and immediately after maximal exercise in human erythrocytes. Experiments were designed to unambiguously answer a clear question: does an acute bout of maximal exercise change PP2A redox state? The answer could pave the way to developing new target-specific systemic redox biomarkers for unravelling the biological roles ROS play in humans.

Results

Experimental approach

Blood samples were collected from adult human males at rest and immediately after an acute bout of maximal exercise. Physiological markers confirmed the maximal nature of the exercise stimulus (Table 1). Blood samples were processed for ALISA. ALISA uses a thiol-reactive fluorescent-conjugated maleimide reporter (i.e., F-MAL) to label reversibly oxidised thiols and a capture antibody to bind the target protein [11]. The target-specific reversible thiol oxidation (i.e., the F-MAL signal) is normalised to the total amount of capture antibody bound target using a biotin-conjugated detector antibody and recombinant protein standard curve (Figure 1). Target-specific redox state is calculated as (F-MAL/[target]).

Table 1. Adult male participant (*n* = 8) data by physiological parameter. SD denotes the standard deviation.

Parameter	Mean	SD	Range
Age (years)	38	9	21-47
Height (cm)	179.3	5.8	174-190
Body mass (kg)	79.9	9.5	60.8-88.8
Body fat (%)	12.3	3.8	4.4-16.5
VO_{2max} (ml/kg/min)	47.4	5.7	41-59
Max work rate (Watts)	365	26	342-408
Exercise time (seconds)	946	78	858-1074
Max Heart rate (bpm)	176	9	158-188

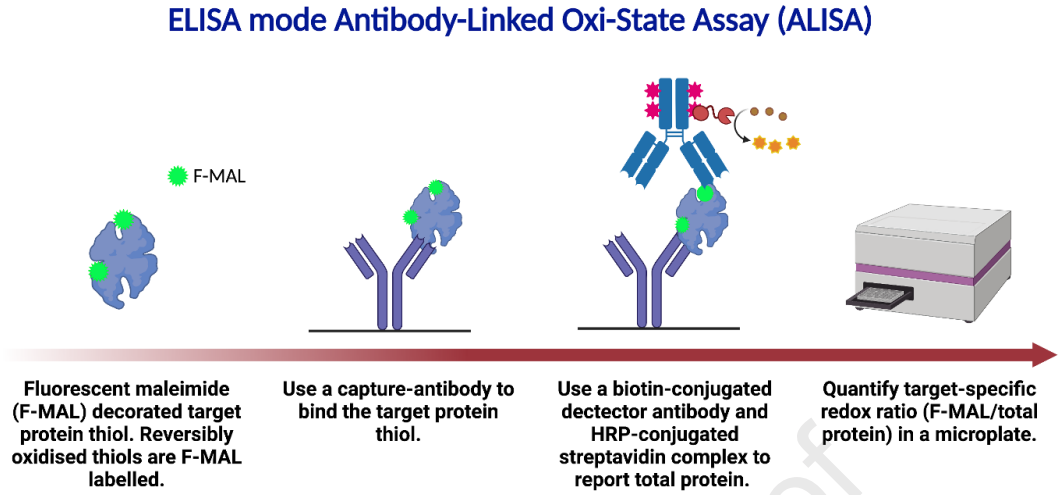

Figure 1. ELISA mode ALISA workflow. Left to right. Reversibly oxidised target protein thiols are decorated with a fluorescent-conjugated thiol-reactive maleimide (F-MAL) reporter using standard labelling protocols (see methods). A microplate immobilised capture antibody is used to bind the F-MAL decorated target protein. After washing (omitted for clarity, see methods), a biotin-conjugated (red groups) detector antibody is used to bind a distinct target protein epitope. Streptavidin-conjugated horseradish peroxidase (HRP) and a turn-on fluorescent HRP substrate are used to quantify total protein relative to a recombinant protein standard curve (omitted for clarity) in a microplate. To terminate the HRP reaction and unmask the F-MAL groups for measurement the antibody-target complex is denatured. Target-specific protein thiol redox state is calculated as: F-MAL/total target.

No maximal exercise-induced change in pan-erythrocyte reversible protein thiol oxidation in humans

Consistent with the presence of many F-MAL decorated proteins, qualitative SDS-PAGE analysis identified several distinct F-MAL positive bands corresponding to proteins containing at least one reversibly oxidised thiol in resting and exercised erythrocyte lysates. (Figure 2A). We quantified their redox state relative to haemoglobin (Hb) protein content in a microplate (i.e., total F-MAL/ Hb). No exercise-induced change in pan-erythrocyte reversible thiol oxidation was observed (Figure 2B). Given the trend towards statistical significance, it might well be that the global analysis was underpowered.

Maximal exercise decreases PP2A-specific reversible thiol oxidation in human erythrocytes

To measure PP2A-specific reversible thiol oxidation, we used ALISA [11]. Exercise significantly decreased PP2A-specific reversible thiol oxidation without changing PP2A content in human erythrocytes (Figure 2C-D). ALISA required only 3-4 µl of sample to measure PP2A-specific redox state and content. The coefficient of variation (CV) of resting and exercise technical replicates for PP2A redox state was 8 and 4.5%, respectively. Note that the CV for ALISA will vary on a target-by-target basis.

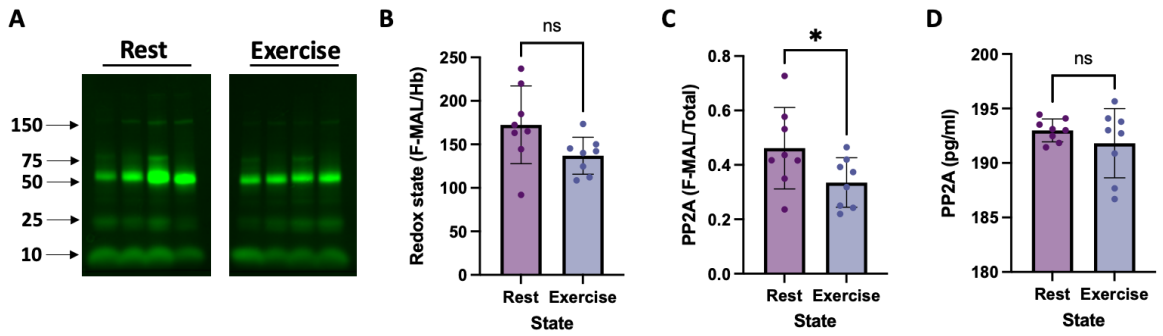

Figure 2. Maximal exercise decreases PP2A-specific reversible thiol oxidation in human erythrocytes. A. Representative qualitative SDS-PAGE gel image showing the presence of several distinct F-MAL positive bands in resting and exercised erythrocyte lysates. Arrows mark various molecular weights (MW) in kilodaltons (kDa). B. Quantifying the F-MAL signal relative to Hb in a microplate revealed no significant (paired t-test, *P* = 0.0832) impact of exercise on global proteome-wide erythrocyte redox state in humans (*n* = 8). C. ALISA revealed a significant (paired t-test, *P* = 0.0367) exercise-induced decrease in PP2A-specific reversible thiol oxidation in human erythrocytes (*n* = 8). D. No significant (paired t-test, *P* = 0.3099) exercise-induced change in PP2A protein content was observed in human erythrocytes (*n* = 8). Panels B to D report the mean and standard deviation.

Chemically reversing protein thiol oxidation increases PP2A activity

To determine whether the exercise-induced decrease in PP2A-specific reversible thiol oxidation is biologically meaningful, we quantified Tris-(2carboxyethyl)phosphine (TCEP)-inducible para-Nitrophenylphosphate (pNPP)-phosphatase activity. To measure TCEP-inducible pNPP activity, we used immunoprecipitation (IP) to "pull-down" PP2A. PP2A pull-down was confirmed by immunoblot (Figure 3A). To prevent artificial oxidation during the IP, PP2A was pulled-down in the presence of *N*-ethylmaleimide (NEM) [43]. IP eluents were split into two aliquots: (1) solvent control and (2) TCEP. Consistent with the ability of NEM to inhibit PP2A [44] and reversible thiol oxidation, TCEP significantly increased PP2A activity compared to the solvent control regardless of state (Figure 3B). Comparing the thiol-reductant induced increase in resting compared to exercise eluents revealed that TCEP-induced a significantly greater increase in PP2A activity at rest compared to exercise (Figure 3C). Chemically decreasing PP2A-specific reversible thiol oxidation increased pNPP-specific PP2A activity. Data are consistent with a canonically "oxidative" stimulus—exercise—activating a selective target-specific and functionally relevant "reductive" response (Figure 3D).

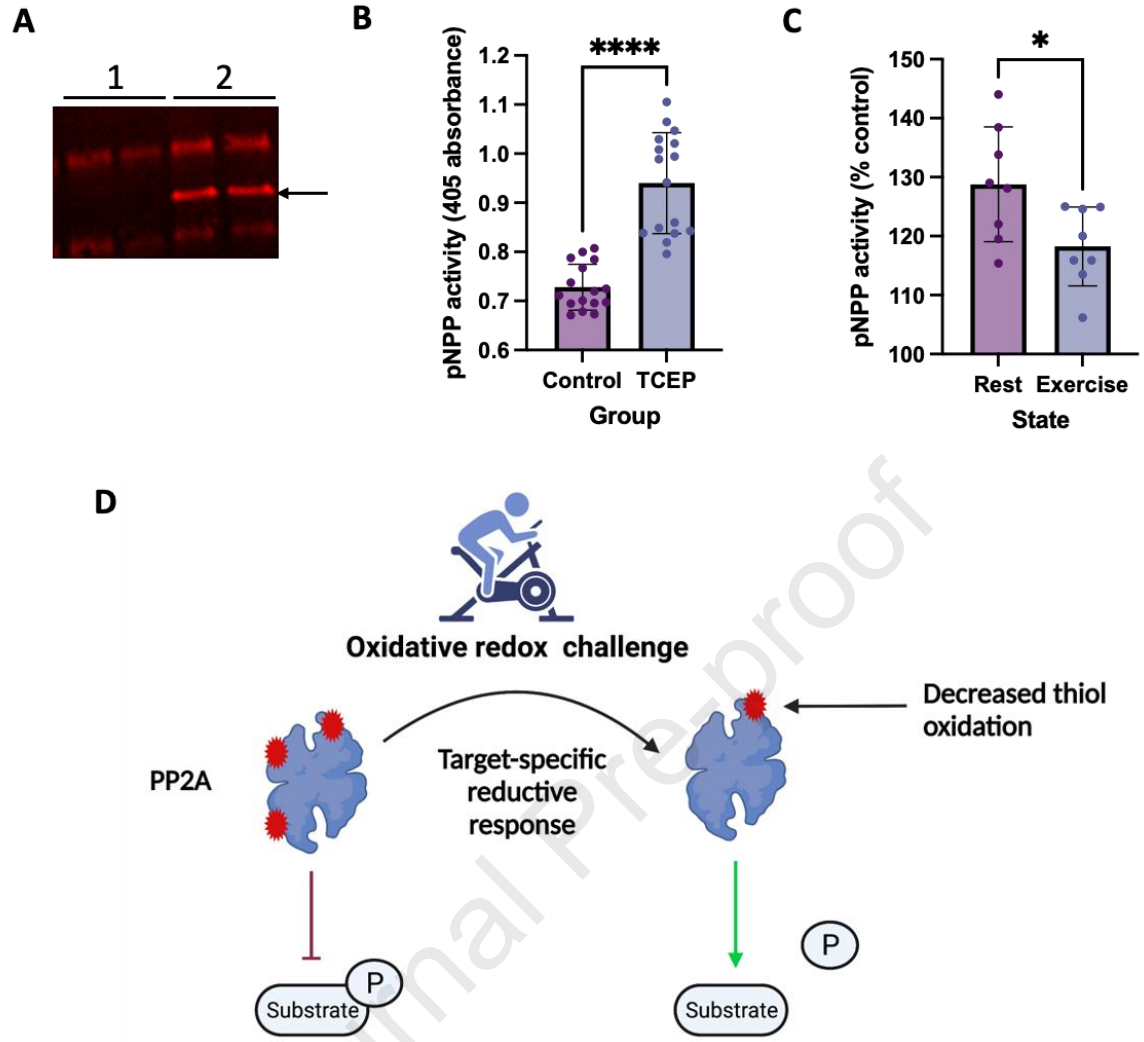

Figure 3. Chemically reversing thiol oxidation increases PP2A activity. A. Immunoblot result showing the presence of a PP2A-specific band at ˜37 kDa (marked by an arrow) in the PP2A capture antibody eluent (2), but not the rabbit "sham" isotype control eluent (1). The image shows both technical replicates. B. A significant (paired t-test, *P* <0.0001) TCEP-inducible increase in PP2A activity compared to the solvent control, as assessed by the monitoring pNPP dephosphorylation at 405 nm, following PP2A IP (*n* = 16). For this experiment, IP eluents (*n* = 16, 8 rest + 8 exercise) were divided into two and treated with either a solvent control (*n* = 16) or TCEP (*n* = 16). Hence the number of data-points are greater than 8 for this panel. C. The TCEP-induced increase in PP2A activity was significantly (paired t-test, *P* = 0.0164) greater in IP eluents from rested (n =8) compared to exercised human erythrocytes (*n* = 8). This experiment refers to the comparison between the rest and exercise +TCEP eluent for each participant. D. Reverse redox regulation model. Exercise a canonical "oxidative" redox challenge induced a PP2A-specific reductive response manifested by decreased reversible thiol oxidation in human erythrocytes. Decreased exercise-induced thiol oxidation increased PP2A activity—depicted by substrate dephosphorylation. Panels B and C report the mean and standard deviation.

Discussion

Superimposing exercise-induced oxidative stress in erythrocyte cells already burdened by chronic Hb autoxidation might be expected to comprise their ability to defend thiol redox homeostasis as manifested by increased reversible thiol oxidation [40,43,45]. Unexpectedly, brief maximal exercise decreased PP2A-specific reversible thiol oxidation in human erythrocytes, which is associated with increased PP2A catalysed pNPP phosphatase activity.

This counterintuitive finding supports a "reversed" redox regulation model wherein PP2A-specific reversible thiol oxidation constrains PP2A activity at rest. Exercise releases a redox break on PP2A activity by decreasing reversible thiol oxidation, which may enable erythrocytes to post-translationally maintain a vital function (e.g., ion homeostasis [42]). Mechanistically, the target-specific "reductive" response is compatible with several possibilities converging on a change in the rate of reversible thiol oxidation formation and/or reduction. Accordingly, we use the word reductive in the sense that the net outcome of several complex processes was a decrease in PP2A-specific reversible thiol oxidation. An interpretationally essential point: The finding does not necessarily mean the enzymes responsible for reversing PP2A oxidation were more active or ROS levels were decreased. Further work is needed to unravel the underlying mechanism. Moreover, studies to decipher the relevant PP2A-substrates and to extend our finding to different exercise types in other cohorts are warranted. A persistent degree of reversible thiol oxidation in the exercised state suggests fine-tuned granular redox control. Data point to a general (i.e., beta subunit independent) and pervasive PP2A regulatory mechanism defined by reversible core catalytic subunit thiol oxidation [46]. Given the potent PP2A inhibitor okadaic acid binds to Cys269 [47], it is entirely possible that oxidising Cys269 is an endogenous "natural" control mechanism, and its redox state may govern the efficacy and IC_{50} of okadaic acid. Targeted redox proteomics is needed to pinpoint the oxidised residues [24,48,49] and future studies should explore the chemotypes involved and their regulation. In particular, ALISA is compatible with new sulfenic acid reactive turn-on fluorescent probes [50]. Our work is consistent with redox regulated PP2A activity [41,51] and the wider value of the erythrocyte for studying protein thiol redox biology [52].

Translational human redox biology research is beset by overtly flawed and/or severely limited assays [36]. Despite hampering current progress, it has been difficult to persuade many to abandon the offending assays because they are easy to use and implement—convenient commercial kits are available. ALISA offers all of their advantages—easy to implement, interpret, and access—without their troubling and rate-limiting drawbacks. ALISA is valid and target-specific. Selected drawbacks of ALISA include (1) the inability to reveal the residues oxidised, (2) fold-change analysis, and (3) the need for a suitable capture antibody, and in this case, an appropriate well-validated ELISA (see [11] for an involved discussion). Nonetheless, the advantages of ALISA allowed us to discover PP2A as a first-in-class systemic protein thiol redox biomarker. Our work and the advent of the state-specific redox array [53] sets the stage to develop protein thiol redox biomarkers for unravelling the biological roles ROS play in humans. At present, PP2A should be treated as a candidate biomarker only because further validation work (e.g., across different cohorts) is clearly required.

In summary, we have discovered a new readily accessible and easily measurable systemic redox state change in PP2A—a key phosphatase—that is associated with an unexpected redox regulation model wherein a physiological "oxidative" stimulus that is well known to induce oxidative stress triggers a biologically meaningful target-specific "reductive" response as defined by a decrease in target-specific reversible thiol oxidation.

Methods

Materials

Supplementary Table 1 details the materials and reagents used.

Participants

Following ethical approval (#OL-ETHSHE-1436) and after obtaining written informed consent, 8 adult male participants provided a venous blood sample before and immediately after a maximal exercise test.

Exercise

Participants completed an incremental exercise test to exhaustion on a bicycle ergometer. Their heart rate and oxygen uptake were recorded throughout (see extended methods).

Thiol labelling

NEM (100 mM) supplemented erythrocyte aliquots were lysed in an equal volume of IP lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, pH 7.1) supplemented with a protease inhibitor tablet. Samples were centrifuged (14,000 *g* for 2 min), soluble supernatants were passed through a 6-kDa spin column to remove excess NEM. Flow throughs were treated with 5 mM neutral-TCEP for 30 min on ice. After removing excess TCEP with a spin column, samples were treated with 1 mM F-MAL (i.e., fluorescin-5-maleimide) for 30 min on ice. Excess (i.e., unreacted) F-MAL was removed with a spin column.

SDS-PAGE

Samples (50 µM Hb) were supplemented with 2X loading buffer and resolved by SDS-PAGE on a precast 4-15% gradient gel. F-MAL signals were measured on a gel scanner.

Global-ALISA

Samples were dispensed in triplicate into microplate wells containing distilled H_2O. F-MAL was measured for 100 ms at 494 and 519 nm in a plate reader. Spectrometric Hb absorbance was measured for 100 ms at 577 nm [54]. After subtracting the background, global reversible thiol oxidation was calculated as: F-MAL/Hb.

PP2A-ALISA

Black MaxiSorp microplates were incubated with 2 µg/ml capture PP2A antibody overnight at 4°C in binding buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6) on a plate shaker at 350 rpm. Wells were blocked (50% PBST, 50% Superblock) for 2 h at RT at 350 rpm and washed (3 x 2 min PBST washes at 400 rpm). The recombinant PP2A standard and samples (diluted to 100 µM Hb in PBS) were added in duplicate and incubated for 2 h at RT at 350 pm. Excess sample was removed, wells were washed (3 x 2 min PBST washes at 400 rpm), and 0.5 µg/ml biotin-conjugated PP2A detector antibody was added for 1 h at RT at 350 rpm. After washing, 0.05 µg/ml of HRP-conjugated streptavidin was added for 1 h at RT at 350 rpm. After

a final wash, wells were incubated with QuantaBlu™ for 10 min at RT at 400 rpm. The QuantaBlu™ signal was measured at 325 and 425 nm for 100 ms. Denaturing buffer (4% SDS) was added to stop the HRP reaction and unmask the F-MAL1 signal. After subtracting the background, PP2A-specific redox state was calculated as: F-MAL/total PP2A. All assay steps were performed under light protected conditions.

PP2A activity assay

Lysates were incubated with anti-PP2A functionalised magnetic protein A beads overnight at 4°C. After washing (3 x 2 min distilled H_2O), PP2A was eluted in gentle IP elution buffer. To normalise protein content, eluents were divided into two aliquots and their pNPP activity was assessed with (aliquot 1) and without (aliquot 2) 1 mM neutral-TCEP at 405 nm in a microplate.

PP2A-Immunoblot

IP eluent PP2A content was checked by immunoblotting as previously described[11,26]. To avoid interference from coeluting capture antibody light and heavy chains, membranes were probed with a fluorescent-conjugated PP2A antibody.

Statistics

After assessing normality, data were assessed using paired t-tests with alpha <0.05 on GraphPad Prism version 9.

Author contributions: Conceptualisation: D.M., D.C., G.W. and J.N.C. Methodology and Resources: J.N.C. and A.T.; Investigation: D.C. and D.M.; Formal data analysis: D.M., A.T., and D.C., Writing original draft: J.N.C., Writing review and editing: All authors, Funding acquisition: J.N.C., I.L.M. and D.C.

Acknowledgements: We acknowledge the participants who made the present work possible and thank the EU INTERREG VA programme (D.C.) and Highlands and Islands Enterprise (HMS 9533763, J.N.C and D.C.) for funding the present work. The graphical abstract, Figure 1, and Figure 3D were created using BioRender (www.Biorender.com) and exported with the appropriate publication license.

Conflict of interest:

There are no conflicts of interest.

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Highlights

- Exercise decreases PP2A-specific reversible thiol oxidation in human erythrocytes.
- Reversible thiol oxidation inhibits PP2A activity in human erythrocytes.
- PP2A enzyme activity is greater after exercise compared to rest.
- Exercise triggers a PP2A-specific “reductive” response.
- PP2A redox state can be measured in an accessible microplate format.