OXIDATIVE STRESS IN LYMPHOCYTES WITH EXERCISE

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ABSTRACT

Vigorous exercise is associated with oxidative stress, a state which involves modifications to bodily molecules due to release of pro-oxidant species. Assessment of such modifications provide non-specific measures of oxidative stress that can be assessed in human tissues and blood, including circulating lymphocytes. Lymphocytes are a very heterogeneous group of white blood cells, consisting of sub-types that have different functions in immunity. Importantly, exercise drastically changes the lymphocyte composition in blood by increasing the numbers of some subsets while leaving other cells unaffected. This fact may imply that observed changes in oxidative stress markers are confounded by changes in lymphocyte composition. For example, lymphocyte subsets may differ in exposure to oxidative stress because of subset differences in cell division and the acquisition of cytotoxic effector functions. The aim of this review is to raise awareness of interpretational issues related to the assessment of oxidative stress in lymphocytes with exercise and to address the relevance of lymphocyte subset phenotyping in these contexts.

INTRODUCTION

During strenuous physical exercise transient alterations in homeostatic control take place. For example, exercise causes muscle respiration to increase 50-100 fold [1] which is accompanied by elevated breathing frequency, greater cardiac output, and increased sympathetic activity [2]. These increases are, amongst others, associated with oxidative stress and changes in the cellular composition of the peripheral blood [3-6]. The latter two processes have been studied in the context of exercise with some overlap: while some researchers have examined lymphocyte kinetics during exercise [3, 7], others have investigated oxidative stress in lymphocytes during exercise [8-13]. This review provides an overview of these research areas, and presents several considerations and pitfalls concerned with studying oxidative stress in lymphocytes with exercise.

EXERCISE AND LYMPHOCYTES

Lymphocytes are a heterogenous group of white blood cells, of which 60-80% comprise of cytotoxic T cells and helper T cells. Natural killer (NK) cells and B lymphocytes constitute the remaining 20-40%. Each sub-population has different functional characteristics: Cytotoxic T cells kill virally infected and cancerous self cells, while helper T cells orchestrate the immune response mainly by secretion of cytokines; the signalling molecules of the immune system [14]. The hallmark of B cells is the capacity to produce antibodies, soluble receptors that bind to immunological targets, marking them for destruction [14]. NK cells have a cytolytic capacity comparable to the cytotoxic T cells, which is similarly used to kill virally-infected and cancerous cells (see Table 1) [14].

[INSERT TABLE 1 AROUND HERE]

One of the effects of exercise is an immediate influx of lymphocytes into peripheral blood, which is variably referred to as lymphocytosis, lymphocyte recruitment, or lymphocyte mobilisation. This mobilisation is driven by two mechanisms. First, shear forces associated with increased cardiac output dislodge lymphocytes adhered to the vasculature, denoted as the 'peripheral pool', which then enter the peripheral blood [2]. Second, the large adrenaline surge with exercise reduces adhesiveness of lymphocytes, further facilitating their release from the vascular endothelium and other reservoirs such as the spleen and lungs [15]. This mechanism operates via activation of the beta-2 adrenergic receptors on lymphocytes, which causes conformational changes in adhesion molecules [16].

Lymphocyte sub-populations show large variation in the expression of beta-2 adrenergic receptors, whereby the highest expression levels are observed on cytotoxic T cells and NK cells. Consequently these subsets show the highest sensitivity to exercise-induced mobilisation. For example, during a bout of strenuous exercise the number of NK cells show a 10-fold increase [5] whereas the number of B and T helper lymphocytes increase only by approximately 50-100% [6]. The upshot is that not only does blood contain higher numbers

of lymphocytes, but also the composition of the lymphocyte pool has now drastically changed [3, 5, 6]. As discussed later in the article, this principle suggests that measurements of oxidative stress in lymphocytes isolated from blood during exercise, are being made in a different population of cells compared to measurements made at rest. Thus, observations of exercise induced oxidative stress in total lymphocytes might be influenced by the cellular composition of blood at the time of sampling.

One to two hours after exercise termination, the lymphocyte composition of blood is once again different compared to rest and exercise. Following vigorous exercise, cytotoxic T cells and NK cells exhibit a large fall in numbers (variably referred to as lymphocytopenia, lymphocyte egress, or lymphocyte extravasation), so that compared to baseline, there are fewer of these so-called effector cells in the circulation [3] (see Figure 1). The functional relevance of this process is thought to represent mobilisation of cells important for immunosurveillance during exercise, which allows for the post-exercise egress of these cells to peripheral tissues, where they are more likely to encounter antigen [17]. Thus, as with blood sampled during exercise, measurements of oxidative stress in lymphocytes being made in the hours following exercise, are being made on a different population of cells.

IDENTIFICATION OF LYMPHOCYTE SUBSETS

Lymphocytes can be identified on the basis of surface expression of so-called cluster of differentiation (CD) molecules (see Table 1). For example, CD19 molecules are uniquely expressed on B cells whereas CD3 identifies T lymphocytes. CD3 in combination with CD8 is used to identify cytotoxic T cells and CD3 in combination with CD4 identifies helper T cells. These T cell subsets can be subdivided, for example identifying subtypes that have certain functional abilities (e.g., cytotoxicity) or differentiating among cells that have encountered antigen (denoted ‘memory’ cells) versus T cells that have not (denoted ‘naive’ cells). An identification method used by many laboratories to identify subtypes of cytotoxic T cells uses CD27 (a member of the Tumour Necrosis Factor receptor super family) and CD45RA expression (an isoform of the pan-lymphocyte marker CD45, which is typically expressed by antigen inexperienced cells) [18, 19]. This strategy yields four populations of T cells; i.e., one naïve subset ($CD27^{-}CD45RA^{+}$), and three memory populations, respectively

denoted as central memory (CM; $CD27^+$ $CD45RA^-$), effector memory (EM; $CD27^-CD45RA^-$), and revertant memory cells, so-called because they have re-expressed CD45RA (EMRA; $CD27^-CD45RA^+$) (see Table 1) [18, 19]. The latter population are also referred to as terminally differentiated T cells. Terminally differentiated $CD27^-CD45RA^+$ EMRA cells are known to exhibit a strong effector potential, characterised by the ability for rapid target killing, inflammatory cytokine production, and tissue migration. However, other combination of CD molecules to identify similar or comparable cytotoxic T cell subsets are also in use [20]. For example, instead of CD27, the co-stimulatory molecule CD28 or the chemokine/lymphoid homing receptor CCR7 can be examined in combination with CD45 isoforms [18, 21].

Compared to naïve T cells, the EMRA sub-population express the beta-2 adrenergic receptor very densely and are therefore highly sensitive to adrenergic stimulation [16, 22]. These cells therefore mobilise to the greatest extent with exercise [3, 5, 7]. These exercise responsive cells exhibit distinct functional and cellular characteristics, and may, as a corollary, also show distinct levels/concentrations of oxidative markers. Thus, it is possible that measurements of oxidative stress in total lymphocytes at rest, when compared to identical measurements during or in the hours following exercise, are confounded by a shift in lymphocyte cell populations present in peripheral blood at the time of sampling (see Figure 1). This phenomenon may impact upon measures of oxidative stress in lymphocytes following exercise, as reported increases in total lymphocyte oxidative markers may not reflect exercise-induced oxidative stress, but instead may represent an influx of cells into blood which already carry increased oxidative biomarkers.

[INSERT FIGURE 1 AROUND HERE]

OXIDATIVE STRESS

Oxidative stress is a state which occurs when pro-oxidants overwhelm antioxidant defences to oxidise proteins, lipids and DNA [23]. Pro-oxidant molecules are naturally occurring species that serve to initiate cellular signalling and adaptive processes, and as such, are

crucial for basic cell function and contribute to the health benefits that occur as result of exercise adaptation [24, 25]. Pro-oxidants are produced from a number of sources, for example: the mitochondrial electron transport chain; peroxisomes; endothelial derived xanthine oxidase; and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase on activated phagocytes [26, 27]. Antioxidant protection is provided by intracellular molecules (e.g., glutathione; GSH, and the enzymes superoxide-dismutase; SOD, catalase, and glutathione peroxidase) and extracellular molecules (e.g., plasma uric acid, ascorbic acid, alpha-tocopherol and albumin) which scavenge and buffer reactive species [24, 28].

EXERCISE AND OXIDATIVE STRESS

Pro-oxidant species are produced in response to exercise [4, 26]. Whether oxidative stress occurs, or adaption takes place, is likely to be related to the magnitude of pro-oxidant formation, which appears to be governed by the intensity and duration of the exercise, and the extent of antioxidant defences present. Oxidative stress has mainly been studied in the context of aerobic exercise: usually running or cycling exercise, ranging in duration from relatively short; < 60 minutes [e.g., 29], to moderate; 6-7 hours [e.g., 30], and finally long duration; up to 33 hours of continuous exercise [e.g., 31]. In general, increases in markers of oxidative stress can be detected immediately after exercise, and with longer and more intense bouts of exercise, oxidative stress can persist for up to 72 hours depending on the biomarker and tissue examined [8, 31-33].

NON-SPECIFIC MEASURES OF OXIDATIVE STRESS

Due to the very short half life of pro-oxidant species, oxidative stress is commonly assessed by measurement of modifications or adducts to bodily molecules [26]. Interaction between pro-oxidants and proteins, in particular the amino acids cysteine and methionine, leads to protein carbonylation [34]. When measured in tissue, plasma or cells, protein carbonyls are robust, stable, and non-specific markers of oxidative stress [35]. Similarly, peroxidation of lipids provides another non-specific measure of oxidative stress that can be assessed in plasma or serum. Lipid peroxidation most likely represents interaction of pro-oxidants with membrane lipids or fatty acids, and has been shown to disturb membrane integrity, structure and function [36].

Complimenting the assessment of oxidative modifications to bodily molecules, antioxidant capacity can be measured in whole blood, plasma or serum [26]. Plasma or serum antioxidant power is largely attributable to uric acid, vitamins A, C, and E, thiols, bilirubin, and albumin [24, 28]. Assessment of thiol groups such as reduced glutathione (GSH), or the ratio of GSH to oxidised glutathione (GSSG), is also frequently used to assess the redox-status and antioxidant capacity of whole blood. Typically exercise is seen to induce a plasma antioxidant response, characterised by increased antioxidant capability [24, 25, 37].

ASSESSMENT OF OXIDATIVE STRESS IN LYMPHOCYTES

In addition to oxidative biomarkers measured in plasma, or whole blood, more specific indices of cellular redox-status and the formation of adducts can be assessed in lymphocytes. Measuring biomarkers of DNA damage in lymphocytes is a common approach, such as the assessment of 8-oxo-7-hydroxyguanosise (8-oxodg), a promutagenic DNA lesion, by high performance liquid chromatography, coupled with electrochemical detection [38]. Another very robust technique is single cell gel electrophoresis, also known as the comet assay, which quantifies strand breaks to DNA [39]. Indeed, damage to lymphocyte DNA has been observed following various forms of exercise [40]. Likewise, protein carbonylation in lymphocytes is seen after intensive swimming and endurance cycling exercise [8-11]. Increases in the activity of enzymatic antioxidants has been observed in lymphocytes following exercise, with or without changes in gene expression and protein transcription for these molecules [8-13]. For example, it has been shown that immediately after intensive cycling exercise, lymphocyte catalase, SOD and glutathione peroxidise activity is increased ~40-60% compared to pre-exercise values, with no changes in gene expression for these molecules [12].

These results must be interpreted with caution. As described earlier, blood sampled during exercise comprises largely of lymphocytes with functional characteristics that are distinct from those of most cells present in the circulation at rest (see Figure 1). It is known that some of these cells exhibit short telomeres due to repeated rounds of antigen-stimulated cell division [41], thus, it might be speculated that these cells could also differ in their oxidative

history, which may manifest as the number of oxidative biomarkers detected or different levels or activities of intracellular antioxidant molecules.

We have undertaken analyses concerned with characterisation of the redox status of lymphocyte sub-populations [42]. In the first analysis of this kind, we examined the effect of vigorous exercise on total lymphocyte protein carbonylation. We assessed whether the observed exercise-induced oxidative stress in total lymphocytes was related to the composition of the lymphocyte pool during exercise. We detected an increase in protein carbonylation with exercise in total lymphocytes, which appeared un-related to the cells present in the circulation at the time of sampling. No correlations were observed between the numbers or proportions of any lymphocyte subset contributing to total lymphocytes and the level of protein carbonylation assessed in these cells. For example, a shift in the lymphocyte pool during exercise towards a higher proportion of EMRA cells had no effect on the degree of protein carbonylation found in total lymphocytes. In support we showed that the cells responsible for lymphocytosis (e.g., NK and EMRA cells) had a higher intracellular thiol content compared to B cells and naïve cells (which show minimal mobilisation with exercise). Cells with high thiol levels are usually associated with low levels of protein carbonylation [43, 44], and it is therefore unlikely that these cells already carried a greater level of protein oxidation prior to entering the blood with exercise. Therefore, we concluded that exercise caused a transient oxidative stress to total lymphocytes. Although not directly assessed in our study, our results suggest that reports of increased antioxidant activity in total lymphocytes following exercise [e.g., 12] could quite feasibly be a product of the influx of cells which already exhibit a greater concentration of antioxidant molecules (e.g., intracellular thiols) prior to entering the blood.

Observations of protein carbonylation in total lymphocytes have been made by others following bouts of exercise [8-11]. Importantly, in our investigation, we ruled out effects of lymphocyte kinetics influencing our measurements of protein carbonylation. Therefore, exercise caused lymphocyte oxidative stress as assessed by protein carbonylation. The consequence of such damage to lymphocyte proteins is unknown, but it is conceivable that this may induce apoptosis or inhibit cell functioning [45], which is appealing given that protein oxidation has been associated with altered protein and receptor function. For example,

carbonylation of the protein moiety of low density lipoprotein is known to increase the uptake into blood monocytes [46]. However, any effects on lymphocyte function related to our observations of protein oxidation with exercise would likely be minimal, considering that protein carbonyls returned to baseline levels fifteen minutes after exercise cessation [42].

FUTURE DIRECTIONS

Tools for the assessment of oxidative biomarkers in lymphocyte sub-populations are lacking. Probes to detect radical species by flow cytometry (e.g., 2',7'-Dichlorodihydrofluorescein) [47] do exist but are subject to limitations. First, these probes detect the production of pro-oxidant species (e.g., hydrogen peroxide), often after stimulation with a mitogen such as phorbol 12-myristate 13-acetate (PMA) [47]. Responses to mitogens, relevant to immune function following exercise, are less relevant in the context of exercise-induced oxidative stress. Second, it is possible that any effects of exercise on spontaneous lymphocyte pro-oxidant release (i.e., direct effects of exercise rather than mitogenic stimulation) might be lost/undetectable during the time taken to process blood for analysis**.** Other methodology for consideration might include sorting of lymphocyte sub-populations using magnetic beads, or fluorescence activated cell sorting (FACS); measuring oxidative biomarkers in the isolated lymphocytes using standard assays. However in reality, isolation of cells using FACS is likely to cause oxidative stress, and isolating cells using magnetic beads also comes with a potentially important limitation: cell separations are rarely 100% pure. In addition, with some lymphocyte populations (e.g., EMRA cells) there are very few cells per µl of blood – often in the region of just 10 or 20 cells. This would make it almost impossible to obtain enough cells for standard assays, such as the assessment of protein carbonylation by ELISA [48].

There is a need for the development of assays which, similar to measurement of telomere length by fluorescence in situ hybridisation (flow FISH) [49], quantify oxidative biomarkers in lymphocytes using small samples, on a cell-by-cell basis, and can be used in combination with cell phenotyping. It would be of great value to examine the oxidative status of lymphocytes which accumulate with ageing or disease, because oxidative stress might be of fundamental importance in understanding altered lymphocyte function. Specifically, this analysis might allow us to understand why certain subsets of T cells are more or less

247 efficacious for genetic engineering and targeting of tumour antigens with adoptive
248 immunotherapy [50].

249

250 **CONCLUSIONS**

251 Vigorous exercise has been shown to cause oxidative damage to lymphocytes, and in the case
252 of protein carbonylation in our recent study [42], it appears that this was un-related to the
253 composition of the lymphocyte pool at the time of sampling. Until biomarkers of oxidative
254 stress can be characterised on a cell-by-cell basis, data showing changes in oxidative markers,
255 or antioxidant capacity in total lymphocyte populations following exercise should be
256 interpreted with caution. Such observations may or may not be related to the lymphocytes
257 present in the circulation at the time of sampling depending on the parameter measured (i.e.,
258 oxidative stress biomarkers *vs*. antioxidant capability of cells).

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406 **FIGURE LEGEND**

407 Total lymphocyte protein carbonylation increases with exercise returning to baseline within
408 15 minutes (solid line). The number of total lymphocytes in blood increases (indicated by the
409 number of cells in boxes) and returns to baseline within 15 minutes. 60 minutes after exercise
410 cessation, the number of total lymphocytes falls below baseline level. During exercise, there
411 is a differential increase in the proportions of certain lymphocyte sub-populations (indicated
412 by the phenotype of cells in boxes, and percentages above): B cells, Naive (NA) and Central
413 memory (CM) cells increase minimally (the non-cytotoxic cells), whereas Effector Memory
414 (EM), Revertant Effector Memory (EMRA) and Natural Killer (NK) cells show very large
415 increases (the cytotoxic cells). The composition of peripheral blood is comparable to baseline
416 15 minutes after exercise termination, but 60 minutes later, most EM, EMRA and NK cells
417 leave the blood, extravasting to peripheral tissue, leaving blood largely occupied by NA, CM
418 and B cells.

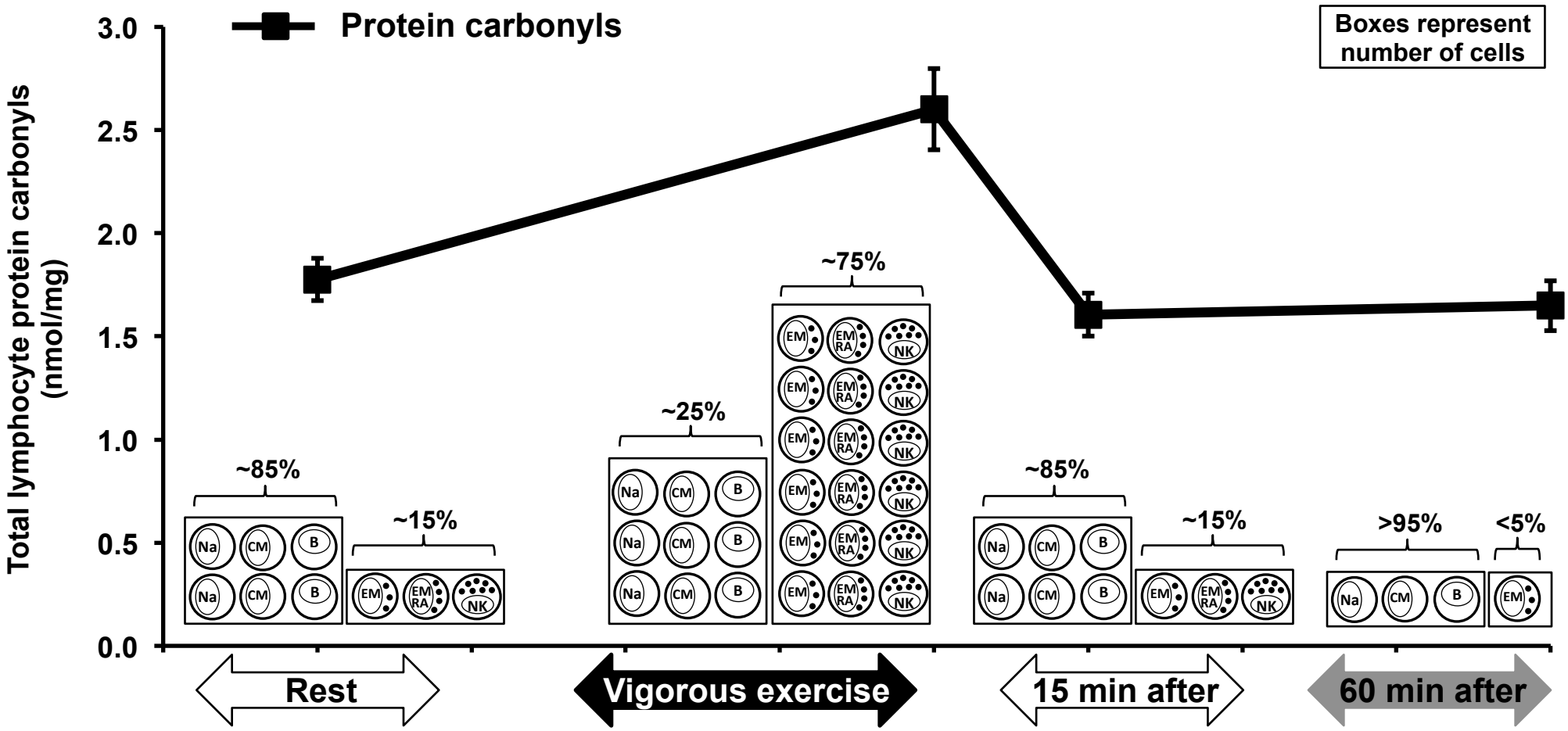
419

420

Table 1.

Identification and functional characteristics of lymphocytes

Cell description	Identification	Exercise responsiveness	Cytotoxic potential	Major role in immunity
B cell	$CD3^{-}CD19^{+}$	+	−	Production of antibodies
Natural Killer cell	$CD3^{-}CD56^{+}$	+++++	+++	Killing cancer or virus-infected cells
Helper T cell	$CD3^{+}CD4^{+}$	++	+	Production of cytokines
Cytotoxic T cell	$CD3^{+}CD8^{+}$	++++	+++	Killing cancer or virus-infected cells
Naïve	$CD45RA^{+}CD27^{+}$	+	−	Recognition of novel antigen
Central memory	$CD45RA^{+}CD27^{-}$	+	−	Long lived memory cell
Effector memory	$CD45RA^{-}CD27^{-}$	+++	+	Killing cancer or virus-infected cells
$CD45RA^{+}$ effector memory	$CD45RA^{+}CD27^{-}$	++++	+++	Killing cancer or virus-infected cells


Protein carbonyls
Boxes represent number of cells
Total lymphocyte protein carbonyls (nmol/mg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
~85%
~15%
~25%
~75%
~85%
~15%
>95%
<5%
Na
CM
B
EM
EM RA
NK
Rest
Vigorous exercise
15 min after
60 min after