Hyperthermia, dehydration and osmotic stress: unconventional sources of exercise-induced reactive oxygen species

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Abstract

Evidence of increased reactive oxygen species (ROS) production is observed in the circulation during exercise in humans. This is exacerbated at elevated body temperatures and attenuated when normal exercise-induced body temperature elevations are suppressed. Why ROS production during exercise is temperature dependent is entirely unknown. This review covers the human exercise studies to date that provide evidence that oxidant and antioxidant changes observed in the blood during exercise are dependent upon temperature and fluid balance. We then address possible mechanisms linking exercise with these variables that include shear stress, effects of hemoconcentration, and signaling pathways involving muscle osmoregulation. Since pathways of muscle osmoregulation are rarely discussed in this context, we provide a brief review of what is currently known and unknown about muscle osmoregulation and how it may be linked to oxidant production in exercise and hyperthermia. Both the circulation and the exercising muscle fibers become concentrated with osmolytes during exercise in the heat, resulting in a competition for available water across the muscle sarcolemma and other tissues. We conclude that though multiple mechanisms may be responsible for the changes in oxidant/antioxidant balance in the blood during exercise, a strong case can be made that a significant component of ROS produced during some forms of exercise reflect requirements of adapting to osmotic challenges, hyperthermia challenges and loss of circulating fluid volume.

Key-words: heat stress, skeletal muscle, oxidative stress, lipoxygenase, taurine
Introduction

Reactive oxygen species (ROS) produced during exercise has been a topic of much interest since its introduction over 30 years ago (16, 18). It is well established that skeletal muscle serves as a source of ROS during contraction (38, 70, 71) but the functional significance of exercise-induced ROS remains unclear and paradoxical. For example, while large amounts of ROS produced during intense contraction can potentially damage cells (70), low to moderate levels of ROS can promote the expression of an oxidative phenotype in muscle (65, 73) and can have important regulatory effects on muscle blood flow (19, 26). Also, a number of environmental and metabolic factors associated with exercise can induce ROS, such as hyperthermia, dehydration, and osmotic stress (34, 35, 42, 43, 51, 59, 67, 93). However, the relative importance of these additional environmental factors in exercise is poorly defined. The aim of this review is to broaden the framework of discussion regarding the sources of ROS production during exercise, specifically focusing on the potential influences of hyperthermia, dehydration and osmotic stress on this phenomenon.

Hyperthermia-induced reactive oxygen: insights from cells, animal models, and human experiments

As an individual exercises, core temperatures increase as a function of exercise intensity and duration, occurring to a greater extent in warm and humid environments. Exercising muscle temperatures can run 0.7-1°C above core temperature during exercise at ~75% of VO2max with duration ranging from 30 to 90 min (63); therefore, during prolonged (~60 min or above) moderate to high intensity (>70% VO2max) exercise in warm environments, muscles can experience temperatures surpassing 41°C. Likewise, passive heating increases ROS production in skeletal muscle. For example, rat diaphragm generates elevated intracellular and
extracellular superoxide (O$_2^{-}$) within a few minutes of exposure to 42°C (93). Although ROS can affect muscle force production, skeletal muscle, fortunately, has a relatively high heat tolerance. This tolerance is specific to muscle type, as intact rat diaphragm is resistant to temperature elevations up to 41°C (59); although mouse soleus has a ≈20% decay in force after 1 hour exposure at this temperature (85). Smaller elevations in temperature may affect long-term muscle function. For example, exposure of mouse extensor digitorum longus fibers to 40°C results in a leaky sarcoplasmic reticulum (SR) Ca$^{2+}$ pump (82). This effect can be blocked by O$_2^{-}$ scavengers and reversed by reducing agents that act on oxidized sulfhydryls (82).

Mechanisms for heat-induced ROS formation in muscle remain elusive. Both mitochondrial and non-mitochondrial sources have been proposed. One popular hypothesis is based on the observation that muscle mitochondria uncouple in a temperature-dependent manner during hyperthermia of >40°C (8). Uncoupling was historically thought to lead to production of mitochondrial O$_2^{-}$ (75). However this concept has been challenged on both theoretical and experimental grounds (66). For example, recent work demonstrates that hyperthermia elevates mitochondrial membrane potential, a state that directly leads to mitochondrial ROS formation (40). This has been shown to be chiefly responsible for elevations in ROS formed from isolated muscle mitochondria when exposed to hyperthermia (41). Whether mitochondria are responsible for ROS, in vivo, is still unknown, and attempts to apply conventional approaches to block mitochondrial ROS have been unsuccessful (94). ROS formation generated in muscle during exercise may not be related to the responses seen in hyperthermia. Growing evidence reveals that mitochondria produce more ROS during state 4 (basal) respiration (typical of a resting state) compared with state 3 (maximal ADP-stimulated respiration) (4, 17, 54). This is important since during aerobic contractile activity skeletal muscle mitochondria are in state 3, thus limiting generation of ROS during contractions (33). What this means during exercise in hyperthermia is unknown, but it is possible that exercise-induced
shifts to state 3 respiration suppress the mitochondrial ROS formation associated with hyperthermia in resting muscle, due to the shift of mitochondria to state 3-ADP-dependent respiration.

Other ROS generating pathways may be equally important. For example, heat exposure in vitro activates phospholipases (PLA), causing release of arachidonic acid and other metabolites (12). Some forms of PLA exist in the cytosol, on plasma, nuclear membranes, and embedded in mitochondrial membranes (69). In general, PLA activity supports ROS formation in muscle (27, 55), and pharmacological inhibition of all phospholipases suppresses skeletal muscle extracellular ROS formation during heat exposure (92). PLAs produce arachidonic acid, which serves as a substrate for generation of prostaglandins, leukotrienes, and the other eicosanoids that have many signaling functions. Blocking lipoxygenase during heat almost completely blocks extracellular $\mbox{O}_2^{•−}$ formation in muscle, whereas blocking cyclooxygenases increases $\mbox{O}_2^{•−}$ formation, suggesting a critical role of specific eicosanoid metabolic pathways in management of extracellular oxidants in heat (92). Furthermore, these pathways are linked to function. By blocking ROS signaling using pharmacological inhibition of eicosanoid forming enzymes, Oliver et al. (59) showed that all of the eicosanoid metabolic pathways studied appear to be functionally important in the contractile response to hyperthermia, i.e. pharmacological blockade of phospholipases, lipoxygenases or cyclooxygenases, resulted in significant reductions in tetanic force and often induced background muscle contracture in heat. In the absence of heat, there were no effects of these pharmacological inhibitors. This may, in part, reflect disturbances in the cell volume regulatory mechanisms of muscle, which are dependent on these pathways, as discussed later in the review.

Skeletal muscle is not the only source of ROS during hyperthermia. For instance, the immune system also plays a pivotal role in oxidative balance following heat exposure. The maximal core temperature reached during exercise is a key factor of protein oxidative damage
in lymphocytes. In fact, the ratio of oxidants to antioxidants in lymphocytes and neutrophils is affected by high intensity exercise (79). When subjects ran for 45 min at 75-80% VO\textsubscript{2}max in the heat (32°C, ~75% relative humidity), reaching maximal core temperatures of 39.8°C, lymphocyte protein oxidative damage was increased. Conversely, when exercise was performed in a cold, temperate environment (10°C, 45% RH) subjects’ core temperature only reached 38°C and this effect was not observed (52). Additionally, antioxidant systems were upregulated in the heat at high core temperatures as evidenced by increases in catalase and glutathione reductase enzyme activities (52). Other evidence that the immune system can be an important source of circulatory oxidants during heat stress comes from an in vitro study showing that human leukocytes display an increase in oxidative damage after incubation at 40°C, following a 2h-treadmill exercise session, where participants jogged at 4-6 km/h for 30-45 min and walked up a 2-10% grade for 75-90 min (74).

Changes in splanchnic blood flow that occur during hyperthermia might be another important source of ROS. After exposure to several days of low-level heat stress, rat intestine shows strong evidence of extensive oxidative damage, and isolated intestinal segments demonstrate oxidation of proteins and elevations in permeability at 41°C that can be blocked with antioxidant treatments (58). This is no doubt a more chronic response. In more acute settings, heat-induced increases in intestinal permeability are in part a consequence of increased ischemia/reperfusion due to local changes in splanchnic blood flow (58). For instance, Hall et al. (30) demonstrated that when core temperature in rats was passively elevated to 41.5°C, splanchnic blood flow was decreased by 40%, leading to the production of ceruloplasmin, semiquinone, penta-coordinate iron (II), and nitrosyl heme. This suggests that reduced splanchnic blood flow is likely a mechanism for increased in ROS generation, which then may exacerbate intestinal and circulatory dysfunction (30). These responses may be amplified during exercise in the heat, as fluid loss in the form of sweat further reduces blood
volume and splanchnic blood flow (25). In support of this hypothesis, Hall et al. demonstrated that ROS formation was locally stimulated in the splanchnic circulation following passive whole body heating in rats (29). Altogether, these data indicate that hyperthermia affects a variety of tissues besides skeletal muscle, which could potentially influence the redox status of the plasma. Additional factors, indirectly related to either exercise or hyperthermia, may also contribute. For example, vascular fluid loss in the form of sweat or muscle swelling can lead to dehydration. As will be discussed later in the review, dehydration may enhance ROS production because of its influence on blood viscosity, vascular shear stress and osmotic gradients (21, 28).

In tissues from more chronic animal studies, oxidant production also leads to an increased activity of antioxidants in order to protect redox homeostasis and build a defense against future insults. Some studies have shown increases in antioxidant enzymes such as superoxide dismutase (SOD) with heat stress alone (31, 47, 53). Similarly, it has been demonstrated that when antioxidants are chronically lowered, particularly SOD, the ability to withstand hyperthermia decreases (60). Collectively, these data support the notion that adaptations to hyperthermia and antioxidant defense mechanisms are upregulated to protect cells or tissues from excessive ROS formation induced by heat or other associated stress-signaling events.

**Hyperthermia and ROS formation in resting and exercising humans**

Human experiments suggest that many of the observations in isolated muscles or other tissues may directly translate to humans, as summarized in Table 1. In one of the first human experiments with passive heat exposure studying biomarkers of ROS-induced damage, Ohtsuka et al. (57) passively submerged subjects in 42°C water for 10 min. Significant decreases in the antioxidant substrate, glutathione (GSH), were observed in red blood cells.
Circulating lipid peroxides (a measure of ongoing oxidative damage) were also elevated. In contrast, immersion in 25°C water increased GSH in red blood cells, with no change in markers of lipid peroxidation in the blood. The authors concluded that oxidative stress is produced by short-term (10 min) passive exposure to a warm environment, while exposure to a cooler environment stimulates blood antioxidant defenses (57).

McAnulty et al. (51) employed treadmill running for ~50 min at 50% VO₂max in humans in a hyperthermic environment (35°C, 75% relative humidity (RH)), compared to a normothermic environment (25°C, 40% RH). F₂-isoprostanes (an arachidonic acid-derived indicator of lipid peroxidation) were significantly elevated in hyperthermia, above those measured in normothermia, providing evidence that increases in core temperature boost ROS production in tissue compartments having access to the circulation. Of note, participants in the hyperthermic group were dehydrated by ~3% of initial body weight. Similar findings were reported by Pilch et al. (64) who determined changes in blood oxidant/anti-oxidant balance when core temperature was elevated by 1.2°C via a passive heat source (sauna) or when core temperature was elevated to the same extent by cycling in a hot environment (33°C/70% RH). Both hyperthermia exposures caused reductions in the antioxidant capacity of blood and elevations in lipid peroxidation, though the reduction in antioxidant capacity was more evident in passive heat exposure than in exercise-induced heat in untrained subjects. Resembling the study of McAnulty et al. (51) the subjects exhibited large reductions in plasma volume during the experiment, with average values ranging from 7-10% between groups (64). Therefore, in both of these studies it is difficult to attribute the elevations in ROS to increased core temperatures alone because of vascular dehydration.

Sureda et al. (80) and Mestre-Alfaro et al. (52) demonstrated the importance of core temperature in augmenting the antioxidant and anti-inflammatory status. In both studies subjects exercised for 45 min at 75-80% of VO₂ max and were exposed to either a hot
environmental condition of 30-32°C (75-78% relative humidity) or a cooler condition of 10-12°C
(40-55% relative humidity), where maximal core temperatures were ≈39.4-39.8°C. It was found
that both circulatory markers of oxidative damage and activity of antioxidant enzymes were
significantly increased following exercise in the heat (80).

To address the possibility that dehydration could be a confounder to the effects of
exercise or exercise in hyperthermia, Laitano et al. (43) tested the effects of hyperthermic
exercise while compensating for net dehydration. Participants were subjected to hyperthermia
via a water-perfused suit and performed one–legged knee extensor exercise at 50% of pre-
established peak power output, compared to resting conditions. Blood samples were taken
before exercise and at the fifth minute of one-legged knee extensor exercise. Subjects
recovered for 15 min and then were passively subjected to an increase in core temperature by
1.2°C and a skin temperature of +6°C. Once the target temperature was reached, a blood
sample was taken, followed by a repeated knee extensor exercise in the heated condition. A
subsequent blood sample was taken after the fifth minute of heated single-legged exercise.
Importantly, subjects were kept euhydrated by ingesting 1.5 L of warm water throughout the
heat stress protocol, matching net water loss. At rest, ~75 min of passive heat increased blood
oxidized glutathione (GSSG), but there was no change in GSH, which led to a decrease in the
GSH/GSSG ratio, an indicator of oxidative stress. However, when heat stress was combined
with one legged-knee extensor exercise, GSH and GSSG were both increased, resulting in no
change in the GSH/GSSG ratio. This suggests passive heat exposure results in increased
oxidant production at rest, but when combined with moderate intensity resistance exercise there
are compensatory responses that may keep blood redox state in relative equilibrium. The
preservation in GSH/GSSG ratio in the face of rising GSSG reflects exercise-induced GSH
release into the circulation, confirmed via direct measurements of net release of GSH across the
exercising limb vascular bed (42). Though rarely discussed, GSH can be actively secreted from
skeletal muscle fibers in response to stress stimuli present in exercise or in heat (15, 42) and this could theoretically provide antioxidant support to the blood and to other organ systems during exercise in stressful environments.

Quindry et al. (67) also assessed the effects of environmental temperature on markers of oxidative stress during exercise while subjects remained euhydrated as measured by body weight. Subjects performed cycle ergometry at a moderate intensity (60% of maximal power output) for 60 min while exposed to 7, 20, and 33°C. Recovery occurred in the same environmental temperature as their respective trials. Subjects were hydrated, during both the exercise and recovery phases, by ingesting approximately 1.2 L of H₂O. Core temperatures were elevated with increased environmental temperatures in both the exercise and recovery phases. The authors measured a panel of oxidative stress biomarkers, which accounted for aqueous and lipid phase antioxidants and several measures of oxidative stress. Results were corrected for changes in plasma volume. Exercise and recovery in a warm environment led to greater oxidative stress responses compared to exercise and recovery in cooler environments. Interestingly, exercise in heated environments resulted in a greater antioxidant capacity in the blood.

The studies reviewed in this section included a variety of heat stress and exercise protocols as highlighted in Table 1. For instance, some studies employed the whole-body passive heat stress through a water-perfused suit (43), sauna (64) or warm water immersion (57) whereas others achieved heat stress by exercising in a warm environment (51, 52, 64, 67, 80). Likewise a blend of exercise types, duration, and intensities were used such as the short-term one-legged knee extensor exercise (43), mid to long-term cycling (64, 67) or running (51). Although it is unlikely that all changes herein reported are taking place under all conditions, it is possible to summarize that hyperthermia in man, with or without exercise, irrespective of hydration status causes the plasma to shift to a more oxidized state (Fig. 1). The response to
exercise hyperthermia appears to be more influenced by hyperthermia than by exercise, and there is accumulating evidence that exercise may superimpose an increase in antioxidant capacity of the blood, potentially serving a protective function.

**Effects of dehydration and hemoconcentration on ROS formation during exercise in man**

There has been considerable interest in the effects of dehydration (acute reductions greater than 2% of body mass) on exercise performance, and it has been fairly well established (not without debate) that higher levels of sweat-induced dehydration can limit exercise performance (76, 86). Many mechanisms for the effects of dehydration on performance have been proposed, but one potential mechanism is through the effects of dehydration on redox status during exercise as suggested by human experiments summarized in Table 2 (35, 42, 62).

Hillman et al. (35) investigated the effects of exercise-induced dehydration, with and without hyperthermia, on oxidative stress. Trained male cyclists completed 90 min of intense cycling exercise at 95% lactate threshold (LT). This was followed by a 5-km time trial in a warm or a thermoneutral environment. They found that oxidized glutathione (GSSG) increased significantly post exercise during a ~3.8% dehydration, while euhydration attenuated these increases, regardless of the environmental temperature. In a follow-up experiment, the same research group compared the effects of hyperhydration on oxidative stress, thermoregulation, and cycle performance (34). Trained males consumed glycerol or water to achieve hyperhydration, followed by a 90 min time trial. Total GSH increased post exercise in all trials, GSSG and protein carbonyl concentrations were increased post exercise for the control trial only. The study concluded that fluid intake attenuated oxidative stress associated with exercise, but did not enhance thermoregulation or performance.

Other investigators have examined the influence of rehydration on oxidative stress following a period of dehydration (42, 62). One particular study by Paik et al. (62) induced 3%
dehydration by passive heating in a sauna, followed by an exhaustive bout of treadmill running. Although these authors did not monitor core temperature they found dehydration increased oxidative lymphocyte DNA damage during treadmill running and this damage was reversed by fluid replacement with either water or a sports drink. In another investigation, Laitano et al. tested one-legged knee extensor exercise in both a dehydrated and rehydrated state in humans (42). Participants underwent graded dehydration by 0, 2, or 3.5 % of body mass, were then exercised and subsequently rehydrated. Dehydration in exercise resulted in significant elevations in a net release of GSH into the blood from the exercising muscle, but without significant elevations in GSSG or indicators of oxidative stress. Venous SOD activity was significantly decreased in the dehydrated state compared to the euhydrated state. These experiments support the concept that vascular dehydration may contribute to the oxidative state of the blood during exercise, particularly when core temperature is elevated.

**Potential mechanisms for increased ROS formation during dehydration**

The total peripheral resistance to blood flow within the vasculature is regulated in part by the caliber of the vessels and the viscous characteristics of the blood (21). During exercise, fluid shifts from the intravascular to both the interstitial and intracellular space. Sweat-induced dehydration results in fluid shifts and increases both blood osmolality and viscosity. This results in increases in shear stress as the blood interacts with the vascular wall. Hyperthermia and exercise can induce a state of hemoconcentration (9) due to the effects of sweat loss, filtration through capillary leakage and uptake of water in exercising muscles (83). Hemoconcentration, increases in viscosity, and changes in shear stress have been shown to occur in both maximal and submaximal exercise (45, 88). Hemoconcentration has been demonstrated repeatedly in exercise, even in the absence of sweat loss (56). This is in accordance with other studies that have demonstrated increases in plasma viscosity and hematocrit, regardless of whether fluid losses are replaced or not (83). Whereas, dehydration of 2% or less has minimal effects on
whole blood viscosity (1) losses equal to 4-5%, which are common during exercise in warm conditions (77), have significant effects (1). Fluid shifts, water loss, water trapping in muscle, along with the aggregability and rigidity of red blood cells during hemoconcentration are all potential mechanisms for acute increases in blood viscosity in exercise (2, 9, 20).

Maximal and submaximal exercise protocols of at least 20 minutes in duration have been shown to increase whole blood and plasma viscosity with corresponding increases in hematocrit and total protein concentrations (10, 11, 22, 48). Increases in plasma proteins such as fibrinogen and globulins, as well as water loss promote the increased plasma viscosity (14). These changes influence shear stress on the wall of the vessel and signal the release of nitric oxide and (14) and ROS release from the vessel wall (44). This occurs by activation of signaling pathways involving phosphatidylinositol-3-kinase, mitogen activated protein kinase 7, and nitric oxide (44). The subsequent oxidant production, if severe enough, can impair the rheological properties of blood (72) by increasing red blood cell rigidity and decreasing red blood cell deformability (3, 72). It is of note that endurance training appears to protect the red blood cell from oxidative damage (14).

Shear stress not only induces ROS production but also is thought to be an important physical factor for hemolysis (46). Hemolysis can itself induce ROS formation via iron redox chemistry with ascorbate and O₂. The degree of hemolysis, and presumably the amount of oxidative damage, is a result of the amplitude and exposure time to shear stress (7) and other factors such as the extent of dehydration (5). Thus, changes in blood rheology and shear stress emerge as two strong mechanistic factors that could account for some ROS production during dehydration associated with exercise.

Osmotic stress in skeletal muscle fibers.
Skeletal muscle fibers are exposed to a number of shifts in intracellular and extracellular solute content during exercise and during heat exposure that can result in osmotic stress. In general, intensely exercising muscles swell by surprising amounts, i.e. as much as 10-35% in fatiguing contractions, as reviewed in (81). In low load resistance exercise to exhaustion in humans (20% of maximum contraction), muscles swell by ≈20%, and remain swollen beyond 60 min (90). Historically this was thought to be due to increases in interstitial or vascular fluid volume in the intact muscle tissue, but a number of exquisitely designed studies have brought a consensus that most of the swelling can be accounted for by osmotic gradients created across the muscle fibers, reviewed in (81). In theory, the swelling in exercising muscles could account for some of the loss of plasma volume and hemoconcentration seen in exercise, even during whole body euhydration (43). Importantly, metabolic water production must also be taken into account as ultimately all macronutrients undergo reactions with oxygen to produce carbon dioxide and water as a consequence of skeletal muscle metabolism during contraction. The total volume of water produced in the muscle is dependent upon the amount of substrate metabolized, but it is estimated that 0.6 ml of water is formed per gram of oxidized carbohydrate whereas 1.1 ml of water is generated per gram of oxidized fat (50). While this metabolic water production is usually neglected when hydration status is estimated from changes in body mass (50), it may account for some skeletal muscle fiber swelling during contraction.

Osmotic changes are also responsible for the acute swelling in exercising muscle and include the splitting of phosphocreatine (PCr), elevations in lactate and H⁺ when arising from muscle glycogen, and shifts in ion gradients due to reductions in membrane potential (37, 81). Skeletal muscle PCr levels can be as high as 30 mM at rest. During volitional exercise to exhaustion, PCr can decrease by 70% (39). Therefore, just through PCr splitting alone, intense exercise can easily result in a >20 mOsm increase in intracellular solute concentration, which
could account for ≈7% elevation in intracellular volume as water moves down its concentration gradient.

Most cell types exposed to acute heat stress also undergo a period of rapid swelling (up to 40%) over an hour or more, but then exhibit a loss of cell volume over several hours (24, 32). Presumably, acute swelling occurs in skeletal muscle fibers during heat exposure, but to our knowledge this has not been directly studied. Cell or fiber volume increases in exercise or hyperthermia are normally countered to some extent by reductions in plasma volume of 3-9% (42). Conceivably, when the plasma is hypertonic in exercise, non-exercising muscles are exposed to opposing forces that tend to induce outward fluid movement, eliciting an entirely different set of stress signaling responses not discussed here, see (37).

The ability of skeletal muscle to compensate for osmotic challenges due to swelling in exercise is dependent, in part, on localized ROS formation. Immediately following exposure to hypotonic extracellular fluid, experimentally causing muscle fibers to swell, there is a rapid and very localized production of ROS near the muscle membrane (49, 61), Fig. 2A. The resulting ROS signal has been linked to localized elevations in intracellular Ca\(^{2+}\) in mouse skeletal muscle, in the form of Ca\(^{2+}\) sparks (49). Both the ROS signal and the Ca\(^{2+}\) sparks can be inhibited by NADPH oxidase (NOX) inhibitors (49). ROS-activated pathways are important mediators of the regulation of muscle volume control because antioxidant treatments or NOX inhibitors block the Ca\(^{2+}\) signal (49) and impair compensatory osmolyte transport mechanisms discussed below, Fig. 2B (61). Localized ROS formation may not function as a specific mediator of osmotic stress adaptation, but rather it may operate by creating a locally oxidizing environment (pink region in Figure 1A) that induces generalized phosphatase inhibition and kinase facilitation, thus prolonging the half-life of phosphorylation events that drive the signaling responses to stress exposure (89).
The current thinking regarding the physiology of the compensatory regulation to muscle cell swelling in heat or exercise and its relationship to ROS formation is summarized as follows and is outlined in Figure 2A:

1. The mechanism by which muscle cells detect increases in fiber volume is unknown but the most likely mechanism involves activation of transient receptor potential channels (TRP), such as TRPV4 (84), which are present on muscle fibers and are sensitive to both mechanical stretch and eicosanoids derived from PLA\textsubscript{2} activity (23, 37). In response to stimulation they elevate cation permeability, most importantly, the permeability to extracellular Ca\textsuperscript{2+}. Interestingly, TPRV2 is required for normal skeletal muscle cell responses to reductions in cell volume (91) and TPR channels play important roles in thermal sensitivity throughout the body, as reviewed in (36).

2. The elevation in subsarcolemmal Ca\textsuperscript{2+} may be responsible for supporting assembly and activation of Rac-activated NADPH oxidase, which is believed to be the primary source of ROS during osmotic stress (49). In addition, it may contribute to Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR). The combination of elevations in local Ca\textsuperscript{2+}, the local ROS formation, and in the distortions of the coupling of the SR to the cell membrane or T tubules due to mechanical effects of swelling, are believed to contribute to the formation of Ca\textsuperscript{2+} sparks at the SR in skeletal muscle (49).

3. The elevation in intracellular Ca\textsuperscript{2+} can also support activation of Ca\textsuperscript{2+}-dependent phospholipases (e.g. PLA\textsubscript{2}), which produce arachidonic acid and other eicosanoid substrates. The phospholipases are also activated by heat (12) and in many ways may be responsible for many overlapping pathways involved with cellular responses to cell swelling, hyperthermia and exercise. Many different eicosanoid products have specific and sometimes opposing effects on cellular osmoregulation (37), but in skeletal muscle the most specific response identified so far are related to products from lipoxygenase-5.
enzyme, LOX-5 (61). LOX-5 produces leukotriene B4 (LKB4) and many other biologically active eicosanoids. Another important product of PLA2 is lysophosphatidyl choline (LPC), which can lead to osmolyte release during osmotic stress, possibly by activation of protein kinase C (PKC) (38, 61).

4. One of the questions regarding the mechanism for ROS production in heat has been why lipoxygenase blockade results in such potent suppression of muscle ROS formation (92). The answer may lie in the discovery that receptors for LOX5 products, such as the leukotriene-B4 (LKB4) receptor (Fig. 2A) induce activation of the GTPase, Rac, which is essential for assembly of NADPH oxidase (13, 87). Therefore, ROS production may depend or be sustained by an autocrine/paracrine signaling pathway of eicosanoids released during osmotic or heat stress (Fig. 2A). Understanding this pathway is complex because each component of the system described in Fig. 2A augments the response of upstream elements. Therefore, there is much uncertainty in defining where the cellular response to osmotic stress starts, how the signaling elements are linked together and how the response is terminated.

5. There are three primary response elements by which cells respond to swelling that together are called the “regulatory volume decrease” (RVD) response (37). The underlying strategy that all cells use is to pass substantial osmolytes out of the cell; causing water to leave down its concentration gradient, and returning the cell volume to near its normal set point. The primary osmolytes released are K⁺, anions (largely Cl⁻ and HCO₃⁻) and the amino acid, taurine (37, 61, 81), Fig. 2B. Each pathway responds to a number of signals generated by the osmotic sensing pathways described in Fig. 2A; ROS, eicosanoids and Ca²⁺ are among the most important activators of these transporters or channels and when blocked, suppress the ability of the fiber to regulate volume.
6. Note that free taurine in skeletal muscle is very high; e.g. in human vastus lateralis muscle it is 50 mM (6), about 1000 times that of plasma, and skeletal muscle abundantly expresses taurine transporters (68). In response to exercise, ischemia and other perturbations that cause muscles to swell, taurine is released very rapidly, thus returning the muscle fiber to osmotic balance (63, 78). It may be one of the most important mechanisms of maintaining volume control in skeletal muscle fibers. The release of taurine is also dependent on activation of phospholipases as well as other downstream pathways of arachidonic acid metabolism driven by PLA₂ and lipoxygenase (61, 78).

These cellular responses to osmotic stress that are predicted to occur in both exercise and hyperthermia are designed to prevent muscle cells from being damaged and are dependent on the formation of localized ROS. Their existence provides insight into another possible mechanism by which exercise induces elevations in circulating ROS.

**Summary and translational implications**

The origin and importance of ROS in exercise remains a controversial topic. The general discussion in the field of exercise physiology is most often restricted to whether ROS, antioxidants or fluid replacement are “good or bad” for performance or for adaptations to training. Considering the complexity of ROS actions throughout the body, it is not surprising that experiments that try to block ROS during exercise often have very mixed and sometimes unpredictable outcomes. Here we attempt to extend the framework of this discussion to environmental factors that, based on solid experimental evidence, clearly are important in ROS formation in exercise. The observations made here regarding the potential influence of these environmental factors come from many sources and many kinds of exercise stimuli. This leads to questions regarding how the nature of the exercise stimulus (e.g. resistance vs. endurance...
exercise) and the duration of the exercise affect the unfolding of oxidant/antioxidant responses in the circulation and how these link to the timing of alterations in osmotic stress, dehydration and temperature in various tissues. Nevertheless, given the limitations of our understanding, the schema in Fig 3 provides a visual summary of the pathways and mechanisms involved, which may serve as a hypothesis generating tool for future studies, both for identifying possible ROS forming mechanisms and for identifying candidates for intervention. Consider the scenario of trying to avoid ROS produced in exercise with a cocktail of antioxidants. Clearly some ROS pathways are necessary for normal muscle fluid volume homeostasis. Blocking this may result in a greater potential for muscle injury. Likewise, some normal hemoconcentration may be necessary to assist water movement out of exercising muscle in order to maintain cross bridge lattice structure. On the other hand, strategies (e.g. hydration) to prevent excessive shear stress or protecting red blood cells from hemolysis could result in positive consequences.
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Figure 1. Summary of the findings in the “acute” plasma responses considering the results of the human studies reported in this review. Note that hyperthermia alone increases ROS formation and decreases antioxidant defense while hyperthermia combined with exercise increases ROS formation and antioxidant defense. Dehydration combined with exercise has a similar response to hyperthermia alone and rehydration can maintain ROS formation while increasing antioxidant defense.

Figure 2. A. Signaling pathways involved in ROS formation during osmotic stress and/or hyperthermia in skeletal muscle. Stretch or solute concentration are sensed at the cell membrane by a poorly understood sensing system, most likely an isoform of transient receptor potential channels (TRP) that function as stress activated Ca\(^{2+}\) channels. The elevation in Ca\(^{2+}\) or other signaling systems can induce ROS formation by stimulation of NADP oxidase activity and by activation of phospholipase (PLA2). Elevations in PLA2 support both protein kinase C (PKC) activity and eicosanoid metabolism such as via lipoxygenase. Lipoxygenase activity supports ROS formation either directly or through autocrine/paracrine signaling to eicosanoid receptors, such as leukotriene B4 receptor, which can activate the GTPase, Rac, the primary signaling pathway for NADPH oxidase. B. Ca\(^{2+}\), ROS, eicosanoid activity, stretch, PKC activity and other signals play integrative roles in opening K\(^{+}\) and anion channels (Cl\(^{-}\)) and in activating taurine transport mechanisms. Taurine transport may be of unique importance in skeletal muscle osmotic homeostasis and is dependent of ROS formed during osmotic stress for its activation.

Figure 3. Working model for sources of ROS during exercise, dehydration or hyperthermia. Both hyperthermia and exercise, alone or in combination, can induce both muscle fiber swelling and whole body evaporative water losses\(^{1}\). Both processes contribute to hemoconcentration\(^{2}\), which can elevate ROS through changes in blood viscosity, associated shear stress\(^{3}\) and hemolysis\(^{4}\). Muscle fiber swelling, caused by elevations in solute content in muscle fibers during exercise induces the “regulatory volume decrease” response (RVD)\(^{5}\), which is dependent on both localized ROS signaling and on eicosanoid metabolism through phospholipase A\(_{2}\)\(^{6}\) and downstream arachidonic acid (AA) metabolism pathways, eventually leading to ROS formation through NOX\(^{7}\). Hyperthermia independently activates PLA\(_{2}\) and produces ROS from either NOX pathways or from mitochondrial sources\(^{8}\). Finally, the RVD response functions to return fiber volume to steady state by activation of solute transport systems that push solutes out of the muscle fiber. This includes the taurine transport system\(^{9}\).
### Table 1. Studies performed in humans on the effects of hyperthermia on exercise-induced oxidative stress.

<table>
<thead>
<tr>
<th>Author/year of publication</th>
<th>Hyperthermia</th>
<th>Tissue</th>
<th>Biomarkers</th>
<th>Protocols</th>
<th>Results/Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ohtsuka et al. 1994 (57)</td>
<td>Body temperature not reported</td>
<td>Erythrocyte Plasma</td>
<td>Glutathione, glutathione peroxidase, glutathione reductase, lipid peroxides.</td>
<td>10 min of hot bath immersion</td>
<td>Heat stress causes oxidative stress and cold stress increases antioxidant defenses</td>
</tr>
<tr>
<td>McAnulty et al. 2005 (51)</td>
<td>39.5˚C rectal temperature</td>
<td>Plasma</td>
<td>Isoprostanes, lipid hydroperoxides.</td>
<td>~50 min of walking/running inside a climatic chamber at 50% VO\textsubscript{2}\text{max}</td>
<td>Hyperthermia increases oxidative stress independent of oxygen consumption</td>
</tr>
<tr>
<td>Mestre-Alfaro et al. 2012 (52)</td>
<td>39.4°C rectal temperature and 36.9°C skin temperature</td>
<td>Blood</td>
<td>Malondialdehyde, protein carbonyls, glutathione reductase, glutathione peroxidase</td>
<td>45 minutes at 75-80% VO\textsubscript{2}\text{max} at either 10-12°C or 30-32°C</td>
<td>Leukocyte and neutrophil antioxidant enzyme activity increased and carbonyl index decreased following exercise in hot environment</td>
</tr>
<tr>
<td>Laitano et al. 2010 (43)</td>
<td>38.3°C rectal temperature 38.7°C Skin temperature</td>
<td>Whole blood, plasma, erythrocytes</td>
<td>Glutathione, glutathione disulfide, superoxide dismutase, isoprostanates.</td>
<td>~75min of heat protocol with water perfused suit followed by 5 min isolated knee extensor exercise</td>
<td>Resting heat stress induces non-radical oxidative stress, but moderate exercise negates the effects of heat through anti-oxidant compensation</td>
</tr>
<tr>
<td>Quindry et al. 2013 (67)</td>
<td>39.2°C rectal temperature</td>
<td>Blood plasma</td>
<td>Lipid hydroperoxide, protein carbonyls, trolox equivalent, ascorbate equivalent</td>
<td>60 min of exercise at 33°C</td>
<td>Increased oxidative stress in blood following moderate exercise and recovery in a warm environment</td>
</tr>
<tr>
<td>Pilch et al. 2014 (64)</td>
<td>1.2°C increase in rectal temperature</td>
<td>Blood plasma</td>
<td>Antioxidant status, peroxidation products</td>
<td>Cycle ergometer at 50% VO\textsubscript{2}\text{max} until hyperthermia vs passive heating in sauna</td>
<td>Passive heating caused a greater increase in oxidative stress indices when compared to physical exercise in elevated ambient temperatures</td>
</tr>
<tr>
<td>Sureda et al. 2015 (80)</td>
<td>39.8 °C rectal temperature</td>
<td>Blood plasma</td>
<td>Malondialdehyde, protein carbonyls, nucleic acid oxidation, enzymatic activity of catalase and superoxide dismutase.</td>
<td>45 minutes at 75-80% VO\textsubscript{2}\text{max} at either 10-12°C or 30-32°C</td>
<td>Hot environmental temperatures induced greater oxidative and cellular damage but also increased post exercise increases in antioxidants</td>
</tr>
</tbody>
</table>
Table 2. Studies performed in humans on the effects of dehydration on exercise-induced oxidative stress.

<table>
<thead>
<tr>
<th>Author/year of publication</th>
<th>Dehydration (change in body mass)</th>
<th>Tissue</th>
<th>Biomarkers</th>
<th>Protocols</th>
<th>Results/Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paik et al. 2009 (62)</td>
<td>3%</td>
<td>Blood plasma</td>
<td>Malondialdehyde, total antioxidant status, lymphocyte DNA damage</td>
<td>3 h sauna followed by treadmill running to exhaustion at 80% of VO2max</td>
<td>Dehydration increases DNA damage during exercise to exhaustion and fluid replacement decreases DNA damage</td>
</tr>
<tr>
<td>Hillman et al. 2011 (35)</td>
<td>3.8%</td>
<td>Whole blood Plasma Monocyte Lymphocyte</td>
<td>Glutathione, glutathione disulfide, TBARS, HSP72, HSP32</td>
<td>90 min of cycling at 95% of LT* followed by 5 km time trial</td>
<td>Exercise induced dehydration increased GSSG concentration and euhydration prevented increases regardless of environment</td>
</tr>
<tr>
<td>Laitano et al. 2012 (42)</td>
<td>2% and 3.5%</td>
<td>Whole blood Plasma Erythrocytes</td>
<td>Gluthione, glutathione disulfide, superoxide dismutase, isoprostanes</td>
<td>60 min cycling in the heat followed by one-leg knee extensor exercise</td>
<td>Mild and moderate dehydration decreased femoral venous erythrocyte SOD activity</td>
</tr>
</tbody>
</table>

*LT = lactate threshold, HSP = heat shock protein, SOD = superoxide dismutase, GSSG = glutathione disulfide
A. Signaling pathways in fiber swelling

B. Osmolyte movements in the regulatory response to cell swelling
EXERCISE + HYPERTERMIA

1. Evaporative water loss + Sweating
   - Muscle fiber swelling
   - Hemoconcentration
     - Increased blood viscosity
     - Increased vascular shear stress
     - Hemolysis
       - ROS
         - Fe
         - PLA
         - AA
       - NOX
         - Mitochondria
         - Solute transport e.g. (taurine)

2. Muscle fiber swelling
   - Hemoconcentration
     - Increased blood viscosity
     - Increased vascular shear stress
     - Hemolysis
       - ROS
         - Fe
         - PLA
         - AA
       - NOX
         - Mitochondria
         - Solute transport e.g. (taurine)