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Thermal tolerance of contractile function in oxidative skeletal muscle: no protection by antioxidants and reduced tolerance with eicosanoid enzyme inhibition

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Abstract

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Mechanisms for the loss of muscle contractile function in hyperthermia are poorly understood. This study identified the critical temperature, resulting in a loss of contractile function in isolated diaphragm (thermal tolerance), and then tested the hypotheses *1*) that increased reactive oxygen species (ROS) production contributes to the loss of contractile function at this temperature, and *2*) eicosanoid metabolism plays an important role in preservation of contractile function in hyperthermia. Contractile function and passive force were measured in rat diaphragm bundles during and after 30 min of exposure to 40, 41, 42 or 43°C. Between 40 and 42°C, there were no effects of hyperthermia, but at 43°C, a significant loss of active force and an increase in passive force were observed. Inhibition of ROS with the antioxidants, Tiron or Trolox, did not inhibit the loss of contractile force at 43°C. Furthermore, treatment with dithiothreitol, a thiol (-SH) reducing agent, did not reverse the effects of hyperthermia. A variety of global lipoxygenase (LOX) inhibitors further depressed force during 43°C and caused a significant loss of thermal tolerance at 42°C. Cyclooxygenase (COX) inhibitors also caused a loss of thermal tolerance at 42°C. Blockage of phospholipase with phospholipase A_2 inhibitors, bromoenol lactone or arachidonyltrifluoromethyl ketone failed to significantly prevent the loss of force at 43°C. Overall, these data suggest that ROS do not play an apparent role in the loss of contractile function during severe hyperthermia in diaphragm. However, functional LOX and COX enzyme activities appear to be necessary for maintaining normal force production in hyperthermia.

Keywords: diaphragm, lipoxygenase, cyclooxygenase, phospholipase A_2, heat stress

THE CELLULAR MECHANISMS RESPONSIBLE for maintaining skeletal muscle function at elevated temperature are not well understood. However, since skeletal muscles are heat generators during

exercise, their ability to resist the effects of heat must be an integral part of their normal physiology. Human limb muscles can attain temperatures up to 41°C during intense exercise in hot ambient environments, (45) and rat limb muscle temperature has been measured as high as 44°C during exhaustive exercise (11). In pathological conditions of heat stroke or malignant hyperthermia, core temperatures can exceed 44°C (2). Surprisingly, we know little about the effects of hyperthermia on contractile function within this temperature range (41–44°C). Therefore, one of the objectives of this study was to identify the temperature at which significant contractile dysfunction is evident in isolated, intact skeletal muscle over a time period that could be relevant to exercise. A second objective was to identify possible mechanisms responsible for the loss of contractile function at this temperature.

In previous work, our laboratory observed that exposing skeletal muscle to 42°C resulted in increases in both intracellular and extracellular reactive oxygen species (ROS) (66). Similar elevations in ROS production during heat stress have been made in a variety of tissues (26, 61) and in whole animals (30), but the functional significance of these findings is not well known. Recently, van der Poel and Stephenson (57) have shown that at elevated temperatures (43–47°C), contractile function was disrupted in isolated rat extensor digitorum longus muscle fibers, a largely fast twitch muscle. ROS were implicated because treatment with DTT, a thiol (-SH) reducing agent, or by Tiron, an antioxidant eliminated or greatly diminished the effects of high temperature on contractile force. However, no such effects of antioxidants were observed in a small group of more oxidative fibers from the peroneus longus muscle (57). Therefore, a second objective of this study was to test the hypothesis that ROS formed during heat are responsible for the loss of contractile function in an intact muscle with a fiber population that is largely oxidative, namely the isolated diaphragm.

The cell membrane is believed to be particularly sensitive to the effects of temperature and has been described as the “temperature transducer” of the cell (4). In a number of isolated cell systems, as temperature increases to 42°C or above, there is a rapid release of AA, through the action of PLA_2 on the membrane phospholipids (12). This suggests membrane repair or remodeling activities are ongoing. Since AA is further metabolized by LOX, COX, and cytochrome *P*-450 enzymes, it is likely that these enzymes also play significant roles in normal membrane responses to hyperthermia. Interestingly, these pathways may also be responsible for some fraction of oxidant production at high temperatures. In previous work, inhibition of global LOX activity almost completely inhibited the extracellular ROS formed during heat exposure (65). Therefore, we hypothesized that by blocking LOX and possibly other pathways of eicosanoid metabolism, we might protect skeletal muscle function during heat exposure and possibly inhibit oxidative stress at the same time.

In summary, this study had three objectives: *1*) to determine the functional responses to brief heat exposure in isolated, relatively intact, and largely oxidative skeletal muscle, where other variables such as blood flow, central neural responses to heat, neuromuscular activation, or whole body inflammation are not present; *2*) to test the hypothesis that increased oxidant production in heat stress is responsible for the loss of contractile function in intact muscle tissue; and *3*) to evaluate the role of eicosanoid metabolism, specifically catalyzed by lipoxygenase and cyclooxygenase, on the loss of contractile function in hyperthermia.

METHODS

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Animal treatment protocols and isolated muscle preparation.

Adult male Sprague-Dawley rats (350–450 g) were used for this study. Animal care and treatment were

performed using protocols approved by The Ohio State University and the University of Florida Institutional Animal Care and Use Committees. Rats were anesthetized by injection of ketamine (100 mg/kg ip) and xylazine (20 mg/kg ip), tracheotomized, and ventilated with room air. The diaphragm was quickly excised from the animal and placed in preoxygenated (95% O_2-5% CO_2) Ringer solution (in mM: 21 $NaHCO_3$, 1.0 $MgCl_2$, 0.6 Na_2HPO_4, 0.45 Na_2SO_4, 2.0 $CaCl_2$, 5.9 KCl, 121 NaCl; 11.5 glucose, and 10 μM D-tubocurarine). Strips of muscle, 5–6 mm wide, were created with the central tendon and rib intact. The central tendon was fixed to a plastic frame with a small amount of cyanoacrylate gel adhesive and was affixed to a force transducer (Grass FT03) via 3-0 silk suture. The rib was attached to a glass frame using a loop of suture around the rib. The strips were placed in 20-ml water-jacketed tissue baths, maintained at 37°C. Four strips were obtained from each animal and studied simultaneously. The optimum length (L_o) and stimulation current, resulting in maximum twitch force, were determined. Throughout the study, force frequency (FF) curves were constructed using the twitch force and the force responses to 400-ms trains of supramaximal stimuli of 0.2-ms duration at 20, 30, 60, and 150 Hz with 20-s rest between contractions (Fig. 1).

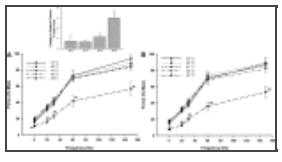

Fig. 1.

A: force frequency relationships obtained at the end of 30 min exposure to 37°C (n = 6), 40°C (n = 9), 41°C (n = 9), 42°C (n = 8), and 43°C (n = 8). Force is expressed as **...**

Protocols.

All protocols for heat exposure and pharmaceutical treatment were similar or are described further in the RESULTS section. The tissues were first equilibrated in oxygenated Ringer solution at 37°C for 30 min. During this time, L_o and optimum stimulation voltage were determined. They were then stimulated at 0.05 Hz throughout the equilibration periods for all experiments. After initial equilibration, a baseline FF relationship was determined. The baths were then changed to either fresh, oxygenated Ringer buffer, preheated to 37°C or buffer containing one of a large number of pharmaceutical preparations of antioxidants, COX, LOX, or PLA_2 inhibitors (Table 1). Matched controls always contained the corresponding vehicles (usually DMSO) in the same concentration as the maximum dosage of the vehicle in the treated baths. After 30 min of equilibration in the treatment buffers, a second FF was obtained to determine the independent influence of the drugs. The baths were changed again with the appropriate drugs or vehicle, and the strips were exposed to either 37°C or one of four elevated temperatures, 40°C, 41°C, 42°C, or 43°C. Temperatures within the baths were monitored and maintained within 0.1°C of the target. Once the target temperature was obtained, it was maintained for a 30-min exposure, at which time another FF was performed. The baths were then replaced with fresh Ringer solution and returned to 37°C for another 30 min after which a final FF was obtained to monitor recovery. The length and weight of each tissue were measured to determine specific force (average value for control tissues: 21.8 N/cm^2 ± 0.5, n = 43). More specific combinations of drug treatments, their doses, and the specific temperature exposures in each experiment will be described in RESULTS.

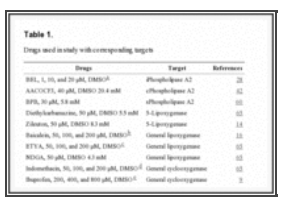

Table 1.

Drugs used in study with corresponding targets

Membrane permeability assessment.

Membrane permeability was assessed using procion orange MX2R dye (Sigma Aldrich, St. Louis, MO, USA), which only crosses damaged membranes and has been used frequently to detect muscle cell membrane damage (59). At the end of some experiments 0.15% wt/vol procion orange MX2R was added to the tissue bath for 45 min, followed by a 15-min rinse with fresh buffer. The tissue was then fixed in 10% formalin, mounted in a 3% agar gel, and later cut in 20- to 60-μm sections with a vibrating tissue slicer (Precisionary Instruments, Greenville, NC). Multiple images of each section were recorded with an epifluorescence microscope using standard filter settings for fluorescein. The numbers of cells containing procion orange MX2R were delineated as a fraction of the total number of cells in the field. Three fields were chosen at random for each sample. This ratio was used to quantify the extent of damage in each specific tissue.

Statistical analysis.

Statistical significance was analyzed using ANOVA, designed for each experimental protocol. Treatment and temperature were used as factors of interest in most studies. Data were expressed as a percentage of maximum baseline force. The effects of animal, i.e., specific experiment on matched tissues were treated as a random variable, resulting in the equivalent of a repeated-measures design. Post hoc analyses (Dunnett's) were done to determine specific effects of treatment from control measurements. Mean contrasts after ANOVA were used in some experiments to compare sample means in complex designs (SAS_{JMP} statistical package). All results are reported as means ± SE; $P < 0.05$ was considered to be statistically significant.

RESULTS

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Thermal tolerance of isolated diaphragm.

As shown in Fig. 1*A*, compared with control measurements at 37°C, exposure of diaphragm to temperatures from 40 to 42°C for 30 min had no significant effect on force developed at all stimulation frequencies. In contrast, at 43°C, maximum force dropped markedly from control with similar relative decreases seen at all lower frequencies, including twitch. After 30 min of recovery, at 37°C, there were no improvements in contractile function (Fig. 1*B*), demonstrating that the effects were not readily reversible. In response to elevated temperature, an increase in passive force was also observed by the end of the 43°C exposure (Fig. 1*A*, *inset*).

Effects of antioxidants and reducing agents on contractile function with heat exposure.

To test the hypothesis that the loss of contractile function during heat stress is due to the effects of increased oxidant production, diaphragms were exposed to 43°C in the presence of antioxidants, Tiron (1, 10, and 20 mM) and Trolox (50 μM, 100 μM, and 1 mM) (Fig. 2). The intermediate dose of Tiron (10 mM) was shown in previous studies to be effective in preserving contractile function during hypoxia (63), and the maximum dose (20 mM) was previously used by van der Poel and Stephenson (57). The lowest dose of Trolox (50 μM) approximated the concentration shown by Betters et al. (7) to reduce oxidant stress in the diaphragm in the whole animal, whereas the highest dose has been shown to be effective in reducing oxidant production in isolated myotubes (64). Contrary to expectations, no effects of these antioxidants on the heat-induced loss of contractile force were observed, although there was a significant reduction in force at the highest concentration of Trolox. There were also no improvements in function during the recovery period in antioxidant-treated tissues (data not shown).

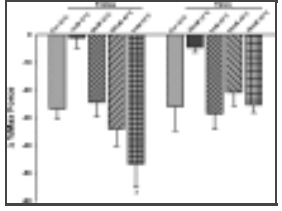

Fig. 2.

Change in %max force (150 Hz) of diaphragm strips exposed to 43°C with antioxidants. Trolox (n = 6) and Tiron (n = 6) data are after 30 min. at 43°C. Dose-responses are shown with time-matched controls. * **...**

To compare our results to observations in isolated fibers (57), we tested whether the heat-induced reductions in force could be reversed by treatment with 5–20 mM DTT, given immediately after the heat exposure and allowed to remain in the bath during the 30-min recovery period (Fig. 3). The dosage range came from several sources. The dose of 5 mM was previously shown to be effective in partially reversing the effects of fatigue in in vitro diaphragm, presumably by reducing protein -SH oxidation (23). Control tissues were performed and found to be similar to Diaz et al.'s (23) findings, which showed that a 5-mM dose of DTT has no effect on baseline muscle function after a full time-matched exposure protocol in 37°C buffer. The 10 mM DTT dose has also been used in mechanically skinned rat skeletal muscle fibers with no adverse reactions on twitch responses (37). Van der Poel and Stephenson (57) previously used the highest (20 mM) dose in isolated fibers. To perform this experiment, the DTT was dissolved directly into buffer in the tissue bath to reduce rapid oxidation of DTT. No significant recovery of function was observed following DTT treatment over the 30-min recovery period.

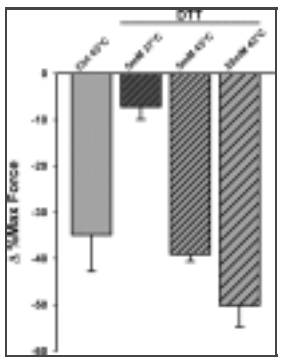

Fig. 3.

Change in %max force (150 Hz) of diaphragm strips exposed to 43°C with dithiothreitol (DTT). DTT (n = 8) data are shown after 30 min of recovery at 37°C (n = 8), shown with control recovery after 43°C and **...**

Effects of lipoxygenase inhibition on the loss of contractile function at 43°C.

Because we previously observed that one source of ROS in heat stress arises from one or more lipoxygenase pathways, we tested whether blockade of LOX activity could protect contractile function at 43°C. Initial doses of LOX inhibitors were determined from previous studies as follows: Zileuton [a 5-LOX inhibitor, 50 μM, (14) Cayman Chemical], diethylcarbamazine (a 5-LOX inhibitor, 50 μM, Sigma Aldrich), baicalein [a 12-LOX inhibitor and partial general LOX inhibitor (16)] (50 μM; Sigma Aldrich, St. Louis, MO) and nordihydroguaiaretic acid (NDGA, general LOX inhibitor) (50 μM, Sigma Aldrich). Contrary to our hypothesis, NDGA treatments greatly increased the loss of force during exposure to 43°C (for data, see the online supplement to this article). The 5-LOX inhibitors, Zileuton and diethylcarbamazine showed no significant effects compared with control. Follow-up experiments with NDGA (data not shown) demonstrated that it caused significant reduction of contractile function in the absence of heat exposure, making it unsuitable for these kinds of experiments.

Effects of lipoxygenase and cyclooxygenase inhibition on heat tolerance to 42°C.

On the basis of the results of the previous experiment, we hypothesized that LOX and possibly other eicosanoid metabolic pathways are important components of the normal heat tolerance mechanisms of skeletal muscle. To test this hypothesis, we exposed diaphragm strips to the highest temperature that does not cause significant loss of contractile function (42°C, Fig. 1*A*) and evaluated the effects of LOX

and COX inhibition (Fig. 4).

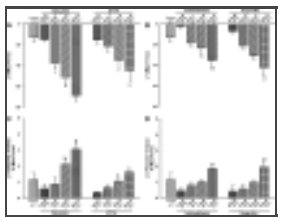

Fig. 4.
Change in %max force (150 Hz) of tissues exposed to 42°C with LOX or COX inhibitor treatment. *A*: general LOX inhibitors, baicalein ($n = 4$) and ETYA ($n = 4$) reduced force in a dose-dependent manner. *B*: general COX inhibitors, **...**

Both treatment with baicalein (12-LOX inhibitor, doses of 50, 100, and 200 μM) and ETYA (eicosatetreanoic acid, general LOX inhibitor, 50, 100, and 200 μM) resulted in significant decreases in contractile function during exposure to 42°C (Fig. 4*A*). In time controls at 37°C, the middle dose of either of these drugs had no significant effect on contractile function compared with time controls (baicelein, −15 Δ%Max Force ± 1.4, ETYA, −15 Δ%Max Force ± 4.0), indicating no measurable pharmacological effects. The decrease in function occurred in a dose-dependent manner and resulted in the greatest loss of function at 200 μM during 42°C heat exposure. Like the influence of heat exposure alone, recovery of function was not observed in these tissues (data not shown). These results suggest that functional LOX activity may be an important factor for heat tolerance in skeletal muscle at 42°C.

A parallel experiment was done using COX inhibition. Since COX also metabolizes AA, it was logical to think that this pathway may also affect heat tolerance at 42°C. The addition of indomethacin at (50, 100, and 200 μM) resulted in a dose-dependent reduction in maximum contractile force during exposure to 42°C. Likewise, ibuprofen (9), given at 200, 400, and 800 μM resulted in a dose-dependent loss in heat tolerance at 42°C (Fig. 4*B*), whereas the time controls showed no effect on contractile function (indomethacin, −2.1 Δ%Max Force ± 2.3, ibuprofen, −6.9 Δ%Max Force ± 1.9). Both drugs had qualitatively similar effects, but at these doses, they did not decrease force to the extent that LOX inhibition did. Interestingly, recovery data showed that loss of function at 42°C in the presence of COX inhibition resulted in partial but modest recovery of force over 30 min (data not shown).

To determine whether the influences of LOX and COX during heat exposure had overlapping or additive effects, we inhibited both enzymes simultaneously (see the online supplement for this article), using baicalein (100 μM) and indomethacin (100 μM) in the same muscle tissue at 42°C. A small loss in function was observed in pharmacological controls done at 37°C (−25.1 Δ%Max Force ± 4.6; see the online version of the this article), but a much larger effect was seen at 42°C. Compared with all other drugs, the combination of the two drugs resulted in the greatest loss in function and suggests that the two enzyme systems do not have overlapping effects but rather that their influence is likely additive.

Treatment with LOX and COX inhibitors resulted in an increase in baseline passive force during 42°C exposure as well (Fig. 4, *C* and *D*). ETYA, baicalein, indomethacin, and ibuprofen showed a dose-dependent increase in baseline passive force with baicalein having the greatest impact.

Effects of inhibition of upstream PLA_2.

On the basis of the preceding studies, we hypothesized that the role of LOX and COX in heat exposure might be simply to metabolize and therefore reduce the concentration of free AA and other lipid products created at elevated temperatures that could be detrimental to function. If so, then by blocking upstream PLA_2 alone, we might cause a reduction of heat-induced contractile function at 43°C. To block $iPLA_2$, a Ca^{2+}-independent PLA_2 ($iPLA_2$) inhibitor, bromoenol lactone (BEL) was used. Previous work has demonstrated that BEL targets functional $iPLA_2$, which has been shown to be

present in skeletal muscle ($iPLA_2$) (28). Control experiments showed a pharmacological effect of 20 μM BEL at 37°C. At 1 and 10 μM, however, there were no significant effects at 43°C, but at 20 μM, a significant decrease in function was observed (Fig. 5*A*). We accredited this decrease at the highest dose to be, in part, a nonspecific pharmacological effect.

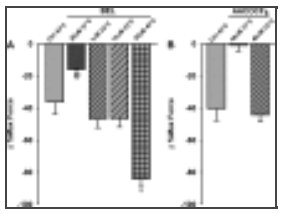

Fig. 5.
Change in %max force (150 Hz) of tissues exposed to 43°C and PLA_2 inhibition. *A*: $iPLA_2$ inhibition by BEL. BEL (20 μM) showed a significant reduction in force compared with control, whereas 1 μM and 10 μM had no **...**

To block the cytosolic PLA_2 ($cPLA_2$) and iPLA2 together, arachidonyltrifluoromethyl ketone ($AACOCF_3$) was used at a dose (40 μM) previously shown to be effective in isolated skeletal muscle (42). $AACOCF_3$ showed no significant effect on contractile function at 43°C (Fig. 5*B*). Finally, 4-bromophenacyl bromide (BPB) was used to inhibit secretory PLA_2 at 30 μM shown to be effective in smooth muscle (60) (see the online version of this article). Control experiments showed a pharmacological effect of BPB during 37°C, which was significantly higher than 37°C control tissues ($P < 0.01$). Also, tissues exposed to BPB at 43°C showed a significantly larger decrease in force compared with untreated 43°C controls ($P < 0.01$). However, these effects can likely be attributed to the pharmacological effects of the drug, as shown in the 37°C controls.

Tests for membrane damage in heat stress.

On the basis of the sensitivity of contractile function to elevated temperatures and particularly to enzymes known to be involved with maintenance of membrane function, we hypothesized that the loss of contractile function in heat stress may reflect a subpopulation of fibers within the intact muscle that have lost membrane integrity. To test this hypothesis, we evaluated the integrity of the sarcolemmal membrane using procion orange leakage into diaphragm cells (32). Examples of the kinds of results from this experiment are shown in Fig. 6. No additional leakage was observed in response to heat stress alone (43°C). However, significant leakage was observed when muscles were incubated with both indomethacin and baicalein at 43°C (17.35% positive cells ± 1.37, $n = 6$) compared with control (0.45% positive cells ± 0.21, $n = 6$). Muscles incubated with indomethacin and baicalein at 37°C showed no significant increase in permeability (2.37% positive cells ± 0.71, $n = 6$) compared with both 43°C (2.32% positive cells ± 0.91, $n = 6$) and 37°C (0.45% positive cells ± 0.21, $n = 6$) controls.

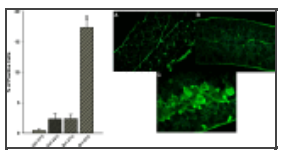

Fig. 6.
Membrane permeability evaluated using procion orange. Baicalein and indomethacin addition at 43°C caused a significant increase in the number of procion orange-positive cells. Procion orange diaphragm tissue: control 37°C (*A*), control **...**

DISCUSSION

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The results demonstrate that oxidative skeletal muscle, as represented by isolated rat diaphragm, is resistant to 30 min of hyperthermia between 40 and 42°C. However, exposure to 43°C causes a marked loss of tetanic force and an elevation in passive force. Although the mechanisms for the loss of normal contractile function at this threshold temperature are not known, we demonstrate that they are not

easily reversible in 30 min and do not appear to result from a response to oxidative stress. In addition, the response does not primarily reflect loss of cell membrane integrity, though when combined with LOX and COX inhibition, loss of the sarcolemmal barrier was widely present. Our data also suggest that a critical aspect of the muscle's ability to sustain normal function in hyperthermia is an intact network of membrane eicosanoid metabolic machinery, requiring functioning COX and LOX enzyme systems. Pharmacological inhibition by many contrasting pharmacological mediators, causes increased sensitivity to hyperthermia and must directly impact contractile function in some, as yet, unknown way.

Critique of approach.

Though it is unlikely that the diaphragm would ever attain temperatures much above body core temperature in hyperthermia, this model is useful as an intact, oxidative skeletal muscle preparation for the purposes of this study. We have little reason to believe that results would differ substantially from oxidative limb muscle, and in essence, our work substantiates results seen in isolated single oxidative limb muscle fibers exposed to heat (57). O_2 delivery is always a concern in nonperfused, intact preparations, but since the muscles were not fatigued or exercised intensely during the protocol, it is unlikely that the results can be attributed to differences in oxygen delivery. A 9% reduction in O_2 solubility occurs between 37°C and 43°C, and equivalent reductions in P_{O_2} have little effect on rat diaphragm contractile function (unpublished observation; Clanton T. L.) or on NADH autofluorescence (67), an indicator of tissue hypoxia. Although pharmacological approaches were used to evaluate the influence of enzymes involved with eicosanoid metabolism, as well as ROS scavenging, we attempted to account for possible nonspecific pharmacological effects of these drugs by *1*) studying their influence on time controls at 37°C, *2*) by studying dose response curves, and *3*) by using multiple drugs that often worked by chemically divergent methods. This approach revealed a significant adverse pharmacological reaction to NDGA, a commonly used LOX inhibitor; confirming recent studies in vitro and in vivo (34, 38).

Mechanisms for the loss of contractile function.

The results show that there is a critical temperature, between 42 and 43°C, in which there is a marked loss of contractile function within 30 min. This is also the range of temperatures generally required for induction of heat shock proteins (HSPs) (43), rapid increases in membrane permeability for small molecular weight molecules (8), and reduced survival in many mammalian in vitro cell types (20). The absolute value of the critical temperature is also, no doubt, a reflection of the duration of heat exposure, as loss of contractile function (57), as well as tissue injury, reviewed by Despa et al. (21), are both time and temperature dependent. The underlying biochemical mechanisms responsible for this critical temperature phenomenon are unknown, but most research points to accumulating alterations in protein structure, membrane integrity, or oxidative stress, as discussed below.

Although the activity of most enzymes increases as a function of their temperature coefficients (Q_{10}), it is generally held that protein configuration changes at some critical and protein-specific temperature, thereby decreasing activity or interfering with protein-protein interactions. Temperature-induced protein misfolding, exposure of hydrophobic moities, or degradation have traditionally been the primary mechanisms attributed to HSP induction in hyperthermia, as reviewed by Kim et al. (35). Although degradation is possible at these temperatures, there is little evidence for it in isolated proteins, as studies have shown that temperatures >50°C are generally needed for denaturation (21). Many investigations have attributed protein degradation to a secondary effect of increased oxidant formation during hyperthermia, (e.g., 52), which may be a common mechanism.

Acute hyperthermia also increases membrane fluidity (4) and is known to disrupt membrane microdomains, such as lipid rafts and the interaction of the membrane with associated channels, proteins, and cytoskeleton (41). Heat-induced changes in the biophysical properties of membranes can also result in alterations in water (48) or solute (8) permeability that could have secondary effects on membrane potential, action potential propagation, or contractile function. For example, swelling of muscle fibers, which has been shown to occur in cells at high temperature, could potentially have an influence on the lattice structure of the contractile machinery, and thus reduce force development (51).

Skeletal muscle hyperthermia has also been shown to cause elevations in ROS production (61, 66), but their complex role is not completely understood. Van der Poel and Stephenson (57) reported that in rat extensor digitorum longus muscle, a fast nonoxidative fiber, loss of maximum Ca^{2+}-activated force during heat could be blocked by cotreatment with the antioxidant Tiron (20 mM). In addition, it could be reversed after heat exposure with 20 mM DTT. Interestingly, these investigators saw no such effects of Tiron in more oxidative fibers from the limb muscle (peroneus longus). Our results for the diaphragm are essentially in agreement with the studies of the peroneus (57), presumably because the rat diaphragm is only 3.5% type IIb fast fibers (25). In addition, we tested for the influence of multiple doses of DTT and Trolox. Trolox is a soluble vitamin E analog and a general antioxidant and has previously been shown to be effective in intact diaphragm, protecting it from oxidative damage induced by mechanical ventilation (7) or from secondary oxidant effects of TNF (31). None of these agents were effective at concentrations equal to or much higher than those previously shown to be effective in diaphragm preparations (7, 23, 31, 63). Therefore, our results, taken together, with those of van der Poel and Stephenson (57) are most consistent with the hypothesis that oxidant production is not the main causative factor for the loss of contractile function in oxidative fibers. Because oxidative fibers are rich in mitochondria, which are believed to be a primary source of ROS in pathological conditions, these conclusions seem, indeed, paradoxical.

It is of physiological interest that the FF relationship was relatively unchanged between 37°C and 42°C. Since large shifts of FF to the right occur as temperature increases from 23°C to 37°C, we expected to see a continuation of this behavior above 37°C. The fact that tetanic force at all frequencies remains relatively constant suggests that skeletal muscle has an inherent mechanism for control of muscle force for a given neural activation within a temperature range that corresponds to the range seen in heavy exercise. How it could do this with varying rates of biochemical reactions (Q_{10}) is a mystery but would function to ensure continuity of motor control during exercise in hyperthermia.

Mechanisms for changes in passive force during heat exposure.

Another mechanical effect of heat exposure was an increase in passive tension (contracture) during 43°C exposure or at 42°C when drugs affecting eicosanoid metabolism were present. In all cases, the elevation in passive force was rapidly reversed during 37°C recovery, during which time the loss of contractile function did not reverse. There are at least two possible reasons for this phenomenon: first, resting cytosolic calcium concentration could increase during heat stress, thus activating the contractile machinery at low levels. To our knowledge, intracellular Ca^{2+} has not been measured in hyperthermic skeletal muscle. However, an increase in passive tension has been attributed to elevations in [Ca^{2+}] based on indirect observations that diaphragms with RyR1 mutations associated with malignant hyperthermia have greater heat-induced contracture than wild-type diaphragms (17). Also, elevated temperature has been shown to increase Ca^{2+} leakage from the SR, via an oxidant-dependent mechanism (58), though this would not necessarily result in elevated cytosolic calcium if the SR Ca^{2+}

ATPase continued to function appropriately.

A second possibility is a mechanism that has been demonstrated in glycerinated fibers at high temperature by Ranatunga et al. (50), which is due to non-Ca^{2+}-activated cross-bridge cycling. This is believed to reflect the heat-induced inactivation of the steric hindrance normally blocking cross-bridge interaction in the absence of Ca^{2+} (27). Interestingly, in all experiments, the observations of significant elevations in passive force were accompanied by proportional losses of maximum contractile function. This suggests that understanding the mechanism for elevation in passive force may provide future insights into the mechanisms for loss of maximum stimulated force. However, at this time, the mechanism is not clear in this muscle fiber population.

LOX, COX, and PLA_2 involvement during heat stress.

Very little is known about the functional roles of eicosanoid metabolism in skeletal muscles, even during normothermic conditions, though literature is emerging. With regard to COX, skeletal muscle cells contain both COX-1 and COX-2, and both of these enzymes are present in oxidative and nonoxidative muscle fibers, as well as in surrounding vessels (55). The primary products produced are prostaglandin PGE_2 and TxB_2, with considerably more PGE_2 being formed (55). Although there is some evidence that products formed by COX can modestly affect contractile function (55), their primary roles in physiology are not known. COX products are able to cause vasodilation or vasoconstriction, depending on which product is produced in higher amounts (15), and it is possible that this balance between products may be altered during heat stress. Thus, it is also possible that production of COX products in heat stress could affect heat dispersal mechanisms by influencing conductance of surrounding vessels. This assumes they are capable of paracrine signaling in this tissue. COX has also been found to be potentially important in the regulation of HSPs (6), as PGA_2, an enzymatic dehydration product of PGE_2, appears important for HSP70 expression over 4–8 h and for activation of c-fos and Egr-1 stress genes within 30 min. This process occurs by a mechanism involving increases in intracellular Ca^{2+} (18).

Even less is known about lipoxygenases in skeletal muscle. LOXs are nonheme iron-containing oxygenases that take polyenoic fatty acids (such as AA) and enzymatically oxygenate them into hydroperoxy-polyenoic fatty acids (53). Zuo et al. (65) found immunohistochemical evidence of both 5-LOX and 12-LOX in diaphragm cells, staining predominantly along the sarcolemma of smaller, presumably oxidative fibers. There are a variety of products made from lipoxygenases and associated downstream enzymes, including the leukotrienes and HETEs, and these are known to have a multitude of functions in immune responses, inflammation, and control of smooth muscle contraction. However, the roles of LOX in normal membrane remodeling, breakdown of subcellular organelles, and cell differentiation may be of equal or greater importance in many cells (36). Blocking global LOX activity, even at normal temperatures, greatly decreases extracellular ROS production and has no effect on intracellular superoxide formation in diaphragm muscle in heat exposure (65). Recent evidence suggests that HETEs, leukotrienes, AA, and other eicosanoid products can directly bind and interact with numerous intracellular proteins, including cytoskeleton components associated with the contractile machinery (10). Such lipid-protein interactions are believed to be important in normal regulatory processes of the cell, such as water balance across the cell membrane (44) and GLUT4 translocation to the muscle cell membrane (56).

No mechanisms have been described that could fully account for the effects of LOX and/or COX inhibition on contractile function during hyperthermia. However, several mechanisms are of potential

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significance. One working hypothesis is that inhibition of any important AA metabolic pathway could allow AA to accumulate in the cell to toxic levels during hyperthermia. Temperatures of 42–45°C are known to cause rapid increases in AA release and prostaglandin synthesis in cell systems (12) and AA alone has been shown to stimulate oxidant formation (24), to activate calcium channels (40), to disrupt the sarcolemmal Ca^{2+}-ATPase (13), and to activate the mitochondrial transition pore (22, 49). The sarcolemmal Ca^{2+}-ATPase denaturation temperature has been found to shift according to the concentration of free fatty acids in the cytosol (40). Therefore, by these mechanisms, blockage of any pathway that allows AA to accumulate in the cell could potentially result in cell dysfunction. A second, related idea is that LOX and COX inhibitors can sometimes act as prooxidants. This is a controversial area because most of the pharmacological agents used in this study have been shown to be chemical antioxidants by this laboratory (65) and others, e.g., baicalein (47), ETYA (54), indomethacin (46), and ibuprophen (19). Nevertheless, there is abundant evidence, particularly in intact tissues or cells, that any of these agents can also act as prooxidants under certain conditions, e.g., baicalein (62), ETYA (3), indomethacin (5) and ibuprophen (39). Since many in vitro chemical studies have ascribed these agents as having antioxidant activities, it could be that frequent observations of oxidant reactions in more intact preparations is secondary to the oxidative effects of accumulating AA. With this in mind, it is possible that application of any eicosanoid enzyme inhibitor could induce an AA-mediated oxidant stress, superimposed over any damage caused by hyperthermia alone.

To test the possibility that AA accumulation could be responsible, we attempted to reduce AA formation during 43°C heat stress by inhibiting PLA_2 with the calcium-independent PLA_2 (specific $iPLA_2$) inhibitor, BEL, as well as by a general cPLA2 and iPLA2 inhibitor, $AACOCF_3$. Though not studied extensively, isoforms of $iPLA_2$ are highly expressed in skeletal muscle cell lines (28) with negligible Ca^{2+}-dependent PLA_2 being expressed. The specificity of BEL has been found to be 1,000-fold greater for $iPLA_2$ compared with the $cPLA_2$ (33) and has been used extensively in muscle (29, 42). $AACOCF_3$ has been reported to inhibit both cPLA2 and iPLA2 at the concentrations used (1). However, contrary to our hypothesis, neither BEL nor AACOCF3 administration protected muscle function in 43°C hyperthermia. The highest concentration of BEL contributed to a further decline in function at 43°C, although this could have been due, in part, to a pharmacological effect that showed up even at 37°C control experiments (Fig. 5). The results for PLA_2 inhibition by BEL and $AACOCF_3$ do not necessarily disprove the hypothesis that AA accumulation is responsible for the toxic effects of LOX and COX inhibition. However, it lends some credence to an alternative hypothesis that LOX and COX activity or products from their activity are somehow necessary for normal responses to hyperthermia.

Finally, it is possible that the effects of each of the eicosanoid enzyme inhibitors may represent nonspecific toxic effects that are minimally evident at normal temperatures but are accentuated at ≥ 42°C. The extreme effects at 43°C and the apparent added insult on muscle membrane integrity (Fig. 6) could represent an additive effect from the toxicity of the drugs at high temperature and direct effects of extreme heat. They may, therefore, be unrelated phenomena. The only circumstantial evidence that would counter this argument is that the same basic observation was seen with a variety of different drugs at a variety of doses, which, in some cases, are within the therapeutic range in humans. It would seem unlikely that we would observe such a uniform toxicity unless it was directly related to the normal physiology of hyperthermia.

Conclusions.

These results support the concept that skeletal muscles have an inherent thermotolerance within the range of temperatures they are likely to experience during intense exercise. However, during extreme conditions, in which the body cannot adequately maintain thermal equilibrium, they are susceptible to loss of function. Furthermore, our results concur with those of van der Poel and Stephenson (57), in that there may be a unique phenotypic response of oxidative skeletal muscles that is not true of fast fibers, i.e., their loss of function may not be related to oxidant production and may not be rapidly reversible with time. Finally, we have demonstrated that, at least in oxidative diaphragm muscles, normal thermotolerance is dependent on an intact network of eicosanoid metabolic machinery.

Perspectives and Significance

We hypothesize that thermotolerance may be another important function of eicosanoid enzyme activity that has largely gone unrecognized. Hyperthermia can affect all cells and essentially all integrative systems within the body and comprises one of the most primordial environmental stresses driving evolutionary diversity. Although we studied one tissue in isolation in this study, we speculate that eicosanoid metabolism may play a large role in thermal adaptation in many other tissues, beyond its better-known influences on local vascular regulation and as a mediator of inflammatory cascades. It is also possible that alterations in gene expression of eicosanoid enzymes, secondary to disease, chronic inflammation, or drug treatment could make tissues more or less vulnerable to heat injury. Future studies are needed to discover the extent of involvement of eicosanoid metabolism in regulation of normal skeletal muscle function, the hyperthermic tolerance of other tissues of the body, and in the overall integrative responses to exertional hyperthermia and environmental heat exposure. Discovering links between eicosanoid metabolism and hyperthermia could create new therapies and treatment paradigms for heat-related illnesses that have the potential of improving thermal tolerance in athletes, soldiers, first-responders, and other vulnerable populations, such as the elderly and very young.

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