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Intra- and extracellular measurement of reactive oxygen species produced during heat stress in diaphragm muscle

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Zuo, Li, Fievos L. Christofi, Valerie P. Wright, Cynthia Yu Liu, A. John Merola, Lawrence J. Berliner, and Thomas L. Clanton. Intra- and extracellular measurement of reactive oxygen species produced during heat stress in diaphragm muscle. *Am J Physiol Cell Physiol* 279: C1058–C1066, 2000.—Skeletal muscles are exposed to increased temperatures during intense exercise, particularly in high environmental temperatures. We hypothesized that heat may directly stimulate the reactive oxygen species (ROS) formation in diaphragm (one kind of skeletal muscle) and thus potentially play a role in contractile and metabolic activity. Laser scan confocal microscopy was used to study the conversion of hydroethidine (a probe for intracellular ROS) to ethidium (ET) in mouse diaphragm. During a 30-min period, heat (42°C) increased ET fluorescence by 24 ± 4%, whereas in control (37°C), fluorescence decreased by 8 ± 1% compared with baseline ($P < 0.001$). The superoxide scavenger Tiron (10 mM) abolished the rise in intracellular fluorescence, whereas extracellular superoxide dismutase (SOD; 5,000 U/ml) had no significant effect. Reduction of oxidized cytochrome *c* was used to detect extracellular ROS in rat diaphragm. After 45 min, 53 ± 7 nmol cytochrome *c*·g dry $wt^{-1}\cdot ml^{-1}$ were reduced in heat compared with 22 ± 13 $nmol\cdot g^{-1}\cdot ml^{-1}$ in controls ($P < 0.001$). SOD decreased cytochrome *c* reduction in heat to control levels. The results suggest that heat stress stimulates intracellular and extracellular superoxide production, which may contribute to the physiological responses to severe exercise or the pathology of heat shock.

cytochrome *c*; ethidium; laser scan confocal fluorescence imaging; exercise

REACTIVE OXYGEN SPECIES (ROS) play an important role in many biological systems. ROS formation is closely related to the body's response to infection, ischemia-reperfusion, and heavy metal and ethanol toxicity, as well as to many other conditions (13, 27). It is also believed to play important roles in normal cell signaling events (10, 16). Skeletal muscles have been shown to generate considerable ROS under normal conditions. For example, in vitro rat diaphragm produces moderate levels of intracellular (29, 35) and extracellular ROS (36) at rest, and production increases markedly with repetitive muscle contractions (22, 36).

In this study, we test the hypothesis that increased ROS production in skeletal muscles is also associated with exposure to mild heat stress. Temperature elevations have potent effects on nearly all biological systems; however, they may have particular relevance to skeletal muscles because these muscles act as natural heat generators during exercise. For example, in rats exercised to exhaustion, average limb muscle temperatures can reach 44°C and core temperatures can reach 42°C (4). These temperatures would result in heat shock or a stress response in most muscle cells, causing upregulation of stress proteins (27, 41). Although stress proteins are upregulated in skeletal muscle during extreme exercise (19, 41), the relationship between ROS generation, exercise, and heat is still unknown.

Results of the current study demonstrate that markedly increased ROS formation, particularly superoxide ($O_2^-\cdot$), is a natural response of the myocyte to heat exposure at temperatures known to be physiologically relevant to exercise. Furthermore, these signals are shown to be blocked by antioxidants specific for $O_2^-\cdot$. The findings may provide insight into understanding the physiological and pathophysiological responses to heat exposure in exercising or nonexercising skeletal muscle.

METHODS

Male adult Sprague-Dawley rats (300–500 g) and Swiss-Webster mice (20–30 g) were used according to the animal care guidelines at Ohio State University.

Surgical Procedures and the Diaphragm Strip Preparation

The animals were anesthetized with intraperitoneal pentobarbital sodium (~40 mg/kg), tracheotomized, and mechanically ventilated. For the rat studies, the animals were first heparinized (~500 U/kg) via direct injection into the jugular vein, and the diaphragm was then perfused and cleared of blood by retrograde perfusion with oxygenated Ringer solution from the inferior vena cava. This was done to

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eliminate possible blood and serum contamination during the cytochrome *c* measurements. Four diaphragm muscle strips (~0.9 cm wide for rats, 0.5 cm for mice) were dissected out with the corresponding rib attachment and central tendon, placed in Ringer solution (in meq/l: 21 $NaHCO_3$, 1.0 $MgCl_2$, 1.2 Na_2HPO_4, 0.9 Na_2SO_4, 2.0 $CaCl_2$, 5.9 KCl, and 121 NaCl, as well as 2.07g/l glucose with 10 μM *d*-tubocurarine), and bubbled with 95% O_2-5% CO_2.

Extracellular ROS Studies

The cytochrome *c* assay was used to measure extracellular $O_2^-\cdot$ release in rat diaphragm, employing a combination of methods as described by Margoliash and Frohwirt (25), Reid et al. (36), and Kolbeck et al. (22). $O_2^-\cdot$ can reduce cytochrome *c* through a one-electron transfer reaction, resulting in an increase of absorbance peak at 550 nm, with an extinction coefficient of $18.5 \times 10\ M^{-1}\cdot cm^{-1}$ (22, 24, 26). To test whether $O_2^-\cdot$ is released from tissue during heat stress, each rat diaphragm strip was loaded into a 2.9-ml water-jacketed minibath (Radnoti, Monrovia, CA) filled with oxygenated Ringer solution and 5 μM cytochrome *c* (Acros Organics). A tension of 2 g was placed on each strip, which in previous studies was shown to approximate optimum length for the size of the muscle utilized and the inherent weight of the transducer attachments for the muscle (12). The experiments were performed in a darkened room (preventing possible photobleaching of cytochrome *c*). The measurement of cytochrome *c* reduction was obtained by taking the difference between the peak absorbance at 550 nm and the average of the peaks at wavelengths of 540 and 560 nm, as described by Kolbeck et al. (22). This procedure avoided errors in measurement due to non-cytochrome-*c*-specific increases in absorbance during these experiments.

Heat treatment only. Two muscle strips from the same animal were studied simultaneously, each fitted into separate minibaths. After a 20-min equilibration at 37°C, one bath was kept at 37°C (control), and the other was heated to 42°C (heat) for 45 min. The reduction state of cytochrome *c* in the baths was measured by removing 1 ml of the bath solution and immediately transferring it to a cuvette and measuring absorbance using a diode-array ultraviolet-visible spectrophotometer (HP 8452A, Hewlett-Packard). The cuvette sample was then immediately returned to the bath. Measurements were made every 15 min.

Heat treatment plus antioxidants. To test for the specificity of the cytochrome *c* assay for $O_2^-\cdot$ release, heat and control experiments were repeated with strips preincubated for 30 min on ice with 5,000 U/ml superoxide dismutase (SOD; Sigma) or catalase (1,000 U/ml, Sigma). SOD is an effective $O_2^-\cdot$ scavenger. Catalase, a hydrogen peroxide (H_2O_2) scavenger, was used to test for possible H_2O_2 release, since H_2O_2 can oxidize reduced cytochrome *c* and therefore potentially affect the quantification of cytochrome *c* reduction (23, 44).

Controls for the cytochrome c assay. These experiments were performed to answer a variety of questions related to the cytochrome *c* assay at both 37°C and 42°C over the 45-min experimental period. Every attempt was made to mimic the biological conditions studied, as described above. The following issues were studied: *1*) autoreduction of cytochrome *c* and *2*) cytochrome *c* chemical interactions with SOD, 1,2-dihydroxybenzene-3,5-disulfonate (Tiron; Aldrich), and catalase. An additional experiment was performed to determine the release of cytochrome *c* from the tissue in heat by soaking the muscle in oxygenated Ringer solution, without cytochrome *c*, before the 45-min heat and control exposure. There was no cytochrome *c* released during heat stress (data not shown).

Intracellular ROS Studies

Confocal microscopy. The hydroethidine (HE)/ethidium (ET) fluorescent probe was used to detect intracellular ROS production in mouse hemidiaphragms. HE (Molecular Probes) is a noncharged fluorescent probe specifically sensitive to $O_2^-\cdot$, peroxynitrite ($ONOO^-$), and hydroxyl radical (·OH) but not to H_2O_2 (1, 6). HE stock was made in *N,N*-dimethylacetamide (Acros Organics). In response to ROS, HE is dehydrogenated, resulting in the formation of ET. ET is positively charged and has better cellular retention and stability compared with HE. For this reason, ET formation was chosen as an indicator of ROS production, which is a common method when using this probe (1, 6, 29). Mouse diaphragms were used because preliminary studies demonstrated better loading of HE in mouse compared with rat. Furthermore, the mouse diaphragm is considerably thinner, compared with the rat diaphragm, which improves the working distance, the quality, and the resolution of the confocal fluorescence imaging analysis. Muscle strips were loaded with 44 μM HE and 4 mM actin-myosin complex blocker 2,3-butanedione monoxime (BDM; Acros Organics) in Ringer solution, in the dark, on ice, for 1 h. BDM was used throughout the experiment to block movement of the tissue during heat exposure. When tissue movement exceeded 10% of the size of the field in any direction during data collection (based on visual landmarks and pattern recognition), the data were discarded. In addition, vertical artifacts due to any residual BDM-insensitive movements were minimized by selecting an appropriately thick optical slice (i.e., ~10 μm). In some experiments, z-sectioning analysis done before and after heat experiments indicated that the intensity of the ET signal after heat was invariably greater than that for any of the vertical optical slices before heat (data not shown). This strongly supports that any changes obtained were due to heat and did not involve vertical movement artifact. After incubation, the muscles were washed for 10–15 min with fresh oxygenated Ringer solution, placed in a flow-through tissue chamber (Biophysica, Ontario, Canada), stretched carefully with wires attached to magnetic holders to ~120% of relaxed length, and put on the stage of a laser scan confocal and spatial imaging analysis system (Zeiss 410; Carl Zeiss, Germany). An inverted confocal microscope was used, and the tissue was excited by the appropriate lasers through a no. 0.0 coverslip (0.11–0.17 mm thick) forming the bottom of the chamber. After a 15-min equilibration at 37°C using constant superfusion of heated, oxygenated Ringer solution (with 4 mM BDM), measurements of ET fluorescence were made every 5 min. Although loss of HE fluorescence was also measured (excitation: 364 nm, emission: 480 nm ± 30 nm), our preliminary data showed that the nonpolar HE probe had a relatively lower cell retention than ET, its fluorescence was more easily influenced by environmental solutions (29), and the background from NADH within the myocyte fluorescence was in the same emission range as HE (17). The setup for laser scan confocal imaging of ROS was the following: argon/krypton laser power, 10; objective, ×63 with 1.5-mm working distance; numerical aperture, 0.75; ET excitation, 568 nm; ET emission, long pass (LP, 590 nm); contrast, 180–250; brightness, 9500–9999; pinhole, 150 (i.e., its corresponding optical section has a thickness of 9.75 μm). The emitted fluorescence signal captured by a photomultiplier tube was presented as an image of 512 × 512 pixels on the laser-scanning microscope (LSM) computer monitor. The LSM 410

software was used to analyze the fluorescence image, representing a small square of ~0.04 mm^2 of the tissue. Most fields contained portions of ~4–7 myocytes. The average fluorescence over the entire field was collected, and the averaged image of four consecutive scans of the same field was saved.

A number of confocal microscopy experiments, which paralleled the cytochrome *c* assay experiments previously described, were performed as described below.

Heat treatment only. In these experiments, the muscle strips were superfused with oxygenated Ringer solution and 4 mM BDM at 37°C for 5 min and then the stage temperature was increased to 42°C for 30 min. Temperatures were measured to within 0.1°C directly in the superfusate bathing the tissue, using a miniature thermistor (YSI 4610; Yellow Springs Instruments, Yellow Springs, OH). Control muscle strips were treated identically, except that the superfusate was maintained at 37°C throughout the experiments.

Heat plus antioxidant treatment. Tiron (10 mM, a membrane-permeable scavenger of $O_2^-\cdot$) or SOD (5,000 U/ml, nonpermeable to membranes) was added to 37°C perfusate, 15 min before the measurement in both heat and control experiments to let the tissues be fully equilibrated with these antioxidants.

Nuclear localization of ET fluorescence. To identify whether ET fluorescence is concentrated within the cell nucleus or cytoplasm, 4,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; 1 μg/ml, Sigma) was used as a fluorescent marker for the nucleus. All other conditions were the same as described in *Heat treatment only*. LSM overlay image analysis was used to correlate regions of ET fluorescence (excitation: 568 nm, emission: LP, 590 nm) overlapping with regions of DAPI fluorescence (excitation: 364 nm, emission: 480 nm ± 30 nm) at the control temperature.

In vitro tests of the ET fluorescence. Biochemical controls were used to test the specificity of the ET fluorescence assay for heat-induced reactive oxygen using a standard cuvette fluorometer (model 5050; Perkin Elmer). These tests were repeated with reagents used in the tissue experiments.

Statistical Analysis

Values are expressed as means ± SE. Data were analyzed with SAS JMP (SAS Institute, Cary, NC) using two-way ANOVA, with heat treatment and time being the factors of interest and the experimental animal treated as a random variable. Post hoc contrasts were used to determine differences in mean values at specific time points and between treatments. $P < 0.05$ was considered statistically significant.

RESULTS

$O_2^-\cdot$ Release From Tissues in Heat Stress

As shown in Fig. 1, heat exposure caused significant increases in cytochrome *c* reduction, as early as 15 min into the test. After 45 min of exposure to heat, a total of 53 ± 7 nmol cytochrome $c\cdot$g dry $wt^{-1}\cdot ml^{-1}$ solution were reduced compared with control (22 ± 13 $nmol\cdot g^{-1}\cdot ml^{-1}$, $n = 8$, $P < 0.001$).

Figure 2*A* illustrates that extracellular SOD treatment blocked the influence of heat treatment on $O_2^-\cdot$ production. After 45 min, the SOD- and heat-treated muscles demonstrated a total cytochrome *c* reduction (21 ± 8 $nmol\cdot g^{-1}\cdot ml^{-1}$) that resembled control, i.e., SOD- and non-heat-treated diaphragm (13 ± 7 $nmol\cdot g^{-1}\cdot ml^{-1}$). Furthermore, there were no significant differences between SOD-treated control and

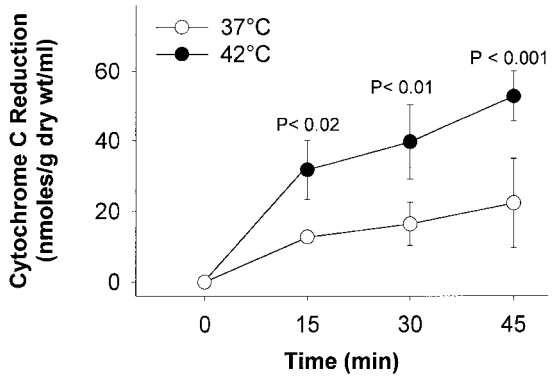

Fig. 1. Effects of heat treatment on extracellular reactive oxygen species (ROS) formation. Cytochrome *c* reduction by diaphragm during heat stress compared with control conditions.

SOD-heated muscle at any time point ($n = 5$). As will be demonstrated later, Tiron caused direct reduction of cytochrome *c*, independent of the presence of diaphragm tissue, thus prohibiting its use in evaluating mechanisms of extracellular cytochrome *c* reduction by muscle tissue.

The catalase experiments are illustrated in Fig. 2*B*. After the 45-min exposure to heat stress, there were no significant differences between catalase-treated (51 ± 5 $nmol\cdot g^{-1}\cdot ml^{-1}$) and non-catalase-treated (63 ± 7 $nmol\cdot g^{-1}\cdot ml^{-1}$) tissues ($n = 6$ from 3 animals), sug-

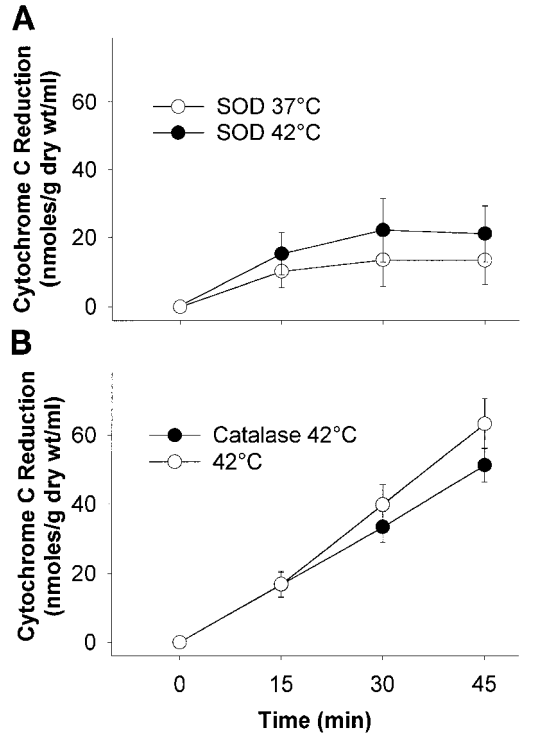

Fig. 2. Effects of antioxidants on extracellular ROS formation. *A*: cytochrome *c* reduction by diaphragm, with and without heat treatment, in the presence of superoxide dismutase (SOD). *B*: cytochrome *c* reduction by diaphragm during heat stress, with and without catalase.

gesting that there was no significant H_2O_2 released from these diaphragms in heat stress.

Biochemical Controls for the Cytochrome c Assay

Potential autoreduction of cytochrome *c* was tested by exposing 5 μM cytochrome *c* in oxygenated Ringer solution to 42°C and 37°C (Fig. 3). After 45 min, cytochrome *c* was reduced similarly in both heat-treated (0.09 ± 0.08 nmol/ml) and control (0.07 ± 0.03 nmol/ml, not significant (NS), $n = 5$), accounting for < 3% of the total cytochrome *c* in solution. SOD treatment (5,000 U/ml) resulted in a reduction of <5% and catalase (1,000 U/ml) of <18% of the total cytochrome *c* in the solution, with no appreciable effects of heat treatment. In contrast, 10 mM Tiron resulted in rapid and immediate reduction of cytochrome *c*, reducing the cytochrome *c* at both 37°C (4.8 ± 0.19 nmol/ml, $n = 3$) and 42°C (4.1 ± 0.13 nmol/ml, $n = 3$) and accounting for >80% of the total cytochrome *c* available for reduction in solution.

Intracellular ROS Formation in Heat Stress

As shown in Fig. 4, *A* and *B*, under confocal microscopy, ET fluorescence increased markedly in heat-treated mouse diaphragm over 30 min (increase in red), with no significant increase in ET fluorescence in control tissue at 37°C (Fig. 4, *C* and *D*). Typically, the ET fluorescence was heterogeneous, both within individual fibers and within the nuclei and cytosol of the myocytes. Grouped data are shown in Fig. 5*A*. After a 5-min preheat measurement (in which ET fluorescence was basically constant), rapid and significant increases in ET fluorescence were observed within 15 min, increasing 24 ± 4% of baseline by 30 min. In contrast, control (37°C) fluorescence decreased 8 ± 1% of baseline during the same period ($n = 6$, $P < 0.001$). It is worth noting that the observed increases in ET fluorescence represented an underestimate of the change, because the entire field was used in analysis rather than individual myocytes. In all experiments, considerable background ET fluorescence was observed in the baseline images. This presumably was due to endogenous ROS formation in resting tissue (36).

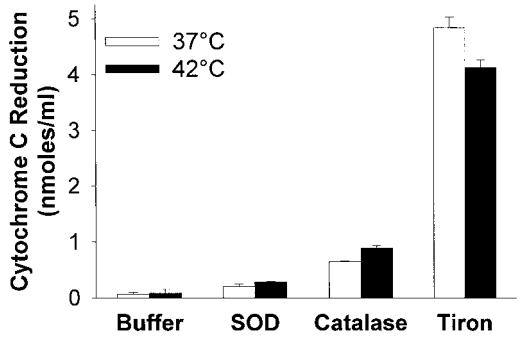

Fig. 3. In vitro cytochrome *c* experiments in oxygenated Ringer solution. Buffer, autoreduction of cytochrome *c*; SOD, reduction of cytochrome *c* with SOD; Catalase, reduction of cytochrome *c* with catalase; Tiron, reduction of cytochrome *c* with Tiron.

Effects of Antioxidants on Intracellular ROS

Treatment with Tiron (10 mM, Fig. 5*B*), a $O_2^-\cdot$ scavenger accessible to the intracellular compartment, completely removed the effect of heat on ET fluorescence in diaphragms. Furthermore, it reduced the ET fluorescence by 7 ± 6% and 12 ± 3% in heated and control tissues, respectively (NS, $n = 3$). In contrast, continuous SOD treatment (5,000 U/ml) in the superfusate did not decrease the ET fluorescence observed during exposure to heat (Fig. 5*C*), suggesting that this ROS signal originated from the intracellular compartment.

Localization of ET Fluorescence

As illustrated in Fig. 4, *E* and *F*, double staining during 37°C with DAPI and HE resulted in colocalization of DAPI (blue fluorescence, Fig. 4*E*) with ET (red fluorescence, Fig. 4*F*). We noticed that >90% of the blue dots (nuclei) superimposed on the red dots in these experiments. Taken together, it is evident that the ET signal localizes in both the nucleus and cytoplasm, although the intensity of the signal in the nuclei is higher than in the cytosol. Whether ET and DAPI also colocalized in the mitochondria was not distinguishable at these magnifications. Note, compared with DAPI, which has a relatively high quantum yield, HE blue fluorescence is almost undetectable at the laser and LSM settings used to detect DAPI (contrast = 213) and therefore did not contribute to the DAPI-blue fluorescence.

Biochemical Controls for Confocal Microscopy In Vitro

Control experiments demonstrated that *1*) heat does not significantly affect the spontaneous conversion of HE to ET, *2*) Tiron does not affect HE or ET fluorescence, *3*) BDM causes <9% decrease by HE baseline fluorescence without significant conversion from HE to ET, and *4*) BDM and Tiron together decrease HE baseline fluorescence by <9%, but without changing ET fluorescence (data not shown).

DISCUSSION

These results demonstrate that exposure to heat stress results in rapid and significant elevations in extracellular and intracellular reactive oxygen production. Although the levels of heat treatment used were within the physiological range measured in exercising rat muscle (4) and in humans during exercise in heated environments (39), exposure to this temperature is sufficient to upregulate heat shock proteins (41) and therefore can appropriately be categorized as heat stress. Treatment with relevant $O_2^-\cdot$ scavengers strongly suggests that the primary or initial species generated during heat stress in muscle is $O_2^-\cdot$, as measured by both extracellular and intracellular probes. The direct production of $O_2^-\cdot$ during heat may have implications with regard to mechanical, pathological, and cell signaling responses to extreme exercise and

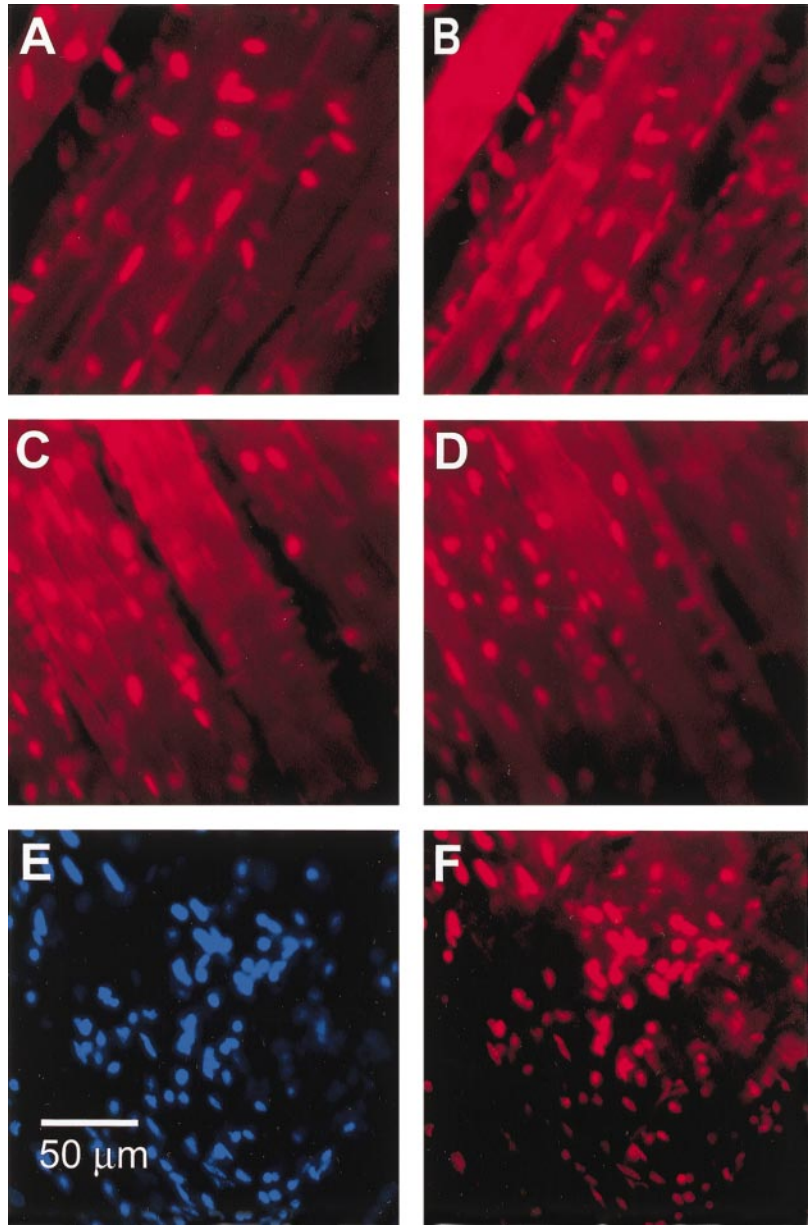

Fig. 4. Intracellular ROS formation in heat stress. Typical ethidium (ET) confocal fluorescent image of mouse diaphragm before (*A*) and after (*B*) 30 min of heat stress (42°C). Typical fluorescent ET images in control diaphragm at baseline (C) and after (*D*) 30 min at 37°C. From *C* to *D*, no increase in ET signal was observed; in fact, a slight loss and redistribution of the ET signal was seen after the control period. In *E* and *F*, tissues were loaded by both 4,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) and hydroethidine (HE). *E*: DAPI staining in the nuclei of the myocytes. *F*: baseline ET produced is localized mainly in DAPI-stained nuclei (see METHODS). (Note: paired *A–B*, *C–D*, and *E–F* represented images from 3 different diaphragms from different mice demonstrating considerable variability in ET baseline signals between diaphragms. For example, differences in contrast between baseline signals in *A* and *C* are typical, and are in part due to different contrast setups to maximize the resolution.)

fatigue, as well as to responses to exercise in elevated environmental temperatures.

Measurement of $O_2^-\cdot$ Release From Diaphragms in Heat Stress

Previous studies by Reid et al. (36) and Kolbeck et al. (22) have used the cytochrome *c* assay to demonstrate $O_2^-\cdot$ generation in resting muscle and have shown marked increases in production during muscle stimulation. The present work extends these findings to conditions of heat stress, suggesting the possibility that extracellular ROS formation is a generalized response to a number of stresses to muscle, which may be related to exercise. Cytochrome *c* is used as an electron acceptor in these studies and, theoretically, any electron donor secreted from the tissue with the appropriate redox potential could reduce cytochrome *c*. However, the inhibition of the heat-induced reduction of cytochrome *c* by SOD provides relative certainty that the assay measured $O_2^-\cdot$ and not another unknown substance released during heat. The cytochrome *c* approach has been used successfully in many other biological models, including but not limited to brain injury during hypoxia (14), dopamine oxidation in the nervous system (21), and menadione-stimulated pyridine nucleotide oxidase activity from neutrophil membranes (30). The large molecular size of cytochrome *c* (12.4 kDa) prevents its penetration through the sarcolemma, making it an exclusively extracellular probe (36). Additionally, in agreement with previous studies (36), we confirmed that it is remarkably stable in physiological, oxygenated buffers, as well as in the presence of SOD and catalase solutions.

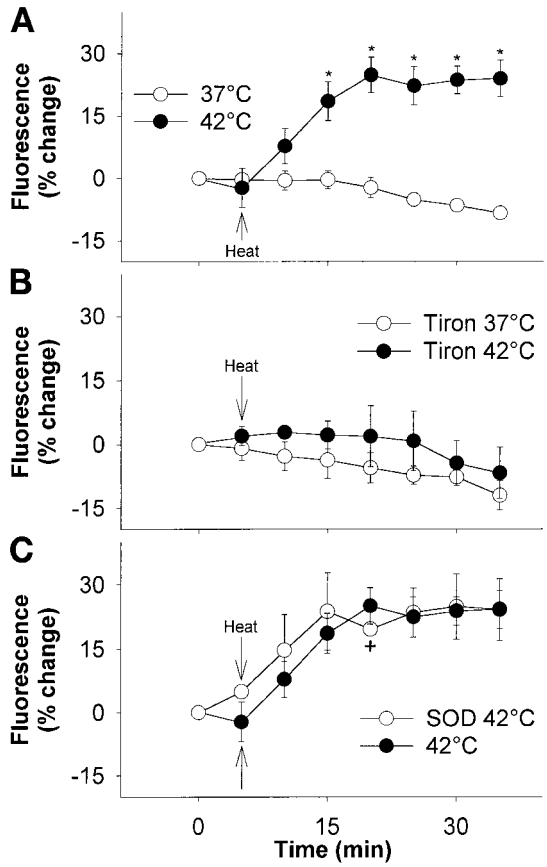

Fig. 5. Grouped data for intracellular ROS formation indicated by changes in ET fluorescence. *A*: effects of heat vs. control conditions. *$P < 0.001$ vs. 37°C control. *B*: same experiment as in *A*, in the presence of Tiron. *C*: effects of SOD treatment on ET fluorescence during heat. Data from heat treatment in *C* (○) are same data shown in *A*, for comparison; $^{+}$one data point removed at this time point because of >10% movement of tissue out of the field ($n = 2$).

Potential Sources of ROS Production in Heat Stress

A number of potential cellular sources for ROS have been proposed in skeletal muscle. Although mitochondria are strong potential candidates, other sources are possible. For example, the diaphragm has a very large capillary bed, which has been estimated to be at least twice that of a comparable muscle fiber population (20). Therefore, the isolated muscle has a large amount of capillary endothelium. Endothelial cells are now understood to be potent potential sources of ROS production via NADH and/or NADPH oxidases on their cell membranes (11, 28). Reactive oxygen production may play a role in vascular responses to sheer stress and other stimuli, which may be important in local vascular regulation (11). Therefore, the extracellular signal observed in these experiments in response to heat stress could conceivably have arisen from endothelial sources as well as myocyte sources. $O_2^-\cdot$ is believed to be capable of diffusing across cell membranes, and it has been postulated that extracellular $O_2^-\cdot$ produced by skeletal muscle may exit the myocyte directly by diffusion (35, 36). This was based largely on the influence of extracellular antioxidants, such as SOD on contractile properties, but also on the inhibition of dichlorofluorescein oxidation by extracellular SOD (35).

In contrast, the intracellular ROS production, as indicated by HE/ET fluorescence, was clearly localized within the myocyte. It is possible that the myocyte may also contain cytosolic or membrane-bound NADPH or NADH oxidoreductases; however, these have not been identified. Furthermore, xanthine oxidase (11), nitric oxide synthase (NOS) (32), or arachidonic acid metabolism (9) have also been presented as potential sources of ROS in skeletal muscle and other tissues. It is possible that the activity of these enzymes may be uniquely affected by heat and/or the influx of intracellular Ca^{2+} during heat stress; however, their role has yet to be identified in muscle.

A likely source of elevated ROS production in heat is the mitochondria. Mitochondria are believed to produce basal levels of ROS in the form of single-electron leakage to oxygen during normal metabolism (8). This can increase dramatically under conditions in which the mitochondria are damaged or exposed to certain toxic conditions. The response of the mitochondria to heat is only partially understood. In the 1970s, a number of studies evaluated the relationships between temperature, muscle function, and oxygen consumption. Oxygen consumption was found to increase markedly, immediately after exercise. Although there are a number of potential mechanisms for this phenomenon, it has been proposed that it may in part be related to increased temperature (4). At high body temperature, skeletal muscles exhibit a lower level of phosphorylation efficiency and a higher respiratory rate (3, 4). Furthermore, higher temperatures have a significant effect on mitochondrial function, although this has not been studied extensively. For example, from 25°C to 45°C, as the mitochondrial oxygen consumption increases ~2.5-fold, mitochondrial-specific ATPase activity increases ~4-fold (3). Finally, elevated temperatures have been shown to result in increased ATP utilization and creatine phosphate depletion (15). The mechanisms for these changes in mitochondrial function and metabolism are not known, but they may be consistent with conditions that promote oxidant production (31). Alternatively, they may reflect the effects of oxidant production on mitochondrial function or both.

Nethery et al. (29) have recently demonstrated that phospholipase A_2 activation appears to be a necessary step in the increase of skeletal muscle reactive oxygen formation during muscle stimulation. The activation of phospholipases may result in reactive oxygen formation by first generating arachidonic acid from available membrane substrates. Arachidonic acid may then interfere directly with electron transport and produce ROS via cyclooxygenase pathways or by direct interaction with the mitochondrial electron transport chain (9).

Another potential source of the increase in both the extracellular and intracellular ROS measurements is the influence of heat on ·NO production. Because ·NO is known to react rapidly with $O_2^-\cdot$ to form $ONOO^-$ and

other down stream metabolites, any change in NOS activity could affect the $O_2^-\cdot$ signal measured by these assays. The current experiment evaluated responses over a period of 1 h or less, making changes in ·NO production due to new NOS gene expression unlikely. However, it is highly probable that NOS activity was directly augmented by increased temperature. For example, Venturini et al. (45) have demonstrated that NOS I and NOS II (i.e., nNOS and iNOS) increase their activity in vitro by ~100% between temperatures of 37° and 42°C. Such an elevation in ·NO would presumably diminish the extracellular cytochrome *c* reduction, because any additional $ONOO^-$ formed would oxidize cytochrome *c* (43). Therefore, our overall conclusions in this paper regarding the effects of heat exposure on extracellular $O_2^-\cdot$ release are qualitatively correct, although possibly underestimated. The effects on intracellular HE oxidation are more complex since both $O_2^-\cdot$ and $ONOO^-$ oxidize HE to ET (1), and both species would be inhibited by Tiron administration. Therefore, it is possible that some of the increased intracellular ET signals we observed could be due to a contribution of augmented NOS activity from $ONOO^-$ in heat. Further experiments are required to address this complex problem.

H_2O_2 Release From the Tissue

Because H_2O_2 oxidizes reduced cytochrome *c* (23, 44), if an appreciable amount of H_2O_2 had been released into the extracellular space, before or during heat stress, we would have expected an increase in the net reduction of cytochrome *c* with catalase treatment during heat stress. Because this did not happen, we speculate that endogenous intracellular H_2O_2 scavengers, such as catalase, the glutathione peroxidase system, or the thioredoxin peroxidase system, were sufficient to remove H_2O_2 before it was able to diffuse out of the tissue in significant quantities. Why $O_2^-\cdot$ was not scavenged by intracellular SOD is a point of some speculation and may reflect unique pathways of $O_2^-\cdot$ excretion from the cell, which have yet to be fully identified.

Measurement of Intracellular $O_2^-\cdot$ Formation in Heat Stress

HE was chosen as the fluorescent probe of choice in these experiments, largely because preliminary experiments using more common probes, such as derivatives of dichlorofluorescein, proved unsuitable at elevated temperatures. At 37°C and particularly at 42°C, the extremely poor retention of these probes made it impractical and perhaps impossible for use in studies of heat stress (data not shown). HE, an uncharged molecule, can enter and stain cells with little or no toxicity. It is easily oxidized by $O_2^-\cdot$, $ONOO^-$, and ·OH to form ET, a polar molecule with a positive charge, which is subsequently trapped inside the cell. The HE/ET probe has been used as a sensitive $O_2^-\cdot$ indicator in studies using endothelial cells (7), cerebellar granule neurons (6, 37), tumor cells (5), lung (1), and inflammatory cells (42). Recently, Nethery et al. (29) demonstrated its use in perfused diaphragmatic muscle, where it was measured by extraction after the experiment. In this study we employed confocal microscopy, which is very useful to observe changes in fluorescence in real time and to provide direct evidence of intracellular localization. The confocal image represents information originating from the optical slice in the plane of focus with unfocused images from other planes excluded, resulting in the improvement of the image's resolution across the cell. In contrast to studies of the cytochrome *c* probe, there was no chemical interaction between the cell-permeable $O_2^-\cdot$ scavenger, Tiron, and the HE/ET probe, thus allowing the use of this antioxidant to block the intracellular signal. The signal, however, was not inhibited by extracellular application of SOD, suggesting an intracellular localization of $O_2^-\cdot$ generation within the myocytes. Although H_2O_2 must also be formed as the result of the reaction between Tiron and $O_2^-\cdot$ (18), HE is relatively insensitive to H_2O_2, and thus the assay is directed largely to $O_2^-\cdot$, $ONOO^-$, and ·OH (1, 6, 29). The DAPI nuclear labeling technique (Fig. 4*E*) provided direct evidence for the localization of the ET signal (Fig. 4*F*), in part, within the nuclei. However, additional ET was always seen in the cytoplasm or perhaps the mitochondria. Localization of ET within the nucleus is not surprising, because the positively charged molecule is a well-known DNA stain and no doubt diffuses into the nucleus regardless of the original source of its intracellular generation. However, further work is required to identify the intracellular sites of ROS production in heat stress.

Functional Implications of Heat-Induced ROS Production

Contractile properties of skeletal muscle at temperatures between 37°C and 42°C have not been studied extensively. Prezant et al. (33) demonstrated that force development and twitch kinetics are relatively well preserved at temperatures of 41°C compared with 37°C. Preliminary experiments from our laboratory have demonstrated small but significant reductions in force production at all stimulation frequencies following exposure of in vitro diaphragm to 15 min of 42°C heat stress (unpublished observations). These effects are sustained over 3 h after the heat stimulus. Another important contractile response to heat stress involves the increases in passive tension and rigidity of skeletal muscle. The causes of this phenomenon are not clear, but may involve increases in resting Ca^{2+} or Ca^{2+}-independent changes in the molecular conformation of actin-myosin filaments and their interactions with tropomyosin (34). Our results suggest the possibility that some of the contractile responses during heat stress may reflect the influence of low-level reactive oxygen production. Reactive oxygen is now believed to play some poorly defined role in depressing muscle function during fatiguing stimulations (12, 35, 40) through possible oxidative modification of proteins involved with excitation-contraction coupling, the contractile ele-

ments, or other cell components. Furthermore, reactive oxygen may be playing an important role as a cell-signaling agent, upregulating stress-induced transcription factors such as nuclear factor-κB (38), upregulating stress proteins such as heat shock protein 70 (41), upregulating antioxidants such as manganese-SOD (46), and altering glucose uptake and metabolism (2).

We speculate that increased temperature may amplify the reactive oxygen production associated with intense muscle stimulation, thus augmenting the influence of ROS on muscle fatigue and possibly on long-term muscle adaptation and viability.

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