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Lipoxygenase-dependent superoxide release in skeletal muscle

Li Zuo,[1] Fievos L. Christofi,[2] Valerie P. Wright,[1] Shengying Bao,[1] and Thomas L. Clanton[1]

[1]*Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Dorothy M. Davis Heart and Lung Research Institute, and* [2]*Department of Anesthesiology, The Ohio State University, Columbus, Ohio 43210*

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Zuo, Li, Fievos L. Christofi, Valerie P. Wright, Shengying Bao, and Thomas L. Clanton. Lipoxygenase-dependent superoxide release in skeletal muscle. *J Appl Physiol* 97: 661–668, 2004. First published April 23, 2004; 10.1152/japplphysiol.00096.2004.—Superoxide anion radical ($O_2^{\bullet-}$) is released from skeletal muscle at rest and is particularly elevated during conditions of heat stress (42°C). Previous studies have shown that in isolated rat diaphragm $O_2^{\bullet-}$ release is not dependent on mitochondrial electron transport, reduced NADP oxidase activity, or the integrity of membrane anion channels. This study hypothesized that $O_2^{\bullet-}$ release, as measured by cytochrome *c* reduction, is linked to metabolism of arachidonic acid. Phospholipase A_2 inhibition with manoalide significantly decreased $O_2^{\bullet-}$ release. In downstream pathways, neither the blockage of cyclooxygenase with indomethacin nor the inhibition of cytochrome *P*-450-dependent monooxygenase with SKF-525A decreased $O_2^{\bullet-}$ release. However, lipoxygenase (LOX) inhibition with general LOX blockers 5,8,11,14-eicosatetraynoic acid and cinnamyl-3,4-dihydroxy-α-cyanocinnamate greatly attenuated the signal. Furthermore, the specific 5-LOX inhibitor diethylcarbamazine also significantly decreased $O_2^{\bullet-}$ release. Immunohistochemistry localized 5- and 12-LOX to the cytosol and sarcolemma of muscle cells. Confocal studies, using the $O_2^{\bullet-}$-sensitive fluorescent indicator hydroethidine, demonstrated that LOX inhibition had no significant influence on intracellular $O_2^{\bullet-}$ formation. When compared with the cytochrome *c* results, this indicates that intra- and extracellular $O_2^{\bullet-}$ must arise from different sources. These data show for the first time that arachidonic acid metabolism through LOX activity, is a major source of extracellular $O_2^{\bullet-}$ release in skeletal muscle.

manoalide; hydroethidine; arachidonic acid; cytochrome *c*; heat stress

EXTRACELLULAR SUPEROXIDE ANION RADICAL ($O_2^{\bullet-}$) is formed by many cell types, most notably inflammatory cells, where it plays a critical role in host defense. In other cell types, the function of extracellular $O_2^{\bullet-}$ is less well understood. Like nitric oxide (NO), it may play roles in regulation of local vascular smooth muscle (30), regulation of the redox tone of the extracellular matrix (53), or local NO bioavailability (34). The discovery of extracellular SOD (12, 22) is another indication that extracellular $O_2^{\bullet-}$ formation may be biologically relevant and under local regulation.

A number of studies have demonstrated that isolated (39) or perfused (18) skeletal muscles produce considerable extracellular $O_2^{\bullet-}$, and the level increases with increased contractile activity (18, 39) in a way that is similar to the formation of extracellular NO (17). In addition, studies from our laboratory have documented a significant rise of $O_2^{\bullet-}$ release from skeletal muscle (diaphragm) under conditions of mild heat exposure, which can be inhibited by SOD (54).

The exit pathway of $O_2^{\bullet-}$ release in skeletal muscle is not well understood. Anion channels do not appear to play a major role in this process (56). We can only speculate that $O_2^{\bullet-}$ may exit the membrane through the formation of perhydroxyl radical or through other redox-active transporters such as ubisemiquinone (56). Furthermore, the organelle or molecular source of $O_2^{\bullet-}$ release in muscle is not known. Our laboratory has previously demonstrated that it is not attenuated by inhibition of electron transport or by inhibition of reduced NADP (NADPH) oxidases (56). Therefore, it seems more likely that the $O_2^{\bullet-}$ generator is associated with the sarcolemmal membrane and that it can pass electrons or $O_2^{\bullet-}$ directly across the membrane by an unknown mechanism. Enzymes associated with arachidonic acid (AA) metabolism appear to be good candidates, because they are present on a variety of cell membranes.

Previous studies in skeletal muscle have indicated that, in some conditions, reactive oxygen species (ROS) formation is linked with the activity of phospholipase A_2 (PLA_2), the enzyme that releases AA and other products such as lysophospatidyl choline (33) from membrane lipids. Manoalide, an effective PLA_2 blocker, can greatly reduce the ROS signal (3, 29, 49, 50). This is consistent with the fact that AA exposure stimulates massive formation of ROS in living cells (50). Downstream of AA metabolism, both cyclooxygenase (COX) (32, 48) and cytochrome *P*-450-dependent monooxygenase (P450) (4, 35) are regarded as candidates for ROS generation. Lipoxygenase (LOX) has also been shown to be capable of producing ROS (21, 23, 25), although it is less well recognized and, to our knowledge, has not been suggested as a source in skeletal muscle. The following hypotheses were tested in this study. *1*) $O_2^{\bullet-}$ release at rest and in heat exposure is related to PLA_2 activity, one of the primary upstream components of AA metabolism. *2*) In downstream pathways of AA metabolism, COX, P450, and/or LOX are important sources of extracellular $O_2^{\bullet-}$ release. *3*) Extracellular $O_2^{\bullet-}$ release is a reflection of intracellular $O_2^{\bullet-}$ formation. All of these hypotheses were tested using the corresponding blockers in each pathway as illustrated in Fig. 1.

METHODS

Surgical procedures. Male Sprague-Dawley rats (350–450 g) were housed and treated according to approved protocols of The Ohio State University Institutional Laboratory Animal Care and Use Committee. The procedures have been described previously (54, 56). In brief, rats were anesthetized, tracheotomized, and ventilated. The diaphragms were retrograde perfused from the inferior vena cava with oxygenated Ringer solution. This was performed to reduce leukocytes and hemo-

Address for reprint requests and other correspondence: T. Clanton, The Ohio State Univ., Pulmonary and Critical Care Medicine, 201 Dorothy M. Davis Heart and Lung Research Institute, 473 W 12th Ave., Columbus, OH 43210 (E-mail: clanton.1@osu.edu).

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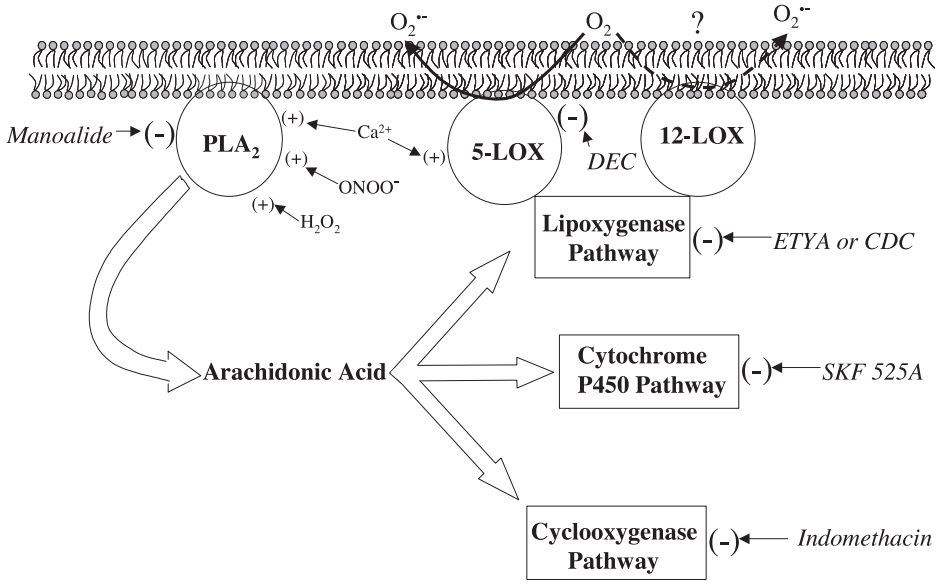

Fig. 1. Schematic diagram of lipoxygenase (LOX) pathways and blocking or stimulating reagents associated with this study. PLA_2, phospholipase A_2; manoalide, PLA_2 inhibitor; Ca^{2+}, potential stimulator of both PLA_2 and 5-LOX; $ONOO^-$, peroxynitrite (potential stimulator for PLA_2); H_2O_2, potential stimulators for PLA_2; DEC, diethylcarbamazine (5-LOX inhibitor); ETYA, 5,8,11,14-eicosatetraynoic acid (general inhibitor of LOX); CDC, cinnamyl-3,4-dihydroxy-α-cyanocinnamate (general inhibitors of LOX); SKF-525A, cytochrome *P*-450 inhibitor; indomethacin, cyclooxygenase (COX) inhibitor. Note the possible formation of extracellular superoxide anion radical ($O_2^{\bullet-}$) by 12-LOX is hypothesized (dashed line) but could not be directly tested with these experiments. −, Inhibition; +, stimulation.

globin in the tissue samples that may possibly affect the cytochrome *c* (cyt c) spectra. Each diaphragm was removed and dissected into several muscle strips (~0.6 cm wide) attached to corresponding central tendon and rib. The strips were kept 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, and 2.07 g/l glucose and 10 μM D-tubocurarine) on ice and bubbled with 95% oxygen-5% carbon dioxide before use.

Cyt c assay. Similar to previous descriptions (54), extracellular $O_2^{\bullet-}$ release was measured with the cyt c assay in a darkened room. Cyt c is highly sensitive to $O_2^{\bullet-}$ and can be easily reduced through one-electron donation from $O_2^{\bullet-}$. To minimize possible non-cyt c-specific interference, spectral measurements of cyt c reduction were calculated by taking the difference between the peak absorbance at 550 nm and the average of the values at 540 nm and 560 nm (18, 54). The extinction coefficient used for cyt c was $18.5 \times 10^3\ M^{-1} \cdot cm^{-1}$ (18, 27, 54).

Treatments. The diaphragm strips were incubated with or without blockers for 30 min on ice. Each muscle strip was then loaded with ~2 g of preload tension to attain approximate optimum length, and equilibrated at 37°C in a 3.0-ml water-jacketed minibath (Radnoti, Monrovia, CA) with or without fresh blocking solution for 10 min. Control strips were exposed to 37 or 42°C for 45 min in 5 μM cyt c solution without blockers. Treated strips were handled identically, except the corresponding blockers were added. The reduction of cyt c in each minibath was monitored by quickly transferring 1 ml of the bath solution to a cuvette in a diode-array ultraviolet-visible spectrophotometer (HP 8452A, Hewlett-Packard) and recording the full spectra. Then, the cuvette solution was immediately transferred back to the tissue bath.

The following blockers were used: manoalide (PLA_2 blocker, Kamiya Biomedical), indomethacin (COX blocker, Sigma Chemical, St. Louis, MO), SKF-525A (proadifen, P450 blocker, Sigma Chemical), 5,8,11,14-eicosatetraynoic acid (ETYA; LOX blocker, Sigma Chemical), cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC; LOX blocker, Biomol), diethylcarbamazine (5-LOX blocker, Sigma Chemical), 9,12-octadecadiynoic acid (ODYA; 15-LOX blocker, Cayman Chemical), and leukotriene B_4 (LTB_4)-dimethylamide (DMA; leukotriene B_4 receptor blocker, Biomol). The stock solutions of manoalide, indomethacin, CDC, ODYA, and ETYA were made with DMSO (Sigma Chemical), the stock of LTB_4-DMA were made with methanol, and all others were made with double-distilled water. For experiments requiring DMSO, the same amount of DMSO was added to matched control tissues. DMSO concentrations were kept below 0.5% vol/vol to reduce possible effects on the muscle (38). Each blocker was pretested using the cyt c assay in the absence of tissues, to check its reactivity with cyt c, and when present, appropriate corrections were made in the final analysis. Based on literature values, concentrations of all blockers were kept higher than those used for cell-loading experiments, because of the necessity for diffusion into whole tissues. All treatments had a relatively low chemical effect on cyt c reduction, i.e., less than ~18% of cyt c fully reduced by ascorbic acid. We found that this degree of effect did not cause artifacts related to the probability of collision between cyt c and ROS (56) and that we were able to fully compensate for these effects with chemical control experiments in the tissue baths. Some LOX blockers, including esculetin, caffeic acid, and baicalein, were found to reduce cyt c much more than the limit above and, therefore, were excluded in our experiments. When necessary, the pH of the buffer was readjusted to 7.4–7.6 before all experiments. All final data were corrected for the direct effects between cyt c and the specific blockers. SKF-525A did not dissolve well in Ringer solution at 50 μM due to precipitate formation with $NaHCO_3$ and Na_2HPO_4. Thus, for this experiment, the buffer solution was prepared without these two components, and the pH of oxygenated buffer was titrated to 7.4–7.6 by addition of NaOH.

Laser-scanning confocal microscopy. Confocal experiments were designed to identify whether the most effective LOX blocker also inhibited intracellular ROS formation. Hydroethidine (HE; Molecular Probes) was used for the detection of intracellular ROS and is particularly sensitive to $O_2^{\bullet-}$, hydroxyl radical, and peroxynitrite (1, 54, 55). HE stocks were prepared in *N*,*N*-dimethylacetamide (Acros Organics). In the presence of ROS, HE becomes oxidized to ethidium derivatives, which were used as the indicator of intracellular ROS production (1, 7, 29, 54, 55). Similar to previous studies (54), mouse

diaphragms were preferred because they are thinner and have better HE loading, confocal detection, and oxygenation when compared with rat tissue under the same conditions. Swiss-Webster mice (20–30 g) were used under the animal care guidelines at Ohio State University. They were anesthetized, and diaphragms were quickly removed. Hemidiaphragms were loaded in a solution of 88 μM HE and 4 mM 2,3-butanedione monoxime (an actin-myosin complex blocker used to reduce movement artifact, Acros Organics), with or without 100 μM ETYA, in oxygenated Ringer solution in the dark on ice for 1 h. After loading, muscles were washed for 10 min with Ringer solution and stretched to ~120% of relaxed length on the stage of a laser-scan confocal microscope (Zeiss 410, Carl Zeiss, Germany). After a 30-min equilibration at 37°C with a constant superperfusion, both treated and untreated groups were exposed to 35 min of 42°C. Measurements of ethidium derivatives fluorescence were taken every 5 min. The setup and analysis for laser-scanning microscopy has been described in detail previously (54).

Test of the in vitro antioxidant effects of 100 μM ETYA on $O_2^{\bullet-}$. Because LOX inhibitors can act as antioxidants, it was necessary to consider that they may behave as direct $O_2^{\bullet-}$ scavengers. To test this possibility, the $O_2^{\bullet-}$-scavenging ability of ETYA was determined. The reaction medium included cyt c (10 μM), catalase (2,000 U/ml; Sigma Chemical), xanthine (0.25 mM; Sigma Chemical), EDTA (2 mM; Acros Organics), and PBS, pH 7.55. Xanthine oxidase (0.75 U/ml; Sigma Chemical) was added to begin the generation of $O_2^{\bullet-}$. Our results demonstrated that 100 μM ETYA had no appreciable scavenging effect on $O_2^{\bullet-}$ (data not shown).

Localization of 5-LOX and 12-LOX with immunohistochemistry. Rat diaphragms were quickly removed and frozen. The tissues were cut into 14-μm-thick sections in both the longitudinal and cross directions. After 60 min of air drying, each slide was fixed in cold acetone (4°C) for 30 min followed by another 30 min of air drying. The samples were equilibrated in PBS for 10 min and then blocked with 5% normal goat serum (Vector Laboratories) for 60 min. Extra serum was removed, and the slides were incubated with primary antibodies for rat LOXs (5-LOX polyclonal antiserum and 12-LOX polyclonal antiserum; host: rabbit; 1:100 dilution; Cayman Chemical) at room temperature for 60 min followed by 15 min of washing in PBS with 1% FBS. Each slide was incubated with a secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit IgG; 1:200 dilution; Sigma Chemical) at room temperature for 60 min followed by washing in PBS with 1% FBS for 15 min. Coverslips were mounted using Vectashield mounting medium (H-1000, Vector Laboratories). Negative controls were prepared by replacing the primary antibody with normal rabbit IgG (R&D System, Minneapolis, MN). The samples ($n = 3$ from 3 rats) were observed with a laser-scanning confocal microscope (Carl Zeiss, Germany) with the following settings: pinhole 1.6 airy units, power 40%, objective ×10, excitation 488 nm, and emission 500–552 nm band pass.

Statistical and graphical analysis. Data were analyzed using a multiway ANOVA and expressed as means ± SE (JMP, SAS Institute, Cary, NC). The animal was treated as a random variable, with drug treatment and time being factors of interest. The differences in mean values between treatment and nontreatment at each specific time point were determined by post-ANOVA contrast procedures available on the SAS JMP software. $P < 0.05$ was considered to be statistically significant.

RESULTS

As illustrated in Fig. 2, after 30 min of PLA_2 inhibition with manoalide (10 μM), there was a significant decrease of cyt c reduction at both 37°C ($n = 6$, $P < 0.02$) and 42°C ($n = 8$, $P < 0.02$). Figure 2 is used to illustrate a complete picture of the typical responses in these experiments over time. From such data, the net changes in cyt c reduction from matched controls were calculated and used to represent the results in Figs. 3 and 4.

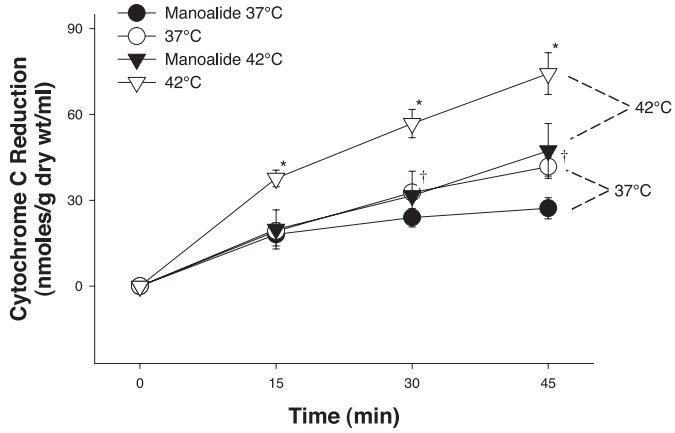

Fig. 2. PLA_2 inhibition with manoalide. After 30 min, manoalide significantly decreased cytochrome *c* (cyt c) reduction at both 37°C (†$n = 6$, $P < 0.02$) and 42°C (*$n = 8$, $P < 0.02$).

Indomethacin (500 μM), a blocker of the COX pathway, had no significant inhibitory effect on cyt c reduction at 37 or 42°C ($n = 6$; Fig. 3). Interestingly, and contrary to our expectations, it significantly increased cyt c reduction at 37°C ($n = 6$, $P < 0.01$; Fig. 3). Similarly, inhibition of the P450 pathway with SKF-525A (50 μM) did not diminish cyt c reduction but rather promoted reduction at both 37 and 42°C ($n = 5$, $P < 0.01$; Fig. 3).

However, Fig. 4 indicates that LOX inhibition (100 μM ETYA) had a significant inhibitory effect on the rate of cyt c reduction at both 37°C ($n = 8$, $P < 0.01$) and 42°C ($n = 6$, $P < 0.01$) at time points of 30 and 45 min. Because our in vitro experiments (METHODS) demonstrated that ETYA has no $O_2^{\bullet-}$ scavenging ability at this concentration, we concluded that the primary pathway of extracellular $O_2^{\bullet-}$ formation is either directly or indirectly through the action of LOX. CDC (5 μM), another general LOX blocker, had effects similar to ETYA ($n = 5$, $P < 0.02$), confirming the crucial role of LOX in this process. Furthermore, although the 15-LOX blocker ODYA (25 μM) had no significant effect ($n = 5$), diethylcarbamazine (50 μM), a 5-LOX inhibitor, also significantly decreased the cyt c reduction ($n = 5$, $P < 0.03$).

Immunohistochemistry studies showed significant levels of expression of both 5-LOX (Fig. 5*A*) and 12-LOX (Fig. 5*B*) distributed throughout cytosol and particularly concentrated at the sarcolemma (typical data from $n = 3$ animals). The staining appears to be greater in cells of smaller diameter for both enzymes. The 15-LOX enzyme was not studied because we could find no significant role for it in extracellular $O_2^{\bullet-}$ release (Fig. 4). Because in other systems $O_2^{\bullet-}$ production has been shown to result from LTB_4 receptor activation (i.e., LTB_4 is a product of 5-LOX activity) (49, 50), we also tested the influence of LTB_4 receptor blocker on heat-induced $O_2^{\bullet-}$ release ($n = 5$ from 3 rats). The LTB_4 receptor blockade with LTB_4-DMA at 5 μM had no significant inhibitory effect on $O_2^{\bullet-}$ release during heat exposure (data not shown).

We further tested whether ETYA inhibited intracellular ROS production. Despite its striking effects on extracellular $O_2^{\bullet-}$ release, Fig. 6 shows that it has no significant effect on

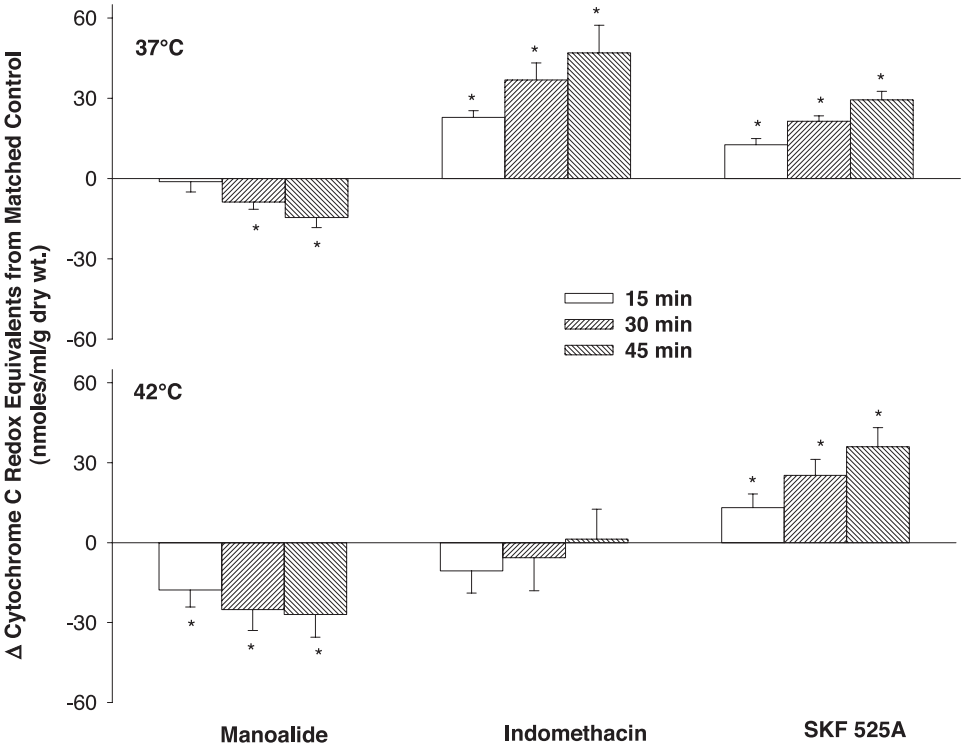

Fig. 3. Summarized data showing net differences from matched controls in response to various blockers in the arachidonic acid (AA) pathway at the given temperature. Δ, Change. *Significant difference between drug treatment and corresponding non-drug treatment ($P < 0.05$).

intracellular ROS formation during heat stress ($n = 5$). This suggests that, although the time courses of intra- and extracellular $O_2^{\bullet-}$ formation appear similar, as shown in our laboratory's previous work (54), they seem to be generated by independent sources or by different molecular mechanisms.

DISCUSSION

The present results strongly suggest that the primary source of extracellular $O_2^{\bullet-}$ release is a reflection of downstream pathways of AA or lipid metabolism and, specifically, the

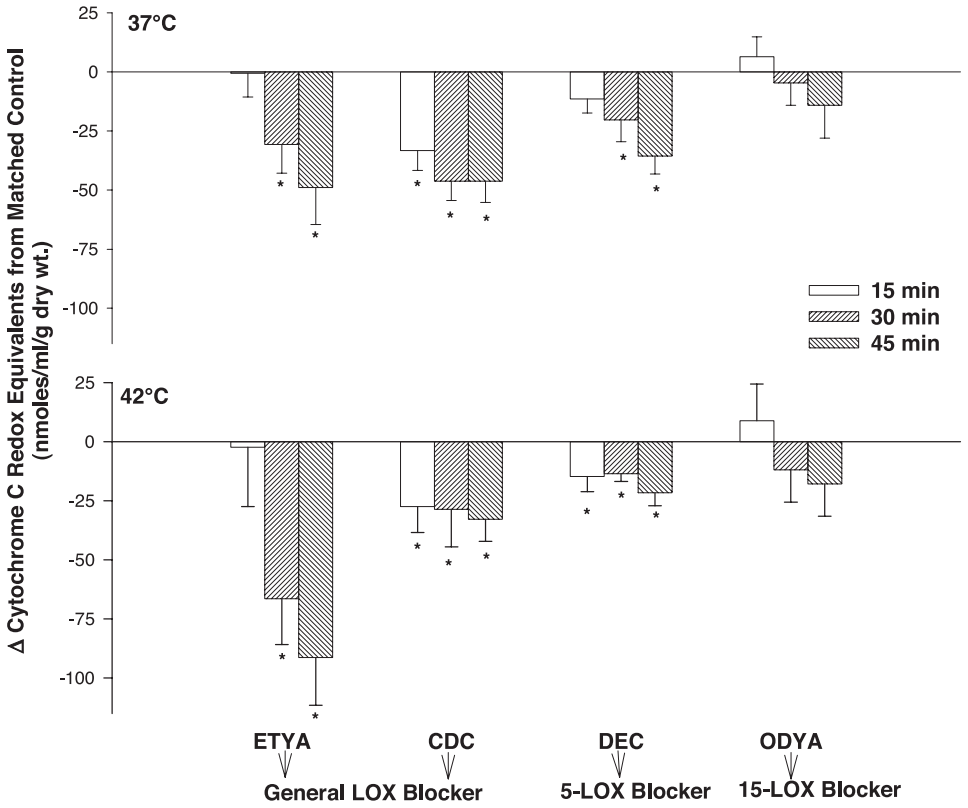

Fig. 4. Summarized data showing net differences from matched controls in response to LOX blockers at 37 and 42°C. *Significant difference between drug treatment and corresponding non-drug treatment at the same temperature ($P < 0.05$).

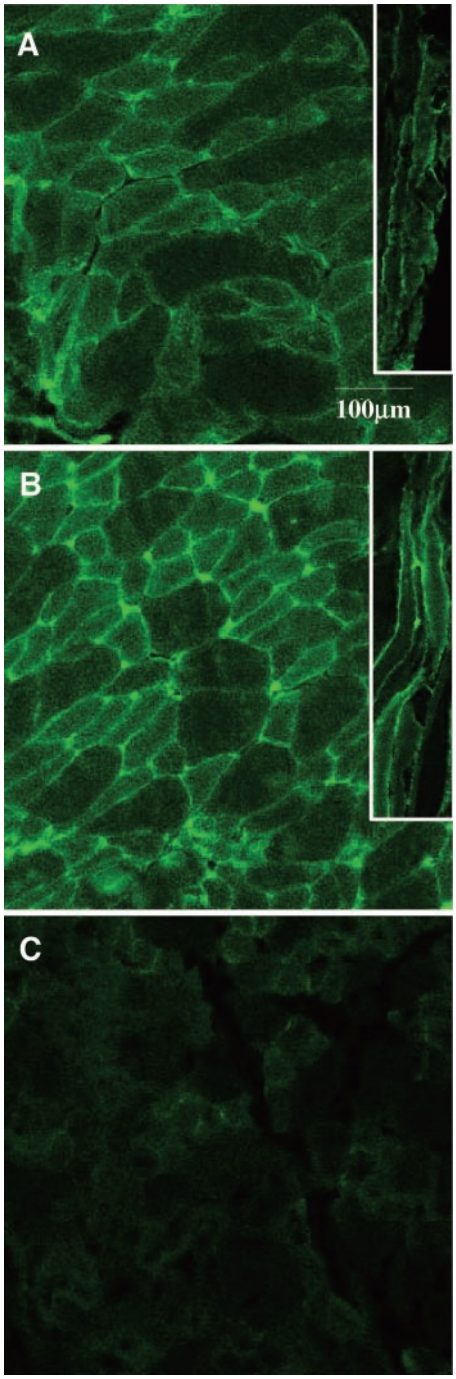

Fig. 5. Localization of 5- and 12-LOX in skeletal muscle with immunohistochemistry microscopy. *A*: 5-LOX immunofluorescence from the cross section of skeletal muscle. *Inset*: longitude section of 5-LOX immunofluorescence. *B*: 12-LOX immunofluorescence from the cross section of skeletal muscle. *Inset*: longitude section of 12-LOX immunofluorescence. *C*: negative controls were performed in a similar way to *A* or *B* with normal rabbit IgG as the primary antibody. All the images are from the same animal.

activity of LOX. Blockage of this pathway caused a significant inhibition of $O_2^{\bullet-}$ release, both at rest and during exposure to mild heat (Figs. 3 and 4). Furthermore, blockage of alternative paths of AA metabolism (COX and P450) stimulated production, which is consistent with known competition for AA substrates between these enzyme systems (19, 28). The confocal experiments suggest that the pathway of extracellular $O_2^{\bullet-}$ formation is separate from the intracellular source. This is consistent with previous data showing that anion pores, known as a major pathway for movement of $O_2^{\bullet-}$ across cell membranes (26), do not play an important role in this setting (56).

Inhibition of AA metabolism. Earlier research has shown that ROS formation can be directly or indirectly related to the activity of PLA_2 in skeletal muscle (29, 49, 50), although the mechanism is not well understood. Exposure of living cells to AA alone can result in the massive formation of ROS (50), suggesting that the pathway may be downstream from PLA_2. Manoalide is an effective blocker of PLA_2 and works by irreversible modification of lysine residues on PLA_2 (3). Our data (Figs. 2 and 3) have shown that ROS release is greatly reduced after PLA_2 blockage but not completely inhibited. It is possible that other phospholipases, not inhibited by manoalide, may have contributed substrates for downstream AA metabolism and consequent $O_2^{\bullet-}$ formation or that some isoforms of LOX-12 are responsible by working on substrates other than AA (52).

LOX pathway as a potential $O_2^{\bullet-}$ generator. Of the three major downstream pathways of AA metabolism, only LOX inhibition significantly blocked cyt c reduction. Blockage of the alternative pathways increased cyt c reduction (Fig. 3). Interestingly, both COX (32, 48) and P450 (4, 35) are well known as ROS generators in other systems. The fact that blockage of both COX and P450 augmented cyt c reduction also provides circumstantial evidence for a role of LOX, since competition for AA substrates between these alternative pathways has been observed in studies on other systems (19, 28).

How does LOX create $O_2^{\bullet-}$? Although less well recognized, LOX has been shown to be capable of contributing to ROS formation (21, 23, 25). However, evidence is lacking with regard to intact tissue and particularly skeletal muscle. The mechanism for extracellular $O_2^{\bullet-}$ release by LOX is not fully understood but has been shown to be NADPH dependent and temperature sensitive (31). There are some indications that the enzymes can behave "as if" located in the extracellular environment because of their sensitivity to extracellular NADH (31). Several possible mechanisms have been proposed for $O_2^{\bullet-}$ formation by LOX. Formation could occur by the single-electron oxidation of NADPH to NADP radical via the reduc-

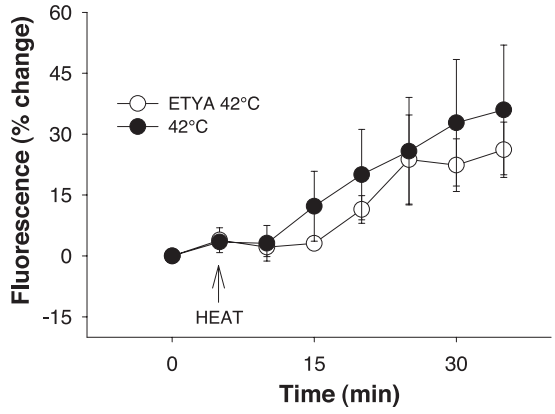

Fig. 6. Confocal study: effect of LOX inhibitor ETYA on intracellular $O_2^{\bullet-}$ formation in heat stress. There is no significant effect of ETYA on $O_2^{\bullet-}$ formation. HEAT, beginning of 42°C superfusion.

tion of the nonheme iron in the enzyme (E) (i.e., E-Fe^{3+} to E-Fe^{2+}). NADP radical can then reduce oxygen to $O_2^{\bullet-}$ radical, forming $NADP^+$ (51). In a related pathway, oxidation of NADPH to NADP radical occurs by an enzyme substrate peroxyl radical intermediate (40). These reactions may be facilitated when the substrate has an imperfect fit in the active site of LOX (5). It is also possible that a high level of oxygen could promote radical formation by LOX. The products of LOX catalysis such as LTB_4 have been shown to play an autocrine or paracrine role in stimulating ROS formation by secondary mechanisms (49, 50). However, our heat-stress experiments have shown no inhibitory effect on $O_2^{\bullet-}$ release by a potent LTB_4 receptor blocker LTB_4-DMA (43), suggesting that the latter mechanism does not appear to be favored (data not shown).

There are some critical issues regarding details in the downstream blockage of AA metabolic pathways. Although indomethacin is a powerful inhibitor of COX, in some conditions, it can also have an inhibitory effect on LOX (48). Our data from COX and LOX blockage (Figs. 3 and 4) suggest, however, that indomethacin had either no significant or a very weak inhibitory effect on LOX in this setting. The next issue is that many LOX inhibitors cannot be used in cyt c experiments due to their strong reduction of cyt c and/or antioxidant effects (METHODS). For example, we were unable to use a popular LOX inhibitor, nordihydroguaiaretic acid, because it strongly reduces cyt c, which greatly interfered with the baseline cyt c spectra. Nordihydroguaiaretic acid is also a well-known antioxidant (24). However, other LOX inhibitors, such as ETYA, had no appreciable antioxidant effect, which is consistent with studies that have reported either no antioxidant function (44) or a weak antioxidant activity requiring an ~30-fold higher concentration than that necessary to inhibit LOX (45). Furthermore, ETYA had a relatively small reducing effect on cyt c (<18%), which can easily be controlled for in this study. Although ETYA has been reported to have some inhibitory effects on COX in certain conditions (11), it seems unlikely to play a major role in our model, since a more potent blocker for COX (indomethacin) had no inhibitory effect on $O_2^{\bullet-}$ release (Fig. 3).

Although the two general LOX blockers used, ETYA and CDC (8), were able to inhibit $O_2^{\bullet-}$ release, a more specific pathway responsible for this process was explored. There are three major categories in the LOX family, 5-, 12-, and 15-LOX. As shown in Fig. 4, ODYA, a 15-LOX inhibitor (42), had no appreciable effect on $O_2^{\bullet-}$ release. This makes some sense because a unique 15-LOX has not been identified in the murine genomic database to our knowledge (5, 20). However, diethylcarbamazine, a specific 5-LOX blocker (9, 46), significantly reduced the amount of $O_2^{\bullet-}$ release, suggesting a key role for this pathway. Many currently available 12-LOX blockers are not specific due to their cross-inhibition of 5-LOX. The most specific 12-LOX blocker available on the market, baicalein, has a large reducing effect on cyt c, eliminating its useful application. Thus we can only speculate at this time that 12-LOX plays a role in our model.

Little information is available regarding the expression or localization of LOX enzymes in skeletal muscle cells. In most cell types studied (e.g., inflammatory cells), 5-LOX exists primarily in the cytosol (13, 36). When the cell is activated or stressed, 5-LOX moves to the perinuclear membrane, where it actively produces specific lipid metabolites (36). Therefore, finding a strong 5-LOX localization in the plasma membrane (Fig. 5) was unexpected. In contrast, the 12-LOX enzyme is often associated with a variety of membranes and is found in many cell types. There are three isoforms of 12-LOX found in humans or animals (52). In rodents, 12-LOX may be the functional equivalent of both 12- and 15-LOX (20, 52). It can work on a broad range of substrates located in the membrane, and the most predominant isoform, the so-called leukocyte isoform, is not dependent on available AA but can work directly on specific membrane lipids (52). Although we were unable to find a suitable specific blocker for 12-LOX in this study, we hypothesize that 12-LOX may have contributed to our measurements of extracellular $O_2^{\bullet-}$, based on the fact that specific blockage of 5-LOX only partially inhibited $O_2^{\bullet-}$ production and the absence of effects of 15-LOX inhibition (Fig. 4).

The absence of an effect of anion channel blockers on extracellular $O_2^{\bullet-}$ release in this model, as shown in a previous study (56), suggests that the mechanism for $O_2^{\bullet-}$ release is likely to involve a molecular system bound to the cell membrane that, by its location, overcomes the hydrophobic barrier for movement of a charged molecule to the extracellular environment. Because immunofluorescence (Fig. 5) techniques illustrated a strong distribution of 5- and 12-LOX at the sarcolemma, LOX appears to fit this role and could possibly produce $O_2^{\bullet-}$ without any coordination of specific channels or other more complicated mechanisms (56). It is also possible that the high environmental oxygen (95%) required of isolated tissue experiments could further amplify the $O_2^{\bullet-}$ signal seen via this pathway.

The function of LOXs in skeletal muscle is also unknown. Although generally regarded as participants in the inflammatory response, it has become increasingly clear that LOXs play other far-ranging roles, such as cell signaling, membrane remodeling, and apoptosis initiation. In skeletal myotubes, they are essential mediators of cell-volume regulation during osmotic stress by influencing taurine transport across the cell membrane (33). In cardiac myocytes, LOXs have been shown to be critically involved in cytoskeleton rearrangement and the translocation of glucose transporter isoform-4 (i.e., GLUT4) (10) and may also play an important role in cardiac preconditioning, because LOX-12 knockout mice show little or no ischemic preconditioning effects compared with wild-type mice (14). Present results suggest that LOXs may play a critical role in the normal physiological responses of muscle to heat exposure. During exercise, rat limb muscle temperatures can exceed 43°C (6). Therefore, the role of LOXs and their involvement in $O_2^{\bullet-}$ formation in skeletal muscles may be particularly important during heat stress.

The regulation of LOX activity is complex. PLA_2, an important upstream enzyme responsible for AA, is under the control of multiple regulatory factors, including intracellular Ca^{2+} concentration ($[Ca^{2+}]$), phosphorylation state, and directly or indirectly by exposure to hydrogen peroxide (2) or peroxynitrite (15) (Fig. 1). It is interesting to speculate that, if LOX produced significant intracellular reactive oxygen, it would comprise a form of positive feedback on PLA_2 that would likely make the enzyme activity unstable and possibly self-destructive. Intracellular $[Ca^{2+}]$ can function in stimulation and translocalization of 5-LOX, but 5-LOX may be

inactivated by reactive oxygen (36). This is particularly important in skeletal muscle, because intracellular [Ca^{2+}] increases in heat stress from known changes in sarcoplasmic reticulum membrane permeability (41), and intracellular reactive oxygen formed during heat stress (54) may further function to stimulate AA production through stimulation of PLA_2 activity (2).

Summary and implications. The results suggest that the AA-LOX pathway is a major source for extracellular $O_2^{\bullet-}$ release at rest and in heat stress. Although not tested under other physiological stimuli, such as muscle contraction (18, 37, 39), the fact that LOX inhibition was effective during resting conditions suggests that it may be of more general relevance, i.e., LOX-generated ROS may play an active role in regulating muscle function in normal conditions. Our results are also supported by Woo et al. (49), who demonstrated that ROS are generated, not through the COX pathway, but through the cytosolic PLA_2-LOX pathway in a study of rat-2 fibroblasts stimulated by tumor necrosis factor-α. Furthermore, in a similar cyt c assay, Turner et al. (47) found that $O_2^{\bullet-}$ release from keratinocytes is dependent on LOX but not on COX or NADPH oxidase, which is highly consistent with our laboratory's present and previous data (56). Hall et al. (16) have demonstrated that, in whole body heat stress, there is a significant and potent elevation in oxidative stress and NO radical formation, which contributes to its pathology. It is possible that AA metabolism may be one of the contributory pathways to the pathology of heat stress. In skeletal muscle, elevations in temperature may be considered a more normal phenomenon because they are natural heat generators (6). Although the direct effect of LOX on heated myocytes is still unknown, it is highly possible that the activation of LOX during heat exposure would potentially influence muscle function by generating ROS and other signaling molecules. Therefore, the role of such mechanisms in skeletal muscles may also be uniquely different from other tissues.

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