ANTIOXIDANTS & REDOX SIGNALING
Volume 5, Number 5, 2003

Original Research Communication

Sources for Superoxide Release: Lessons from Blockade of Electron Transport, NADPH Oxidase, and Anion Channels in Diaphragm

LI ZUO,[1] SILVIU PASNICIUC,[1] VALERIE P. WRIGHT,[1] A. JOHN MEROLA,[2] and THOMAS L. CLANTON[1]

ABSTRACT

Isolated diaphragm releases low levels of superoxide ($O_2^{\cdot-}$) at rest and much higher levels during heat stress. The molecular source is unknown. The hypothesis was tested that heat stress stimulates mitochondrial complex activity or NADPH oxidases, resulting in increased $O_2^{\cdot-}$ release. The mitochondria within intact rat diaphragm were inhibited at complex I (amobarbital or rotenone) or complex I and II (rotenone plus thenoyltrifluoroacetone). NADPH oxidases were blocked by diphenyliodonium. None of these treatments inhibited $O_2^{\cdot-}$ release. Conversely, most blockers stimulated $O_2^{\cdot-}$ release. As intracellular $O_2^{\cdot-}$ generators require a mechanism for $O_2^{\cdot-}$ transport across the membrane, anion channel blockers, probenecid and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, were also tested. Neither blocker had any inhibitory effect on $O_2^{\cdot-}$ release. These results suggest that $O_2^{\cdot-}$ released from diaphragm is not directly dependent on mitochondrial complex activity and that it is not a reflection of passive diffusion of $O_2^{\cdot-}$ through anion channels. Although the molecular source for extracellular $O_2^{\cdot-}$ remains elusive, it is clearly sensitive to temperature and conditions of "chemical hypoxia" induced by partial or complete mitochondrial inhibition. *Antioxid. Redox Signal.* 5, 667–675.

INTRODUCTION

PREVIOUS RESEARCH FROM OUR LABORATORY has shown that significant reactive oxygen species (ROS), especially superoxide ($O_2^{\cdot-}$), are released from rat diaphragm in heat stress, which follows a pattern very similar to intracellular $O_2^{\cdot-}$ production (42). The molecular source, or organelle, responsible for $O_2^{\cdot-}$ release in heat stress, or even at normal temperatures, is unknown. Mitochondria are believed to be one of the possible generators of ROS under many pathophysiological conditions (7). In normal mitochondria, the charge on $O_2^{\cdot-}$ presumably precludes diffusion through mitochondrial membranes, and it is believed to be largely dismutated by mitochondrial superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2), which can pass through membranes. However, it is not known whether mitochondria can significantly contribute to extracellular $O_2^{\cdot-}$ release from intact cells in normal or stressed conditions such as heat exposure. To do so would not only require a mechanism to move $O_2^{\cdot-}$ across mitochondrial membranes, but also a mechanism for exiting the cell through the cell membrane. Anion channels have been shown to play a role in $O_2^{\cdot-}$ release from intracellular sources in isolated red blood cells (22) and are believed to be important for mitochondrial $O_2^{\cdot-}$ transport (16, 38). Thus, it is possible that $O_2^{\cdot-}$ may exit the mitochondrion or cytosol via membrane anion channels.

Another potential source of $O_2^{\cdot-}$ release could be membrane-associated oxidoreductases, *e.g.,* NADPH oxidases (10, 11, 20, 40). NADPH oxidases may not require anion channels for $O_2^{\cdot-}$ release, because it is possible that they transport electrons and reduce O_2 directly on the outside of the membrane (31). NADPH oxidases are normally known to be present in

The Ohio State University Medical Center, [1]Department of Internal Medicine, Dorothy M. Davis Heart & Lung Research Institute, Division of Pulmonary and Critical Care Medicine, and [2]Department of Molecular and Cellular Biochemistry, Columbus, OH 43210.

endothelial cells (9, 26); however, a recent study has shown their presence in skeletal muscles, acting as intracellular ROS generators (17).

In this study, the following hypotheses were tested: (a) Leakage of electrons from the mitochondrial electron transport chain is a primary source of $O_2^{\cdot-}$ release at rest and increased $O_2^{\cdot-}$ release in heat stress. This source can be inhibited by blockers of electron transport. (b) Activation of membrane-associated NADPH oxidases is responsible for increased $O_2^{\cdot-}$ release in heat stress. (c) The release of $O_2^{\cdot-}$ into the extracellular environment from any intracellular source requires open anion channels at the cell and/or mitochondrial membrane.

MATERIALS AND METHODS

Animals

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.

Diaphragm strip preparation

Animals were anesthetized with sodium pentobarbital (~40 mg/kg) or the combination of ketamine (~76 mg/kg) with xylazine (~15 mg/kg), tracheotomized, and ventilated with room air. The blood was heparinized (~500 U/kg) by injection through the jugular vein. The diaphragm was cleared of blood by retrograde perfusion, through the inferior vena cava with oxygenated Ringer's solution, to lower the blood contamination that may affect the cytochrome *c* (cyt *c*) assay. The diaphragm was then surgically removed and dissected in oxygenated Ringer's solution into several muscle strips, each with its corresponding central tendon and rib. These strips were kept in Ringer's 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, 121 NaCl; 2.07 g/L glucose, and 10 μ*M* D-tubocurarine) on ice, and bubbled with 95% O_2/5% CO_2 prior to the data collection period (42).

Cyt c assay

$O_2^{\cdot-}$ release from rat diaphragm strips was measured with a cyt *c* assay as described previously (42). In brief, cyt *c* (oxidized) can be easily reduced through one-electron donation from $O_2^{\cdot-}$, and we previously demonstrated that the primary source of reduction during heat stress is via $O_2^{\cdot-}$, because the signal is essentially completely inhibited by extracellular SOD (42). To minimize possible non-cyt *c*-specific absorbance interference, measurement of cyt *c* reduction was 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). The extinction coefficient of cyt *c* used was $18.5 \times 10^3\ M^{-1}cm^{-1}$ (18, 23, 42).

Treatment groups

All diaphragm strips were incubated in oxygenated Ringer's solution, with or without blockers, for 30 min on ice. In this way, we expected to keep the muscle relatively fresh and subject to a minimum of nonspecific drug effects. Then each muscle strip was loaded with ~2 g of tension, to approximate optimum length. Prior to any readings, tissues were equilibrated for 10 min at 37°C in a 3.0-ml water-jacketed tissue bath (Radnoti, Monrovia, CA, U.S.A.) filled with Ringer's solution. Control strips were exposed to 5 μ*M* cyt *c* solution at 37°C for 45 min, with or without corresponding blockers; heated strips from the same animal were exposed to 5 μ*M* cyt *c* solution for 45 min at 42°C, with or without corresponding blockers. The reduction of cyt *c* in the bath was monitored by transferring 1 ml of bath solution to a cuvette in a diode-array ultraviolet-visible spectrophotometer (HP 8452A, Hewlett-Packard) for an absorbance measurement every 15 min. After each measurement, the solution was immediately transferred back to the tissue bath.

The concentration used for each blocking agent was comparable to values reported in the literature for whole-tissue experiments. However, this was often limited by the solubility of the agent and the maximum solvent concentration that could be used without causing significant adverse effects. Blockers used included amobarbital (5 m*M*, Eli Lilly & Co., Indianapolis, IN, U.S.A.), rotenone (50 μ*M*), antimycin A (50 μ*M*), thenoyltrifluoroacetone (TTFA; 1 m*M*), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS; 0.5 m*M*), probenecid (1 m*M*) (all from Sigma) and diphenyliodonium (DPI; 1 m*M*; Acros Organics). Stock solutions of most chemicals were made in dimethyl sulfoxide (DMSO; Sigma). Amobarbital and DIDS stocks were made in water, and probenecid stock was prepared in a 1 *M* NaOH solution. In all experiments, DMSO concentrations in tissue baths were kept below the level (<0.8%, vol/vol) that would significantly affect muscle contractility (28). Upon the addition of blockers, the pH of the Ringer's solution was readjusted to 7.4–7.6 in all experiments. Corresponding solvent concentrations were used in all matched control experiments.

Each blocker was pretested with the cyt *c* solution in the absence of tissues to check for independent chemical reactivity with cyt *c* over time. In most cases, the effects of blocking agents were not obvious (<10% of the fully reduced cyt *c*). However, we found that DIDS caused a significant reduction of cyt *c* (~18–38% of the fully reduced cyt *c*, depending on DIDS and cyt *c* concentrations), and thus corresponding adjustments in cyt *c* concentration were made in DIDS experiments. Nevertheless, all data were corrected based on the measured *in vitro* chemical reduction of cyt *c* by respective solvents and blockers.

For SOD (Sigma) experiments, muscle strips were first incubated in oxygenated buffer with SOD (5,000 U/ml) for 30 min on ice. Then blockers (rotenone plus TTFA, or DPI) were added to the incubation solution for another 30 min. After this, tissues were washed and equilibrated at 37°C for 10 min before the first measurement. SOD concentrations in the baths were continuously kept at 5,000 U/ml throughout the whole experiment. Controls were done in a similar way, but in the absence of SOD.

Measurements of oxygen consumption

As will be shown in Results, most mitochondrial blockers stimulated $O_2^{\cdot-}$ release, when detected by cyt *c* reduction. The conditions of two critical blocker experiments in the cyt *c* assay, including the rotenone series and rotenone plus TTFA series, were repeated to test their effectiveness on the inhibi-

tion of mitochondrial electron transport in the intact tissue, as monitored by oxygen consumption. Specifically, each muscle strip was suspended in a 3-ml reaction chamber with an integrated oxygen electrode unit (Oxygraph System, Hansatech Instruments, U.K.). The strip was mounted on a plastic frame and loaded with 2 g of tension, to approximate optimum length. To reduce sensitivity so that O_2 consumption could be measured in the 95% O_2 range, specially designed electrodes were utilized. The baths were calibrated and equilibrated to near 95% O_2, and closed. Magnetic stirrers were used to avoid diffusion gradients within the baths. Protocols of muscle treatment were identical to those used for the cyt *c* assay at 37°C. To avoid hypoxia, when bath O_2 concentrations dropped below 75–80% O_2, the bath chamber was opened, reequilibrated with 95% O_2, and closed before continuing the measurement. In all cases, one control tissue was run in parallel with a treated tissue (both from the same animal), and was used to calculate percentage inhibition by the drug. The data were analyzed using the Oxygraph software. The oxygen consumption rate (nanomoles per minute per gram wet weight) was calculated by averaging the slope of the decrease in oxygen concentration during 2-min periods, every 15 min.

Statistical and graphical analysis

In most cases, data were analyzed with multiway ANOVA using SAS JMP (SAS Institute, Cary, NC, U.S.A.) and expressed graphically as means ± SE. The individual "rat" was treated as a random variable with drug treatment and time being the primary factors of interest. The *p* values listed in Results represent the overall treatment effect of the chemicals used. The statistical differences in mean values at specific time points between treatment and nontreatment groups were determined by contrast procedures as illustrated in the associated figures (JMP software). Where appropriate, corrections were made for repeated measures done on separate strips from the same animal. $p < 0.05$ was considered to be statistically significant.

Considerable between-group and between-animal differences in responses, independent of drug treatment, were observed. These may have reflected different basal levels of ROS production in each animal, varied responses of animal groups to concentrations of the solvents used in each series [*e.g.,* DMSO ranged from 0 to 0.77% (vol/vol)], or other nonspecific differences in the groups, such as their age or length of stay in the vivarium. Such differences were controlled in the analysis by randomization, by matching solvents in control strips, and by matching controls and experimental treatments from the same animal. In addition, to simplify the data, results were summarized in a graphical form as differences from sham controls in the same animal with corrections for *in vitro* chemical reactions.

RESULTS

Complex III inhibition with antimycin A (50 μM)

In all experiments, heat treatment significantly increased cyt *c* reduction, as previously reported (42). As a typical example, changes in the absolute cyt *c* reduction, with and without antimycin A treatment at the given temperature, are shown in Fig. 1. Antimycin A-treated tissues caused more cyt *c* reduction than non-drug-treated tissues at both 37°C ($n = 6$, $p < 0.001$) and 42°C ($n = 6$, $p < 0.001$). Furthermore, this pattern, *i.e.,* the absolute changes of cyt *c* reduction over time, is typical of most responses to the blockage of mitochondria and NADPH oxidases, which are summarized in Fig. 2. Results from all treatments expressed in Fig. 2 reflect the change from matched control at the same temperature. For example, the zero delta line (*x* axis) is equivalent to a zero difference between drug treatment and non-drug treatment at that temperature.

Complex I inhibition with amobarbital (5 mM) or rotenone (50 μM)

As shown in Fig. 2, amobarbital significantly increased cyt *c* reduction at 37°C ($n = 10$ from seven rats, $p < 0.01$), similar to preceding experiments with complex III inhibition. However, at 42°C, amobarbital had no statistically significant effect on cyt *c* reduction in tissue experiments ($n = 10$ from seven rats). Rotenone treatment resulted in no significant stimulation of cyt *c* reduction at either 37°C or 42°C over the time course of the tissue experiment ($n = 8$). This raised the question: did the inhibitors used, such as rotenone, get to the mitochondria in sufficient concentrations to block electron transport in our experimental conditions? Figure 3 shows that under similar tissue conditions, rotenone significantly reduced the measured O_2 consumption at each time point at normal temperature ($n = 6$ from five rats, $p < 0.05$). This working model was tested to be valid by additional experiments showing that the cyanide-inhibited rate of respiration in this preparation was <10% of the oxygen consumption in controls (data not shown).

Combined complex I and complex II inhibition with rotenone (50 μM) plus TTFA (1 mM)

As mitochondria, in their intact state within the cell, can provide a variety of substrates for electron transport through complex I and/or II, it is necessary to block simultaneously

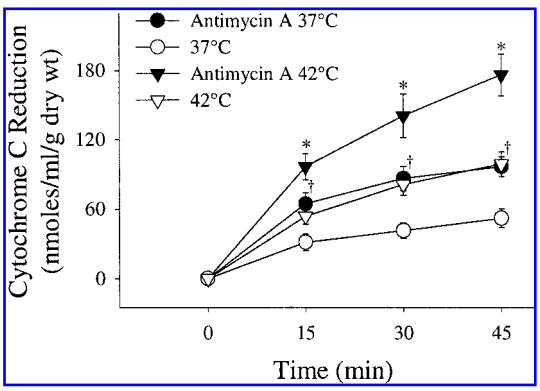

FIG. 1. Complex III inhibition with antimycin A. Results are expressed as absolute cyt *c* reduction. Antimycin A significantly increased cyt *c* reduction at 37°C and 42°C ($n = 6$). *$p < 0.05$ (ANOVA contrasts), between drug treatments and controls at 42°C; †$p < 0.05$ (ANOVA contrasts), between drug treatments and controls at 37°C.

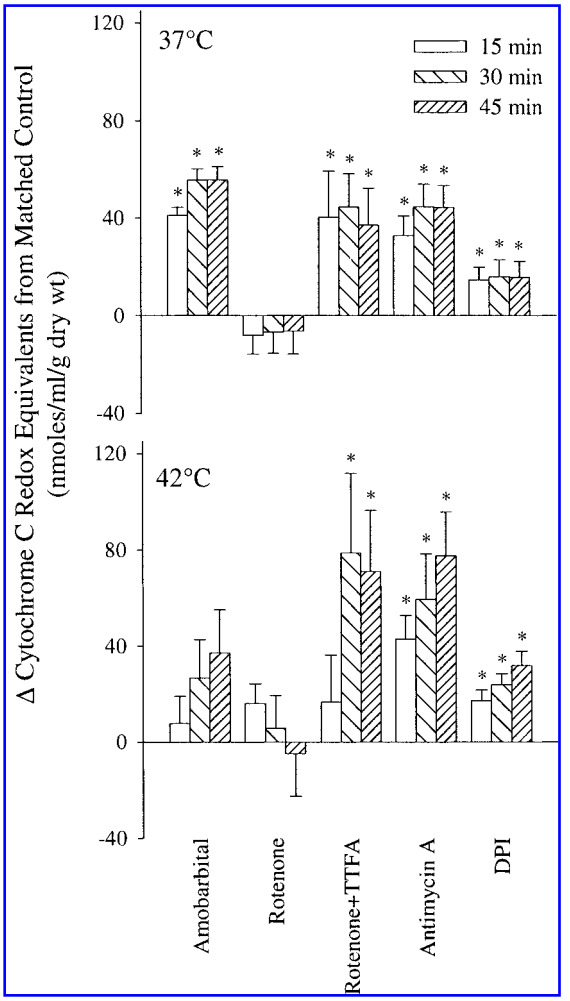

FIG. 2. Summarized data showing net differences from matched controls in response to blockers of mitochondria and NADPH oxidases at 37°C and 42°C. *$p < 0.05$ (ANOVA contrasts), between drug treatment and corresponding non-drug treatment at the given temperature.

both complex I and II to test the role of mitochondria in $O_2^{\cdot-}$ release. Tissues treated with rotenone plus TTFA caused more cyt *c* reduction compared with nontreated tissues at both 37°C ($n = 6$, $p < 0.01$) and 42°C (Fig. 2; $n = 6$, $p < 0.01$), thus exhibiting results similar to those of antimycin A. To test the effectiveness of complex I and II blockade in tissue experiments, changes in O_2 consumption induced by these treatments were measured. Figure 3 shows that rotenone plus TTFA caused a greater inhibition of O_2 consumption than rotenone alone at both 30 min and 45 min at normal temperature ($n = 6$ from five rats, $p < 0.05$).

Membrane-associated NADPH oxidases: inhibition with DPI (1 mM)

Similar to preceding experiments, DPI did not block cyt *c* reduction (Fig. 2) and, in fact, promoted its reduction at both 37°C and 42°C ($n = 9$, $p < 0.001$).

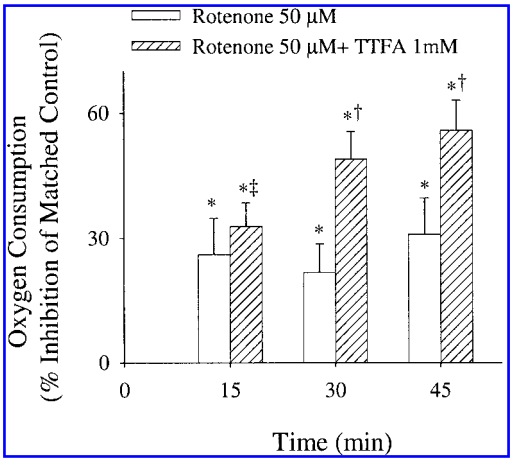

FIG. 3. Inhibition of oxygen consumption with rotenone alone and rotenone plus TTFA in intact tissues. *$p < 0.05$, between drug-treated tissues and non-drug-treated tissues ($n = 6$ from five rats); †$p < 0.05$, between rotenone alone and rotenone plus TTFA ($n = 6$ from five rats); ‡$n = 5$ for this group due to the removal of one data point determined by an outlier test. Inhibition was calculated from measures of oxygen consumption per gram wet weight.

SOD control experiments (5,000 U/ml)

As shown in Fig. 4, SOD significantly inhibited the cyt *c* reduction induced by rotenone (50 μ*M*) plus TTFA (1 m*M*) treatment ($n = 3$, $p < 0.01$). Similar results were obtained in tissues treated with DPI (1 m*M*) ($n = 3$, $p < 0.05$).

Determination of critical concentrations of cyt c

For the majority of this study, cyt *c* was used at a level of 5 μ*M* (42). Other researchers have used 10 μ*M* cyt *c* (22, 24).

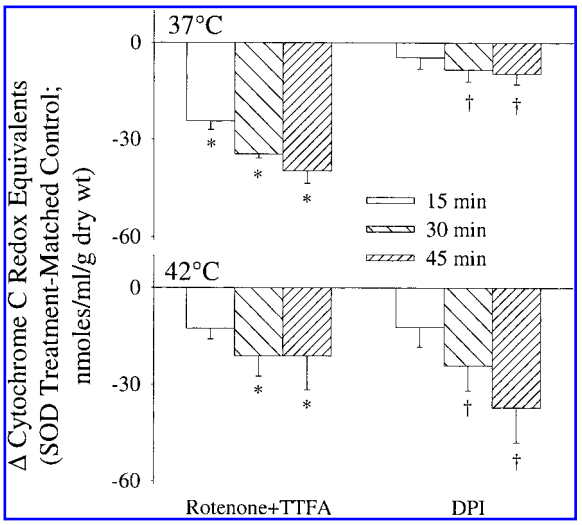

FIG. 4. Summarized data showing net differences from matched control regarding inhibition of cyt *c* reduction by SOD in either mitochondrial blockage (rotenone plus TTFA) or NADPH oxidase blockage (DPI). *$p < 0.01$, †$p < 0.05$ (ANOVA contrasts), between SOD treatment and corresponding non-SOD treatment at the given temperature.

We found that there was no significant difference between 5 and 10 μ*M* cyt *c* assay in our system with the exception that the application of a lower concentration of cyt *c* helps to decrease the absorbance background, increases the sensitivity of the assay, and lowers the foaming during oxygen bubbling (data not shown). However, the drugs added to the cyt *c* solution may have more or less effect on cyt *c* reduction, even in the absence of tissues. Therefore, the concern was raised whether such a low concentration of cyt *c* after the drug addition would be enough to adequately assay $O_2^{\cdot-}$ molecules released by the tissue. Thus, a series of control experiments was performed to test whether the collision probability between cyt *c* (oxidized) and $O_2^{\cdot-}$ is dependent on the concentration of cyt *c* in the bath. This was done by evaluating the difference between reduction measurements at cyt *c* concentrations of 4 (simulating 80% of 5 μ*M* cyt *c* after drug addition) versus 5 μ*M,* and 3 (simulating 60% of 5 μ*M* cyt *c* after drug addition) versus 5 μ*M,* in paired heat-stressed tissues. At each time point, 3 and 4 μ*M* cyt *c* data were expressed as percentage of 5 μ*M* cyt *c* ± SE (Table 1). No significant difference was observed between 4 and 5 μ*M* cyt *c* in heat stress experiments ($n = 6$ from three rats), but significant differences were observed between 3 and 5 μ*M* cyt *c* ($p < 0.05$ at 30 and 45 min, $n = 6$ from three rats). Therefore, the 4–5 μ*M* level of cyt *c* left in the bath after drug addition in most experiments appeared sufficient to ensure that the cyt *c* was not saturated in the reduced form. These experiments were critical controls, because >20% cyt *c* reduction was observed by pure chemical interactions alone with 0.5 m*M* DIDS in subsequent experiments. Thus, the cyt *c* concentration was raised to 10 μ*M* in these experiments to ensure a sufficient level of oxidized cyt *c* to react with $O_2^{\cdot-}$.

*Inhibition of anion channels with probenecid (1 m*M*) and DIDS (0.5 m*M*)*

In Fig. 5, probenecid had no significant effect on cyt *c* reduction at 37°C and 42°C ($n = 6$). For DIDS experiments, as mentioned above, 10 μ*M* cyt *c* was used for ROS detection. Similar to probenecid, DIDS had no significant effect on cyt *c* reduction at 37°C ($n = 6$). However, at 42°C, a trend of increased inhibition was observed. This may be related to factors such as animal variation. In half of the rats, DIDS caused a small increase of cyt *c* reduction, whereas in the other half, DIDS had an inhibitory effect on cyt *c* reduction. As a whole, the DIDS-treated tissues did not show any significant inhibition of $O_2^{\cdot-}$ release from the tissue ($n = 6$, $p = 0.36$ at 30 min and $p = 0.19$ at 45 min). Therefore, $O_2^{\cdot-}$ release did not appear to be dependent on the integrity of anion channels across the cellular or mitochondrial membranes when blocked by these agents.

TABLE 1. TEST OF COLLISION PROBABILITY BETWEEN CYT *C* AND ROS

Min	*% (4 to 5 μM cyt c)*	*% (3 to 5 μM cyt c)*
15	93 ± 9 (NS)	74 ± 14
30	92 ± 8 (NS)	69 ± 10*
45	92 ± 10 (NS)	75 ± 10*

$n = 6$ in each group from three rats. NS, not significant.
*$p < 0.05$, significantly different from controls (5 μ*M*).

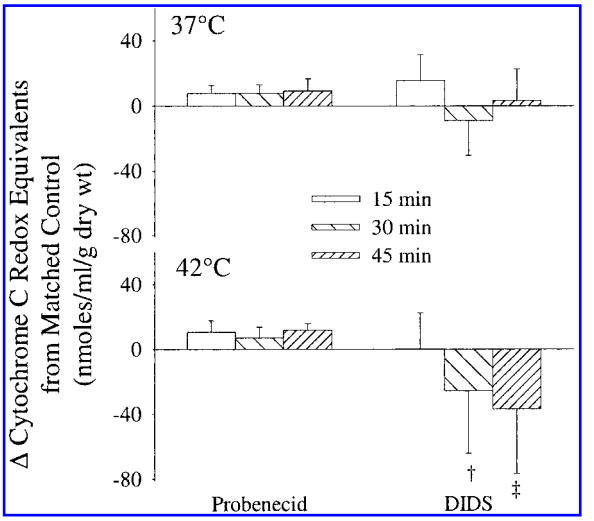

FIG. 5. Summarized data showing net differences from matched controls in response to anion channel blockers at 37°C and 42°C. Neither probenecid nor DIDS had any significant effect on cyt *c* reduction at both 37°C and 42°C ($n = 6$). †$p = 0.36$; ‡$p = 0.19$, between drug treatment and corresponding non-drug treatment at the given temperature.

DISCUSSION

These results are consistent with the hypothesis that $O_2^{\cdot-}$, released from the diaphragm, both at rest and in heat stress, does not seem to originate directly from an intracellular, cytosolic compartment because blockage of membrane anion channels, the only well known pathway for the passive diffusion of $O_2^{\cdot-}$ across the membranes, has no influence on extracellular measurement. Furthermore, the absence of an inhibitory effect of the NADPH oxidase inhibitor, DPI, suggests that extracellular $O_2^{\cdot-}$ does not arise primarily from membrane-associated oxidoreductases that have been identified in endothelial and muscle cells (17, 26, 40). Finally, inhibition of electron transport, both before and after the Q cycle, results in paradoxical elevations in extracellular $O_2^{\cdot-}$ release. This observation suggests that the yet unidentified source of extracellular $O_2^{\cdot-}$ might be sensitive to conditions of disordered metabolism or "chemical hypoxia," and that simply attempting to block mitochondrial electron transport in whole tissues or cells in order to determine the potential role of mitochondria as a source of ROS can lead to complex problems in data interpretation.

Influences of electron transport on extracellular $O_2^{\cdot-}$ formation

Our previous studies have demonstrated that increased cyt *c* reductions measured in heat-stressed skeletal muscle can be almost entirely blocked by SOD (42), suggesting that the sig-

nal is indicative of $O_2^{\cdot-}$ release and that other reducing equivalents such as nitric oxide ($^{\cdot}NO$) (3) or oxidizing agents such as H_2O_2 (39) are not important components of the signal measured by cyt *c*. This is also consistent with the present study (Fig. 4), showing that SOD can significantly inhibit cyt *c* reduction during either mitochondrial blockage or NADPH oxidase blockage, although only partial inhibition by SOD was observed at some time points and conditions in mitochondrial blockage or DPI treatment. The latter suggests that in such conditions other extracellular reducing agents may also contribute to cyt *c* reduction. Of the known intracellular sources of ROS, mitochondria are well recognized and seemed to be the most likely candidates because mitochondrial oxygen consumption and ATPase activity are increased in skeletal muscle during heat exposure (5). Furthermore, increases in temperature can markedly influence membrane fluidity, which is known to be of critical importance for mitochondrial formation of $O_2^{\cdot-}$ by ubisemiquinone at the inner mitochondrial membrane (12). Assuming mitochondrial $O_2^{\cdot-}$ could exit the mitochondria in the intact cell (16, 38), these conditions would improve the probability of electron leakage, promoting reduction of O_2 to $O_2^{\cdot-}$ (35).

Effective blockade of NADH as a substrate through complex I can potentially be overcome in intact tissues by their ability to provide reducing equivalents to complex II through succinate or by the oxidation of fatty acyl coenzyme A through the flavin-linked electron transferring flavoprotein at the level of ubiquinone. The measured metabolic rate, used to calculate the data in Fig. 3, may be variable and is a reflection of resting ATPase activity rather than actual mitochondrial function. Metabolic demand can vary depending on the state of the cell (*i.e.,* membrane potential, Ca^{2+} leakage, myofibrillar tone, etc.) and is no doubt altered to some extent by the stress of mitochondrial poisons. At any given time, resting O_2 consumption may comprise a small proportion of the tissue's total potential to consume O_2 (*i.e.,* far away from V_{max} for cyt *c* oxidase) as skeletal muscle can increase its metabolic rate by well over an order of magnitude as ADP and other regulators increase. This means that if a given inhibitor has blocked 90% of all potential mitochondrial electron transport, sufficient pathways of electron transport may still be available to sustain overall ATP and creatine phosphate concentrations at near normal levels. This is a major difference between studies of intact tissues and isolated mitochondria, where in the latter case substrates and [ADP] are controlled and where usage of electron transport blockers become most effective tools. Rotenone blocked only 25% of resting O_2 consumption and rotenone plus TTFA blocked only 55% (Fig. 3). We can conclude that in intact tissues, it is extremely difficult to load sufficient concentrations of rotenone to block mitochondrial electron transport without inducing nonspecific effects of solvents or that the mitochondria of skeletal muscle are extremely flexible in their ability to effectively shuttle alternate sources of electrons, thus bypassing our specific manipulations of electron transport. As very few articles have addressed oxygen measurements with intact skeletal muscle, this provides valuable lessons for future studies regarding application of inhibitors to tissues.

Nevertheless, the data are insightful and seem to point to a more general conclusion. In virtually every attempt to block mitochondrial electron transport (with the exception of complex I inhibition by rotenone alone, which only inhibited respiration by 25% in our system), there was a paradoxical and marked stimulation of extracellular $O_2^{\cdot-}$ formation, both at rest and during heat stress. Although blocking complex I alone by rotenone (29, 33) or amobarbital (1) has usually been shown to decrease ROS formation, on occasion complex I inhibition with rotenone has been shown to increase ROS, during certain experimental conditions (37). Therefore, at this point we are not sure whether it is possible that these two opposite effects on ROS formation by rotenone blockage may counteract each other in our system (Fig. 2). Thus, it was necessary to perform experiments with blockade of complex I and II together (rotenone plus TTFA), which showed an almost identical effect on $O_2^{\cdot-}$ formation when compared with blocking complex III. Thus, it seems that by poisoning electron transport through multiple pathways we have either unveiled a new extracellular $O_2^{\cdot-}$ generator that is sensitive to the metabolic state of the cell, or somehow stimulated the existing extracellular generators that are active in the tissue at rest or in heat stress. Furthermore, based on the early work of Boveris *et al.* (4), complex III inhibition (antimycin A) would increase $O_2^{\cdot-}$ formation because of its effect on increasing the electrical potential at the Q site. However, in our system, increased cyt *c* reduction by complex III blockage may not necessarily arise from ROS formation in mitochondria because anion channels, the only well known $O_2^{\cdot-}$ exit pathways, were not shown to play a role in our model (Fig. 5). In summary, coupled with the results for anion channel blockade, we see no clear evidence that the extracellular $O_2^{\cdot-}$ generator at rest or during heat stress could arise directly from the mitochondria.

What could be responsible for the influence of metabolic blockade on extracellular ROS formation? One potential source in the intact cell could be the xanthine oxidase system. A buildup of purine nucleotide catabolic products such as hypoxanthine can provide substrate for xanthine oxidase, producing $O_2^{\cdot-}$ and H_2O_2 (13). Other sources could involve release or relocalization of Ca^{2+} from mitochondria or other organelle stores in response to metabolic blockade, possibly activating Ca^{2+}-dependent ROS-generating systems such as the phospholipase A_2 pathway (27). An increase in free Ca^{2+} could also stimulate proteolytic conversion of xanthine oxidase from xanthine dehydrogenase to produce $O_2^{\cdot-}$ (14). Thus, further studies on these complex mechanisms are necessary.

Blockage of membrane-associated NADPH oxidases

Membrane-associated NADPH oxidases provide important sources of ROS during a variety of normal and abnormal conditions (10, 20, 40). DPI and its analogues are most commonly used as potent inhibitors of these oxidases (11) via the iodonium modification of heme b at the flavocytochrome b (10). As extracellular $O_2^{\cdot-}$ did not appear to diffuse across membrane anion channels, a reasonable alternative hypothesis was that a membrane NADPH oxidase might be responsible. Such oxidases do not require channels for $O_2^{\cdot-}$ release and are capable of moving an electron directly across the cell membrane to reduce extracellular O_2 in the study of inflammatory cells (31). Of particular importance for this study is the potential

existence of NADPH oxidases in endothelial cells (36) because the diaphragm contains a large capillary endothelium. Furthermore, a recent study has shown that NADPH oxidase can also function as an intracellular ROS generator in skeletal muscle (17). However, our results show that similar to mitochondrial blockade, general inhibition of NADPH oxidases with DPI did not inhibit, but rather stimulated $O_2^{\cdot-}$ release, and can be inhibited by SOD (Fig. 4). Therefore, these results show that membrane-bound NADPH oxidases are not the primary source of extracellular $O_2^{\cdot-}$ release in a resting control state or in heat stress. This study of extracellular source of ROS is not inherently contradictory to studies of the intracellular sources of ROS in skeletal muscle, which have shown a dependence of NADPH oxidase (17). As DPI analogues in sufficient concentrations can also block complex I of the mitochondria (20), we speculate that the same phenomenon observed for mitochondrial blockade could have occurred with our studies of DPI administration. It is still possible that an underlying NADPH oxidase mechanism is acting during some conditions with this preparation, but it is unlikely to be the only or even the primary source in this model. The results emphasize the complexity of attempting to block specific pathways of ROS formation in the intact tissue, as there could be secondary influences on ROS formation by alternative pathways. This point is largely overlooked in studies of this nature.

Another important NADPH-dependent enzyme is nitric oxide synthase (NOS), which could have many contrasting influences on the measurements. First, NOS can be a $O_2^{\cdot-}$ generator under some conditions of substrate limitation (41). Second, $^{\cdot}NO$ can directly react with $O_2^{\cdot-}$, making it less available for cyt *c* reduction (2). Third, peroxynitrite (formed from $O_2^{\cdot-}$ and $^{\cdot}NO$), a strong oxidant, is likely to directly oxidize cyt *c* (2). Experiments with DPI may address these complexities because a sufficient concentration (1 m*M*) can also inhibit NOS activity (25). As the influence of DPI in cyt *c* reduction was modest (Fig. 2), it is not apparent that NOS played a biologically significant role in these measurements.

Membrane anion channel blockage

Both probenecid (6) and DIDS (34) have been used extensively as anion channel blockers. The most conclusive results are the lack of effects on the measured variables using high concentrations of these agents. This makes mechanisms of passive diffusion for extracellular $O_2^{\cdot-}$ release from intracellular sources unlikely in this model. The role of anion channels in $O_2^{\cdot-}$ release has been studied extensively in isolated red blood cells (22) and are also believed to provide a pathway for $O_2^{\cdot-}$ release from mitochondria in cardiomyocytes (38). However, our data suggest that there must be an alternative electron flux system, which works either by a yet unidentified anion channel or by directly conducting electrons across cell membranes (31). Furthermore, we also speculate the possibility of more complex mechanisms for $O_2^{\cdot-}$ transport. One potential mechanism requires the protonation of $O_2^{\cdot-}$ to form the perhydroxyl radical ($HO_2^{\cdot}$) at the Gouy–Chapman–Stern boundary layer on membrane polyanionic surfaces (15). $HO_2^{\cdot}$ is a neutralized molecule that is more cell-permeable (8, 21). Thus, it is possible for $HO_2^{\cdot}$ to diffuse across the membrane and resynthesize $O_2^{\cdot-}$ extracellularly. Another mechanism would involve redox-active molecules such as ubisemiquinones, which have also been shown to be possible "$O_2^{\cdot-}$ carriers" across the membrane through a series of chain reactions (32). However, issues regarding how these mechanisms are working in our system are currently poorly understood in the field.

Conclusions and speculations

The results suggest that extracellular $O_2^{\cdot-}$ release may not simply reflect a spillover of reduced oxygen from intracellular sources such as mitochondria, because the only well known mechanisms for $O_2^{\cdot-}$ transport across either cell or mitochondrial membranes (*i.e.,* anion channels) do not appear to be involved in this preparation. The stimulation effects on $O_2^{\cdot-}$ release by the variety of blockers used in this study (usually used to demonstrate the inhibition of ROS formation) appear puzzling, but reveal that the mechanisms for ROS production in the intact cell or tissue are very complex and are probably much more interactive than is often appreciated. In a general way, mitochondrial blockage or possibly DPI administration can increase the reducing potential of the cell, thus increasing the probability for intracellular reduction of O_2 to $O_2^{\cdot-}$. How this occurs, we can only speculate. Some $O_2^{\cdot-}$ generating systems, such as xanthine oxidases, lipoxygenases, or cyclooxygenases, require NAD(P)H. As the level of NADH is elevated in chemical hypoxia, they could influence the rate of a variety of redox reactions (19). Alternatively, other transition metals in the cell could be made more susceptible to participating in single electron transfer reactions to O_2 in a highly reducing environment.

It is also possible in the intact living cell that regulation of redox tone is an ongoing process with alternative back-up mechanisms. Thus, when one generator responsible for pushing forward the intracellular or extracellular oxidizing environment is inhibited, another may become up-regulated to maintain local $O_2^{\cdot-}$, peroxide, or thiol "tone." This intriguing idea, which has been the subject of a recent review (30), would suggest that intracellular redox sensors are actively engaged in a normal regulatory pathway through the possible orchestration of multiple ROS-generating sites. Such a possibility makes finding unique sources of ROS formation in a specific biological condition, such as heat stress, extremely challenging in intact tissues.

ACKNOWLEDGMENTS

We thank Dr. Fievos Christofi and Mark Julian for their kind suggestions and Dr. Haidady Nagaraja for statistical help during the preparation of the manuscript. This work was supported by NHLB 53333.

ABBREVIATIONS

cyt *c,* cytochrome *c*; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; DMSO, dimethyl sulfoxide; DPI, diphenyliodonium; $HO_2^{\cdot}$, perhydroxyl radical; H_2O_2, hydrogen peroxide; $^{\cdot}NO$, nitric oxide; NOS, nitric oxide synthase; $O_2^{\cdot-}$, superox-

ide; ROS, reactive oxygen species; SOD, superoxide dismutase; TTFA, thenoyltrifluoroacetone.

REFERENCES

1. Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP, Tritto I, Cirillo P, Condorelli M, Chiariello M, and Flaherty JT. Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *J Biol Chem* 268: 18532–18541, 1993.
2. Beckman JS and Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am J Physiol* 271: C1424–C1437, 1996.
3. Bell LC and Ferguson SJ. Nitric and nitrous oxide reductases are active under aerobic conditions in cells of *Thiosphaera pantotropha*. *Biochem J* 273: 423–427, 1991.
4. Boveris A and Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* 134: 707–716, 1973.
5. Brooks GA, Hittelman KJ, Faulkner JA, and Beyer RE. Tissue temperature and whole-animal oxygen consumption after exercise. *Am J Physiol* 221: 427–431, 1971.
6. Busch AE, Schuster A, Waldegger S, Wagner CA, Zempel G, Broer S, Biber J, Murer H, and Lang F. Expression of a renal type I sodium/phosphate transporter (NaPi- 1) induces a conductance in *Xenopus* oocytes permeable for organic and inorganic anions. *Proc Natl Acad Sci U S A* 93: 5347–5351, 1996.
7. Chance B, Sies H, and Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59: 527–605, 1979.
8. De Grey AD. HO_2^-: the forgotten radical. *DNA Cell Biol* 21: 251–257, 2002.
9. De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, and Greindling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. *Circ Res* 82: 1094–1101, 1998.
10. Doussiere J, Gaillard J, and Vignais PV. The heme component of the neutrophil NADPH oxidase complex is a target for aryliodonium compounds. *Biochemistry* 38: 3649–3703, 1999.
11. Fukuto JM and Chaudhuri G. Inhibition of constitutive and inducible nitric oxide synthase: potential selective inhibition. *Annu Rev Pharmacol Toxicol* 35: 165–194, 1995.
12. Gille L and Nohl H. The ubiquinol/bc1 redox couple regulates mitochondrial oxygen radical formation. *Arch Biochem Biophys* 388: 34–38, 2001.
13. Granger DN. Role of xanthine oxidase and granulocytes in ischemia–reperfusion injury. *Am J Physiol* 255: H1269–H1275, 1988.
14. Granger DN, Hollwarth ME, and Parks DA. Ischemia–reperfusion injury: role of oxygen-derived free radicals. *Acta Physiol Scand* 548: 47–63, 1986.
15. Haines TH. Anionic lipid headgroups as a proton-conducting pathway along the surface of membranes: a hypothesis. *Proc Natl Acad Sci U S A* 80: 160–164, 1983.
16. Han D, Antunes F, Canali R, Rettori D, and Cadenas E. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *J Biol Chem* 278: 5557–5563, 2003.
17. Javesghani D, Magder SA, Barreiro E, Quinn MT, and Hussain SNA. Molecular characterization of a superoxide-generating NAD(P)H oxidase in the ventilatory muscles. *Am J Respir Crit Care Med* 165: 412–418, 2002.
18. Kolbeck RC, She ZW, Callahan LA, and Nosek TM. Increased superoxide production during fatigue in the perfused rat diaphragm. *Am J Respir Crit Care Med* 156: 140–145, 1997.
19. Kukreja RC, Kontos HA, Hess ML, and Ellis EF. PGH synthase and lipoxygenase generate superoxide in the presence of NADH or NADPH. *Circ Res* 59: 612–619, 1986.
20. Li Y and Trush MA. Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production. *Biochem Biophys Res Commun* 253: 295–299, 1998.
21. Liu SS. Cooperation of a "reactive oxygen cycle" with the Q cycle and the proton cycle in the respiratory chain—superoxide generating and cycling mechanisms in mitochondria. *J Bioenerg Biomembr* 31: 367–376, 1999.
22. Lynch RE and Fridovich I. Permeation of the erythrocyte stroma by superoxide radical. *J Biol Chem* 253: 4697–4699, 1978.
23. Margoliash E and Frohwirt N. Spectrum of horse-heart cytochrome *c*. *Biochem J* 71: 570–572, 1959.
24. McCord JM and Fridovich I. Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). *J Biol Chem* 244: 6049–6055, 1969.
25. Miesel R, Kurpisz M, and Kroger H. Suppression of inflammatory arthritis by simultaneous inhibition of nitric oxide synthase and NADPH oxidase. *Free Radic Biol Med* 20: 75–81, 1996.
26. Munzel T, Hink U, Heitzer T, and Meinertz T. Role for NADPH/NADH oxidase in the modulation of vascular tone. *Ann N Y Acad Sci* 874: 386–400, 1999.
27. Nethery D, Stofan D, Callahan L, DiMarco A, and Supinski G. Formation of reactive oxygen species by the contracting diaphragm is PLA2 dependent. *J Appl Physiol* 87: 792–800, 1999.
28. Reid MB and Moody MR. Dimethyl sulfoxide depresses skeletal muscle contractility. *J Appl Physiol* 76: 2186–2190, 1994.
29. Reinhart P, Taylor WM, and Bygrave FL. Studies on alpha-adrenergic-induced respiration and glycogenolysis in perfused rat liver. *J Biol Chem* 257: 1906–1912, 1982.
30. Schafer FQ and Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 30: 1191–1212, 2001.
31. Schrenzel J, Serrander L, Banfi B, Nube O, Fouyouzi R, Lew DP, Demaurex N, and Krause KH. Electron currents generated by the human phagocyte NADPH oxidase. *Nature* 392: 734–737, 1998.
32. Schultz JR, Ellerby LM, Gralla EB, Valentine JS, and Clarke CF. Autoxidation of ubiquinol-6 is independent of superoxide dismutase. *Biochemistry* 35: 6595–6603, 1996.
33. Shanley P, Brezis M, Spokes K, Silva P, Epstein FH, and Rosen S. Differential responsiveness of proximal tubule

segments to metabolic inhibitors in isolated perfused rat kidney. *Am J Kidney Dis* 7: 76–83, 1986.
34. Spira B and Breitbart H. The role of anion channels in the mechanism of acrosome reaction in bull spermatozoa. *Biochim Biophys Acta* 1109: 65–73, 1992.
35. Taylor DE, Ghio AJ, and Piantadosi CA. Reactive oxygen species produced by liver mitochondria of rats in sepsis. *Arch Biochem Biophys* 316: 70–76, 1995.
36. Tolando R, Jovanovic A, Brigelius-Flohe R, Ursini F, and Maiorino M. Reactive oxygen species and proinflammatory cytokine signaling in endothelial cells: effect of selenium supplementation. *Free Radic Biol Med* 28: 979–986, 2000.
37. Turrens JF and Boveris A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* 191: 421–427, 1980.
38. Vanden Hoek TL, Becker LB, Shao Z, Li C, and Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* 273: 18092–18098, 1998.
39. Vandewalle PL and Petersen NO. Oxidation of reduced cytochrome *c* by hydrogen peroxide. *FEBS Lett* 210: 195–198, 1987.
40. van Klaveren RJ, Roelant C, Boogaerts M, Demedts M, and Nemery B. Involvement of an NAD(P)H oxidase-like enzyme in superoxide anion and hydrogen peroxide generation by rat type II cells. *Thorax* 52: 465–471, 1997.
41. Xia Y, Dawson VL, Dawson TM, Snyder SH, and Zweier JL. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc Natl Acad Sci U S A* 93: 6770–6774, 1996.
42. Zuo L, Christofi FL, Wright VP, Liu CY, Merola AJ, Berliner LJ, and Clanton TL. Intra- and extracellular measurement of reactive oxygen species produced during heat stress in diaphragm muscle. *Am J Physiol Cell Physiol* 279: C1058–C1066, 2000.

Address reprint requests to:
Thomas Clanton, Ph.D.
The Ohio State University
Pulmonary and Critical Care Medicine
201 Dorothy M. Davis Heart and Lung Research Institute
473 W. 12th Ave
Columbus, OH 43210

Email: clanton.1@osu.edu

Received for publication June 2, 2003; accepted June 9, 2003.

This article has been cited by:

1. Li Zuo, William J. Roberts, Rosa C. Tolomello, Adam T. Goins. 2012. Ischemic and hypoxic preconditioning protect cardiac muscles via intracellular ROS signaling. *Frontiers in Biology* . [CrossRef]
2. Ma#gorzata Budzi#ska, Hanna Ga#ga#ska, Andonis Karachitos, Ma#gorzata Wojtkowska, Hanna Kmita. 2009. The TOM complex is involved in the release of superoxide anion from mitochondria. *Journal of Bioenergetics and Biomembranes* **41**:4, 361-367. [CrossRef]
3. Chris van der Poel, Joshua N Edwards, William A Macdonald, D George Stephenson. 2008. EFFECT OF TEMPERATURE-INDUCED REACTIVE OXYGEN SPECIES PRODUCTION ON EXCITATION-CONTRACTION COUPLING IN MAMMALIAN SKELETAL MUSCLE. *Clinical and Experimental Pharmacology and Physiology* . [CrossRef]