PLA$_2$ dependence of diaphragm mitochondrial formation of reactive oxygen species

D. NETHERY, L. A. CALLAHAN, D. STOFAN, R. MATTERA, A DiMARCO, AND G. SUPINSKI

Pulmonary Division, Department of Medicine, Case Western Reserve University, and MetroHealth Medical Center, Cleveland, Ohio 44109

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Nethery, D., L. A. Callahan, D. Stofan, R. Mattera, A DiMarco, and G. Supinski. PLA$_2$ dependence of diaphragm mitochondrial formation of reactive oxygen species. J Appl Physiol 89: 72–80, 2000.—Contraction-induced respiratory muscle fatigue and sepsis-related reductions in respiratory muscle force-generating capacity are mediated, at least in part, by reactive oxygen species (ROS). The subcellular sources and mechanisms of generation of ROS in these conditions are incompletely understood. We postulated that the physiological changes associated with muscle contraction (i.e., increases in calcium and ADP concentration) stimulate mitochondrial generation of ROS by a phospholipase A$_2$ (PLA$_2$)-modulated process and that sepsis enhances generation of ROS by upregulating PLA$_2$ activity. To test these hypotheses, we examined H$_2$O$_2$ generation by diaphragm mitochondria isolated from saline-treated control and endotoxin-treated septic animals. We found that free radical formation in intact contracting skeletal muscle is modulated by the activity of phospholipase A$_2$ (PLA$_2$) and that contraction-related ROS formation, and a resultant amplification of PLA$_2$-dependent ROS generation during contraction and sepsis may be critically dependent on PLA$_2$ activity.

Address for reprint requests and other correspondence: G. Supinski, MetroHealth Medical Center, 2500 MetroHealth Dr., Cleveland, OH 44109.
H$_2$O$_2$ formation was measured under both basal conditions and after addition of calcium or ADP (in concentrations that approximated those achieved during muscle contraction). PLA$_2$ dependence of H$_2$O$_2$ formation was assessed by examining the effect of administration of selective inhibitors and activators of various PLA$_2$ isoforms. Mitochondrial PLA$_2$ enzymatic activity levels were assayed by using a previously described technique (26). The contribution of the electron transport chain to PLA$_2$-dependent free radical formation was determined by examining the effect of specific inhibitors of complex I and complex IV on mitochondrial H$_2$O$_2$ formation.

**METHODS**

**Animal preparation.** All studies were performed by using tissues excised from adult male rats (Harlan) weighing between 350 and 400 g. Before experimentation, rats were housed in the Case Western Reserve University Animal Resource Center and cared for by Case Western Reserve University veterinarians in accordance with American Association for Accreditation of Laboratory Animal Care guidelines. Food and water were allowed ad libitum.

When called for, sepsis was induced in rats by the intraperitoneal injection of endotoxin (lipopolysaccharide from *Escherichia coli*; 8 mg/kg in 0.5 ml saline). After 18 h, animals were killed, and the diaphragm was removed and processed, as described in *Diaphragm mitochondria isolation procedure*. An intraperitoneal injection of saline (0.5 ml) was also administered to control rats. In both endotoxin-treated and control groups, 5 ml/kg of saline were injected subcutaneously to prevent dehydration (in endotoxin-treated animals) and to serve as a control (in non-endotoxin-treated animals).

At the time of study, animals were anesthetized with nembutal (50 mg/kg). The abdomen was opened, and the abdominal aorta was isolated and then cannulated by using an 18-gauge angiocatheter. Saline (30 ml), followed by mitochondrial isolation buffer (30 ml), was retrograde perfused through the aorta to flush all blood from the diaphragm.

Diaphragm mitochondria were isolated following the procedure of Humphries et al. (12). Diaphragm tissue was rinsed in cold isolation buffer (180 mM KCl, 5 mM MOPS, and 2 mM EGTA, pH 7.25 at 4°C), blotted dry, weighed, and placed in fresh isolation buffer. The muscle was then coarsely chopped, filtered through one layer of cheesecloth, and added to another volume of cold isolation buffer. After being minced finely with scissors, diaphragm pieces were homogenized for two 10-s periods by using a Polytron homogenizer set at one-half speed. The homogenate was filtered through one layer of cheesecloth into a clean centrifuge tube. A portion of this homogenate was saved and stored on ice to be used for determination of H$_2$O$_2$ generation; the remaining homogenate was centrifuged at 500 g for 7.5 min at 4°C. The resulting supernatant was centrifuged at 6,000 g for 10 min at 4°C. The isolated mitochondrial pellet was washed five times with 1-ml volumes of isolation buffer and then gently resuspended in isolation buffer to yield a final mitochondrial protein concentration of 10–30 mg/ml. Mitochondria were kept on ice until determination of H$_2$O$_2$ generation rates, which were completed the same day.

**Protein assay.** Protein concentrations were determined by using the biuret method, as previously described (8).

**Measurement of formation of H$_2$O$_2$ by isolated mitochondria.** Production of H$_2$O$_2$ by mitochondrial suspensions was assessed by measuring the rate of 4-hydroxyphenyl acetate (4-HPA) oxidation by the H$_2$O$_2$ generated by isolated diaphragm mitochondria. This method follows the coupling of DTNB to free coenzyme A, which is released from acetyl-CoA during the enzymatic synthesis of citrate. DTNB (0.1 mM), acetyl-CoA (0.33 mM), and ~50 μg of mitochondrial protein were added to a spectrophotometer cuvette, and absorbance was measured for 3 min at 412 nm to assess acetyl-CoA deacetylase activity. The citrate synthase reaction was then initiated by the addition of 0.5 mM oxaloacetate; the change in absorbance was measured for an additional 3 min. Activity was calculated by using an extinction coefficient of 13,600 and was expressed as micromoles per minute per milligram protein. This technique has a lower detection limit of ~0.1 μmol citrate synthase min$^{-1}$mg protein$^{-1}$.

**Citrate synthase activity assay.** The purity of the mitochondria isolated from the diaphragm was assessed by determining the activity of citrate synthase, an enzyme located exclusively in the mitochondria, by the procedure of Srere (28). This method follows the coupling of DTNB to free coenzyme A, which is released from acetyl-CoA during the enzymatic synthesis of citrate. DTNB (0.1 mM), acetyl-CoA (0.33 mM), and ~50 μg of mitochondrial protein were added to a spectrophotometer cuvette, and absorbance was measured for 3 min at 412 nm to assess acetyl-CoA deacetylase activity. The citrate synthase reaction was then initiated by the addition of 0.5 mM oxaloacetate; the change in absorbance was measured for an additional 3 min. Activity was calculated by using an extinction coefficient of 13,600 and was expressed as micromoles per minute per milligram protein. This technique has a lower detection limit of ~0.1 μmol citrate synthase min$^{-1}$mg protein$^{-1}$.

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PLA₂ isoforms; 8) diphenylene iodonium (20 μM), an inhibitor of NADPH oxidase; and 9) inhibitors of complex I (rotenone, 5 μM) and complex IV (potassium cyanide, 5 μM) of the electron transport chain. The doses used for the various inhibitors listed above were chosen based on previous reports indicating that the selected concentrations were optimal for inhibiting the specific enzymes being targeted (3, 5, 10, 16, 24, 35). Because studies were performed with phosphate-free buffer, mitochondria were in state 2 for all of these experiments.

Mitochondrial oxygen consumption. Mitochondrial oxygen consumption was assessed by using standard techniques (20). In brief, mitochondrial samples were suspended at a protein concentration of 0.5 mg/ml in buffer (120 mM KCl, 5 mM KH₂PO₄, 5 mM MOPS, and 1 mM EDTA, pH 7.25). State 2 respiration was initiated by adding malate (2.5 mM) and pyruvate (10 mM); after 2 min, ADP (0.5 mM) was added to initiate state 3 respiration. Oxygen consumption rate after completion of ADP conversion to ATP was taken as the state 4 rate of respiration. Respiration rates were measured by using a Clark-type electrode. The amount of oxygen consumed during ADP-stimulated respiration was calculated and used to determine the ADP-to-O ratio.

PLA₂ activity assay. PLA₂ activity was assessed spectrophotometrically by using an assay that employs the 1,2-dithio analog of diheptanoyl phosphatidylcholine as a substrate for PLA₂ (26). With the addition of a sample containing PLA₂, the thio ester bond at the sn-2 position of this substrate is hydrolyzed. DTNB is added, and the liberated free thiols are measured over time at 412 nm on a spectrophotometric plate reader. The reaction rate of PLA₂ can then be calculated by determining the change in absorbance per minute of sample wells, subtracting the change in absorbance per minute of nonenzymatic control wells, and using this difference in a formula that utilizes the corrected extinction coefficient for DTNB (10.66 mM⁻¹) and takes sample dilution into account. Because the 1,2-dithio analog of diheptanoyl phosphatidylcholine used in this assay does not serve as a substrate for the 85-kDa or calcium-independent family of isoforms of PLA₂, this assay specifically measures the activity of 14-kDa PLA₂.

Statistical analysis. Comparison of measurements made on a single parameter (e.g., generation of H₂O₂) between two or more experimental groups (e.g., for control and sepsis mitochondrial isolates) were made by using ANOVA analysis, with post hoc testing (Student-Newman-Keuls) to determine statistical differences between individual groups. A P value of <0.05 was taken as indicating statistical significance. Parameter values are presented as means ± SE.

RESULTS

Generation of H₂O₂ by diaphragm mitochondria from control animals. Addition of either calcium or ADP markedly stimulated H₂O₂ formation by mitochondria from control animals when malate and pyruvate were used as substrate (Fig. 1; P < 0.001 for comparison of H₂O₂ formation with and without addition of calcium, P < 0.001 for comparison of H₂O₂ formation with and without ADP). In contrast, neither calcium nor ADP had any effect on H₂O₂ formation by mitochondria from control animals when succinate was used as substrate. Specifically, H₂O₂ generation rate with succinate averaged 0.21 ± 0.02, 0.17 ± 0.03, and 0.16 ± 0.03 nmol·min⁻¹·mg protein⁻¹ for control mitochondria without calcium or ADP, with calcium added, and with ADP added, respectively (not significant). It was possible to block the stimulatory effects of calcium and ADP on control mitochondrial H₂O₂ formation by using manoalide, a 14-kDa PLA₂ inhibitor, as shown in Fig. 1.

To examine the selectivity of the action of manoalide to suppress calcium-stimulated H₂O₂ generation with malate and pyruvate as substrate, the effects of inhibitors of a variety of different PLA₂ isoforms, as well as an inhibitor of NADPH oxidase, were examined and compared with the suppressive effect of manoalide (Fig. 2). We found that CDC, another inhibitor of the 14-kDa family of PLA₂ isoforms, mimicked the effect of manoalide to suppress calcium-stimulated H₂O₂ formation. ACOCF₃ (an inhibitor of 85-kDa PLA₂), HELSS (a selective inhibitor of calcium-independent PLA₂), and diphenylene iodonium (an inhibitor of NADPH oxidase), however, had no effect on mitochondrial H₂O₂ formation rate (Fig. 2).

To rule out the possibility that PLA₂ only modulates H₂O₂ formation by playing a permissive role in facilitating another calcium-stimulated action on mitochondria, we examined the effect of directly stimulating PLA₂ activity with melittin on mitochondrial H₂O₂ formation. As shown in Table 1, melittin administration resulted in a marked increase in mitochondrial H₂O₂ formation rate (this response mimicked the effects of calcium and ADP stimulation; P < 0.005 for comparison of H₂O₂ formation under basal and melittin-added conditions). To demonstrate the selectivity of this action of melittin, we also examined the effect of simultaneous administration of manoalide and melittin and found that manoalide completely suppressed melittin-induced H₂O₂ formation (Table 1). The effect of administration of arachidonic acid, the principal metabolite resulting from PLA₂-catalyzed degradation of membrane lipids, on mitochondrial H₂O₂ formation...
with malate and pyruvate as substrate was also studied (Table 1). Arachidonic acid markedly stimulated H$_2$O$_2$ formation ($P < 0.001$ for comparison of basal and arachidonic acid-stimulated values). This latter action was not inhibited by manoalide, suggesting that the “downstream” effects of arachidonic acid are responsible for PLA$_2$-modulated H$_2$O$_2$ formation.

**Effect of electron transport chain inhibitors on arachidonic acid-stimulated H$_2$O$_2$ formation by control mitochondria.** The above data suggest that 14-kDa PLA$_2$ modulates calcium- and ADP-stimulated skeletal muscle H$_2$O$_2$ formation by mitochondria and that this effect of PLA$_2$ is mediated by an action of arachidonic acid levels. In theory, arachidonic acid could promote free radical formation by a number of mechanisms (i.e., by supplying substrate for cyclooxygenase pathways, supplying substrate for lipooxygenase, or interacting with the electron transport chain). To test the possibility that arachidonic acid acts by altering electron transport chain generation of H$_2$O$_2$, we examined the effect of administration of inhibitors of various electron transport chain complexes (rotenone, an inhibitor of complex I, and cyanide, an inhibitor of complex IV) on arachidonic acid-induced H$_2$O$_2$ formation with malate and pyruvate as substrate (Table 2). Rotenone completely blocked arachidonic acid-stimulated mitochondrial H$_2$O$_2$ formation ($P < 0.001$), but cyanide did not affect H$_2$O$_2$ formation under these conditions. These results are consistent with a mechanism by which arachidonic acid interacts with the electron transport chain at a site between complexes I and IV. Recent work by Cocco et al. (7) is also consistent with this finding; these authors have shown that rat heart mitochondrial generation of H$_2$O$_2$ by the electron transport chain is markedly increased after addition of arachidonic acid. Arachidonic acid can also generate superoxide by the cyclooxygenase system; the role of this pathway was not evaluated in the present study.

**Effect of sepsis on H$_2$O$_2$ formation by diaphragm mitochondria.** For our experiments examining free radical formation by mitochondria from septic animals, we first examined the “purity” of mitochondrial suspensions isolated from control and septic animals. We found mitochondrial suspensions from control and septic animals had similar citrate synthase activities (1.26 ± 0.09 and 1.33 ± 0.17 μmol·min$^{-1}$·mg protein$^{-1}$, respectively; not significant). This finding argues that the mitochondrial samples used for assessment of H$_2$O$_2$ generation by control and septic animals had comparable compositions. We also found that ADP/O for mitochondria from control and septic animals were similar, excluding the possibility that nonspecific “uncoupling” of control or septic mitochondria was present in our experiments (Table 3).

H$_2$O$_2$ formation by mitochondria from septic animals differed in several important respects from that observed with control animals. We found that "basal"...
H₂O₂ formation rates (i.e., in the absence of calcium or ADP) by mitochondria from septic animals were significantly higher than H₂O₂ formation rates by control mitochondria with malate and pyruvate as substrate (Fig. 3; $P < 0.001$ for comparison of septic and control values). Manoalide administration (i.e., a 14-kDa PLA₂ inhibitor) reduced this heightened basal H₂O₂ formation for mitochondria from septic animals, bringing the level down to the H₂O₂ formation rate of control mitochondria (Fig. 3). To test the specificity of this effect of manoalide to reduce H₂O₂ formation under basal conditions for mitochondria from septic animals, we also examined the effects of other PLA₂ inhibitors in this condition (see Fig. 4). Inhibitors of other PLA₂ isoforms (i.e., inhibitors of 85-kDa PLA₂ or calcium-independent PLA₂), and an inhibitor of NADPH oxidase, had no effect on basal H₂O₂ formation by mitochondria from septic animals.

As for mitochondria from control animals, H₂O₂ formation for mitochondria from septic animals was also stimulated by administration of calcium or ADP (Fig. 5). The absolute magnitude of H₂O₂ formation by mitochondria from septic animals, however, greatly exceeded that observed for control animals under both calcium- and ADP-stimulated conditions ($P < 0.03$ for comparison of H₂O₂ formation between control and septic with calcium, and $P < 0.05$ for comparison of H₂O₂ formation between these groups with ADP). As
for control mitochondria, manolide suppressed calcium- and ADP-stimulated H$_2$O$_2$ formation by mitochondria from septic animals, as shown in Fig. 5.

With succinate as substrate, H$_2$O$_2$ formation by mitochondria from septic animals was not elevated above the rate seen for mitochondria from controls (Fig. 6). We found, however, that both calcium and ADP increased H$_2$O$_2$ formation by mitochondria from septic animals with succinate as substrate ($P < 0.05$ for comparison of values during calcium and ADP stimulation to basal levels), whereas no such stimulation was seen for either calcium or ADP with succinate when control mitochondria are studied. In fact, with succinate as substrate, ADP reduced H$_2$O$_2$ formation by control mitochondria but increased H$_2$O$_2$ formation by mitochondria from septic animals (Fig. 6). Manolide completely suppressed both calcium- and ADP-stimulated H$_2$O$_2$ formation by mitochondria from septic animals with succinate as substrate ($P < 0.02$ for comparison of H$_2$O$_2$ formation by mitochondria from septic animals in the presence and absence of manolide during succinate incubation with either calcium or ADP).

The above findings suggest that there is heightened PLAr-dependent H$_2$O$_2$ formation by diaphragm mitochondria from septic animals. To further assess this relationship, we directly measured 14-kDa PLA2 activity in mitochondrial suspensions with an activity assay that measures enzymatic breakdown of an exogenous substrate (Fig. 7). We found marked increases in mitochondrial PLAr activity levels for mitochondria from septic animals ($8.59 \pm 1.8$ nmol·min$^{-1}·$mg protein$^{-1}$) compared with PLAr levels for control mitochondria ($3.19 \pm 0.7$ nmol·min$^{-1}·$mg protein$^{-1}$; $P < 0.02$ for comparison of samples from septic and control animals).

**DISCUSSION**

The major findings of this study were as follows: 1) both calcium and ADP stimulated H$_2$O$_2$ formation by diaphragm mitochondria from both control and septic animals; 2) mitochondria from septic animals demonstrated substantially higher H$_2$O$_2$ formation rates than mitochondria from control animals under basal, calcium-stimulated and ADP-stimulated conditions; and 3) in all conditions in which mitochondrial H$_2$O$_2$ formation was elevated above the basal rate observed for mitochondria from control animals (i.e., during calcium or ADP stimulation of control mitochondria and for basal, calcium-stimulated and ADP-stimulated septic mitochondria), administration of inhibitors of 14-kDa PLAr blocked the enhanced ROS generation. Moreover, we found that administration of arachidonic acid (the principal metabolic product of PLAr activation) also increased mitochondrial H$_2$O$_2$ generation and did so by interacting with the electron transport chain.

**Skeletal muscle free radical generation by control, nonseptic animals.** Both our group and others have shown that normal skeletal muscle generates small quantities of ROS under basal conditions but far greater levels of ROS during strenuous contraction (8, 19, 25). This past work also indicates that contraction-associated ROS generation contributes to the development of a long-lasting form of muscle fatigue. This phenomenon has been demonstrated in limb skeletal muscle but may play a far more important role in the respiratory muscles (1, 2, 31). Studies have suggested that a sudden increase in the workload placed on the respiratory muscles (as would occur during the acute development of lung disease) is associated with the development of ROS-induced respiratory muscle fatigue, with fatigue, in turn, contributing to the development of respiratory failure (31).

In recent work, we have made several observations that provide clues regarding the cellular mechanisms responsible for increased free radical formation in contracting muscle. Specifically, in studies of intact muscle, we found that it was possible to ablate contraction-related free radical formation, assessed by using a fluorescent-indicator technique, by administration of inhibitors of the 14-kDa isoform of PLAr (19). The present study extends these previous observations and provides a potential explanation for contraction-related free radical generation in skeletal muscle. Muscle contraction is associated with an increase in cytosolic calcium and ADP concentrations. In the present study, we found that addition of physiologically relevant concentrations of calcium and ADP (i.e., concentrations similar to those achieved during strenuous muscle contraction) to mitochondria isolated from skeletal muscle (diaphragm) elicited a substantial increase in mitochondrial H$_2$O$_2$ formation in the presence of a complex I substrate (malate and pyruvate). In addition, we found that addition of inhibitors of 14-kDa PLAr resulted in complete ablation of calcium- and ADP-stimulated H$_2$O$_2$ formation by mitochondrial isolates incubated with malate and pyruvate. Selectivity of the role of 14-kDa PLAr isoforms in mediating this effect was supported by the fact that administration of inhibitors of NADPH oxidase or other PLAr isoform families (i.e.,
of calcium-independent and 85-kDa PLA$_2$) failed to affect calcium- or ADP-stimulated H$_2$O$_2$ formation.

If, as suggested by this first group of results, calcium- and ADP-induced augmentation of free radical generation by mitochondria is mediated by PLA$_2$ activation, it should be possible to stimulate mitochondrial free radical generation by directly activating PLA$_2$. This possibility was borne out by our observation that melittin, a potent PLA$_2$ activator, markedly increased mitochondrial H$_2$O$_2$ formation.

Our experimental results also argue that the effects of PLA$_2$ stimulation are likely mediated by arachidonic acid, which, in turn, interacts with and augments electron transport chain production of ROS (7, 17). It is also important to note that calcium and ADP failed to stimulate H$_2$O$_2$ formation by mitochondria isolated from control animals when succinate was used as substrate. Succinate generates FADH$_2$, which acts as an electron donor to complex II (with subsequent electron flow to complexes III and IV) (4). This latter finding suggests that complex I was the source of the augmented ROS formation seen with calcium and ADP addition to mitochondria in the presence of malate and pyruvate. This conclusion is consistent with the observation that arachidonic acid-stimulated free radical formation was inhibited by complex I blockade and suggests that complex I is the likely source of ROS evoked by PLA$_2$ activation in mitochondria of normal skeletal muscles.

Generation of H$_2$O$_2$ by skeletal muscle mitochondria from septic muscles. A series of reports indicate that free radicals (i.e., ROS, nitric oxide) play a central role in producing skeletal muscle dysfunction in sepsis (13, 14, 27, 29, 33). This past work found that nitric oxide synthase inhibitors, superoxide scavengers, and H$_2$O$_2$ scavengers reduce sepsis-induced muscle dysfunction (13, 14, 27, 29, 33). The increased nitric oxide generation observed in skeletal muscle in sepsis has been attributed to an upregulation in the activity of both inducible and constitutive nitric oxide synthase (13, 14). The source of the increased superoxide generation by skeletal muscle in sepsis has not previously been identified.

The findings of the present study provide a potential explanation for the heightened free radical formation (i.e., superoxide and H$_2$O$_2$ generation) observed in skeletal muscle in sepsis (18). Specifically, we found that diaphragm mitochondrial free radical formation for septic animals was greater than that observed for mitochondria from control animals under several conditions. First, with malate and pyruvate as substrate, basal H$_2$O$_2$ formation by mitochondria from septic muscles was higher than basal H$_2$O$_2$ generation by control mitochondrial samples. Second, levels of H$_2$O$_2$ formation, after calcium and ADP administration with malate and pyruvate as substrate, were higher for mitochondria from septic animals compared with controls. Third, when succinate was used as substrate, administration of ADP failed to increase H$_2$O$_2$ generation by mitochondria from septic animals. Complex I appeared to be the source of the heightened basal H$_2$O$_2$ formation by septic mitochondria with malate and pyruvate as substrate because basal H$_2$O$_2$ by mitochondria from septic and control animals did not differ when succinate was used as substrate. On the other hand, the effect of ADP to increase H$_2$O$_2$ formation for mitochondria from septic animals with succinate as substrate must have been derived from another site in the electron transport chain (most likely, complex III) because FADH$_2$ generated by succinate enters the electron transport chain at complex II, bypassing complex I.

Most importantly, administration of 14-kDa PLA$_2$ inhibitor eliminated all three of these differences between mitochondria from control and septic animals, indicating that all three of these phenomena were PLA$_2$ modulated. We also found that PLA$_2$ activity was increased for mitochondria from septic compared with control animals, as assessed by using an assay that directly measures 14-kDa PLA$_2$ enzymatic capacity. These latter data suggest that sepsis results in an increase in free radical generation in diaphragm skeletal muscle by eliciting an increase in mitochondrial PLA$_2$ activity and that this increased PLA$_2$ activity is responsible for the increased ROS generation observed in skeletal muscle during sepsis.

The present work did not determine the mechanism responsible for the increase in skeletal muscle PLA$_2$ activity. Studies in other tissue types, however, indicate that cytokines (tumor necrosis factor-α in particular) evoke an increase in PLA$_2$ mRNA, PLA$_2$ protein levels, and PLA$_2$ activity (15, 34). The majority of this work has examined the stimulatory effects of cytokines on endothelial and white blood cells, although limited data would indicate cytokines influence PLA$_2$ activity in astrocytes and glomerular mesangial cells (15, 34). It seems reasonable to postulate that cytokines may also be responsible for producing the elevated diaphragm PLA$_2$ activity observed in the present study.

Relationship of the present findings to previous studies of mitochondrial formation of ROS. The present findings are consistent with numerous previous reports indicating that small quantities of ROS are continuously generated by the mitochondrial electron transport chain in normal tissues (6). This basal ROS formation may be important for maintaining normal cellular homeostasis (22). In several pathophysiological conditions (i.e., increased muscle temperature, ischemia-reperfusion), mitochondrial ROS generation can increase to levels that have deleterious effects on cell function (11, 23).

The present findings add to these observations and suggest that the following mechanistic sequence may account for contraction-related increases in muscle free radical formation. 1) Increased cytosolic calcium levels during contraction result in an increase in mitochondrial calcium concentrations and activation of mitochondrial PLA$_2$. 2) Activation of 14-kDa PLA$_2$ (the activity of this isof orm is calcium dependent) elicits an increase in mitochondrial arachidonic acid concentrations. 3) Arachidonic acid interacts with complex I of
the electron transport chain to increase superoxide and, subsequently, H$_2$O$_2$ generation. The present data also indicate that contraction-related increases in cytosolic ADP may stimulate PLA$_2$-dependent mitochondrial free radical generation and may be a second factor influencing free radical formation during contraction. In sepsis, skeletal muscle mitochondrial PLA$_2$ activity is increased, resulting in an amplification of PLA$_2$-dependent free radical generation, with heightened free radical generation under basal, calcium-stimulated, and ADP-stimulated conditions.

**Potential implications.** Respiratory muscle dysfunction is a contributor to the morbidity of critically ill patients with a variety of illnesses. The difficulties associated with “weaning” this group of patients from invasive and noninvasive ventilation are due not only to intrinsic lung disease but also to reductions in respiratory muscle force-generating capacity. Accumulating data indicate that ROS play an important role in mediating both the development of respiratory muscle dysfunction secondary to infection and dysfunction resulting from an increase in the workload of breathing (1, 8, 25, 27, 29, 31–33). As a result, development of therapies that inhibit ROS-induced muscle dysfunction may ultimately prove useful as a means of preventing or reversing respiratory failure in these patients.

The present findings suggest a new strategy to potentially prevent ROS-mediated muscle dysfunction with workload-induced increases in respiratory muscle contractile activity and during infection. Our findings suggest that it may be possible to prevent excessive ROS formation in skeletal muscle in these conditions by inhibiting 14-kDa mitochondrial PLA$_2$. Such an approach offers a theoretical advantage over conventional approaches to reducing ROS-mediated tissue dysfunction, which usually involve administration of agents that react with and scavenge ROS only after these species have had an opportunity to alter intracellular organelle structure and function. In contrast, inhibition of 14-kDa PLA$_2$ should theoretically prevent formation of these toxic substances before they have a chance to interact with and damage cellular constituents.

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