Calcium-independent phospholipase A$_2$ modulates cytosolic oxidant activity and contractile function in murine skeletal muscle cells

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Gong, Ming C., Sandrine Arbogast, Zhenheng Guo, Jeremy Mathenia, Wen Su, and Michael B. Reid. Calcium-independent phospholipase A$_2$ (PLA$_2$) modulates cytosolic oxidant activity and contractile function in murine skeletal muscle cells. J Appl Physiol 100: 399–405, 2006. First published September 15, 2005; doi:10.1152/japplphysiol.00873.2005.—Phospholipase A$_2$ (PLA$_2$) activity supports production of reactive oxygen species (ROS) by mammalian cells. In skeletal muscle, endogenous ROS modulate the force of muscle contraction. We tested the hypothesis that skeletal muscle cells constitutively express the calcium-independent PLA$_2$ (iPLA$_2$) isoform and that iPLA$_2$ modulates both cytosolic oxidant activity and contractile function. Experiments utilized differentiated C$_2$C$_{12}$ myotubes and a panel of striated muscles isolated from adult mice. Muscle preparations were processed for measurement of mRNA by real-time PCR, protein by immunoblot, cytosolic oxidant activity by the dichlorofluorescein oxidation assay, and contractile function by in vitro testing. We found that iPLA$_2$ was constitutively expressed by all muscles tested (myotubes, diaphragm, soleus, extensor digitorum longus, gastrocnemius, heart) and that mRNA and protein levels were generally similar among muscles. Selective iPLA$_2$ blockade by use of bromoenol lactone (10 $\mu$M) decreased cytosolic oxidant activity in myotubes and intact soleus muscle fibers. iPLA$_2$ blockade also inhibited contractile function of unfatigued soleus muscles, shifting the frequency-relationship rightward and decreasing force production during acute fatigue. Each of these changes could be reproduced by selective depletion of superoxide anions using superoxide dismutase (1 kU/ml). These findings suggest that constitutively expressed iPLA$_2$ modulates oxidant activity in skeletal muscle fibers by supporting ROS production, thereby influencing contractile properties and fatigue characteristics.

resting muscles, Zuo and colleagues (42) showed that basal rates of superoxide anion release into the extracellular space are also PLA$_2$ dependent.

Muscle-derived ROS have a physiological role, promoting contractile function and force production by unfatigued muscle (26). To the extent that PLA$_2$ supports endogenous ROS production, PLA$_2$ would also be expected to promote contractile function of skeletal muscle. This expectation is supported by data from vascular smooth muscle. Studies by Guo et al. (10) indicate that PLA$_2$ mediates the increased force of contraction stimulated by phenylephrine or serotonin. Specifically, the increase in force appears to be mediated by the iPLA$_2$ isoform. Guo and associates found that iPLA$_2$ is constitutively expressed by vascular smooth muscle and that agonist-induced increases in the force of contraction could be inhibited by selective iPLA$_2$ inhibition.

The biological importance of iPLA$_2$ in skeletal muscle cells is largely unstudied. Existing data imply that iPLA$_2$ might modulate oxidant production by muscle cells and thereby influence contractile function. The present study evaluated this postulate by testing three hypotheses. 1) iPLA$_2$ is ubiquitously expressed by murine skeletal muscles and muscle-derived cell lines. Differentiated C$_2$C$_{12}$ myotubes and a panel of striated muscles (limb, respiratory, cardiac) from adult mice were tested for iPLA$_2$ mRNA and protein levels. 2) iPLA$_2$ contributes to intracellular oxidant activity in resting muscle fibers. The 2′,7′-dichlorofluorescein (DCFH) oxidation assay was used to measure oxidant activity in the cytosol of differentiated muscle cells with and without iPLA$_2$ inhibition. 3) iPLA$_2$ promotes force production by skeletal muscle. Contractile properties and fatigue characteristics of mouse limb muscles were measured in vitro with and without iPLA$_2$ blockade.

MATERIALS AND METHODS

Reagents. 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) was dissolved in 100% ethyl alcohol, diluted in Krebs-Ringer solution, and stored at −80°C for later use. Bromoenol lactone (BEL; Biomol International, Plymouth Meeting, PA) was dissolved in 100% dimethyl sulfoxide (DMSO). Cu-Zn superoxide dismutase (SOD; Oxis International, Portland, OR) was dissolved in Krebs-Ringer solution. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Myogenic cell cultures. Myotubes were cultured from the murine skeletal muscle-derived C$_2$C$_{12}$ myoblast line (American Type Culture Collection, Rockville, MD) as described previously (16). Briefly, C$_2$C$_{12}$ cells were cultured in DMEM supplemented with 10% fetal calf serum and gentamycin at 37°C in the presence of 5% CO$_2$. Myoblast differentiation was initiated by replacing the growth me-

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Animals and were approved by the Institutional Review Board of the University of Kentucky. Adult male HSD-ICR mice (Harlan Sprague Dawley, Indianapolis, IN) were deeply anesthetized and killed by exsanguination after which the diaphragm, soleus, extensor digitorum longus (EDL), gastrocnemius, and heart muscles were surgically isolated for analysis.

Real-time PCR determination of iPLA₂ mRNA. Differentiated myotubes and individual muscles were placed in RNAlater solution immediately after isolation. As described previously (10), RNA was extracted using Tri Reagent (Molecular Research Center, Cincinnati, OH). The cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase using random hexamers (Invitrogen, Carlsbad, CA). The following real-time PCR primers were used: 5′-AGATGGTCTTCTGCCCAAGCAA-3′ (forward), 5′-ACTGTGGCAAGATCCGAGAACG-3′ (reverse), which included sequence from two adjacent exons from mouse iPLA₂; and 5′-AGAAAGGCTTACACCATCCAA-3′ (forward), 5′-GGGTCGGGAAGTGTTATAATT-3′ (reverse) for mouse 18S rRNA. The real-time PCR reactions were performed using a QuantiTect SYBRgreen PCR kit (Qiagen, Valencia, CA) in an ABI Prism 7000 Sequence Detection System. Specificity of the PCR was verified by dissociation-curve analysis and agarose gel electrophoresis. Each sample was analyzed in triplicate in every experiment. iPLA₂ mRNA was normalized to the 18S rRNA signal; relative quantification was by standard curve analysis. Template controls were not included in each real-time PCR run.

Immunoblot of iPLA₂. Isolated muscle preparations were immediately frozen in liquid nitrogen for processing by established methods (10). In brief, after pulverization and lysis, protein concentrations were determined by bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Fisher). Nonspecific binding sites were blocked on the PVDF membrane were blocked by 4% nonfat milk in PBS buffer (PBS plus 0.1% Tween 20). iPLA₂ was detected by using anti-iPLA₂ antibodies from Cayman (Ann Arbor, MI; 1:7,500 dilution). The immunoreactive bands were blotted using horseradish peroxidase-conjugated goat anti-rabbit antibodies (Jackson Immunoresearch Laboratories) and detected by enhanced chemiluminescence. iPLA₂ protein levels were quantified by Kodak 1D image analysis software (Eastman Kodak, Rochester, NY).

iPLA₂ assay. iPLA₂ activity was measured using the method of Yang et al. (40) as adapted previously (10). In brief, the reaction was conducted in Ca²⁺-free medium containing 4 mM EDTA, 1 mM ATP, and 2 mM diithiothreitol. The membrane fraction was isolated from muscle homogenates by 100,000×g centrifugation at 4°C; ~100 μg membrane proteins were incubated with a mixture of unlabelled and ³⁵Cl-labeled 1,2-dipalmitoyl-sn-3-glycero phospholyl choline for 90 min at 40°C. Free fatty acids were extracted using modified Dole reagents; radioabeled free fatty acids were quantified by liquid scintillation counting. Specific iPLA₂ activity was expressed as picomoles of free fatty acid released per milligram of protein in 1 min (pmol min⁻¹ mg⁻¹).

Intracellular oxidant activity. As described previously (2), oxidant activity was measured by use of a diffusable fluorochrome probe, DCFH-DA. The acetate group is cleaved by cytosolic esterases to yield DCFH, a polar nonfluorescent molecule retained by the cell. DCFH is converted by cytosolic oxidants, including ROS (15), to its fluorescent derivative, 2′,7′-dichlorofluorescein (DCF; 480-nm excitation, 520-nm emissions). The rate of DCF accumulation is proportional to net cytosolic oxidant activity. Fiber bundles were loaded with DCFH by in vitro incubation with DCFH-DA (50 μM) for 45–60 min, time periods that enable probe equilibration. Accumulation of oxidized DCF was measured from representative areas of the fiber bundle surface (0.27 mm²) by use of an epifluorescence microscope (Labophot-2, Nikon Instruments, Tokyo, Japan) and charge-coupled device camera (Series 72, Dage-MTI, Michigan City, IN). A mechanical shutter in the excitation light pathway was controlled by computer using commercial data acquisition and analysis software (Optimas 4.02; Bioscan, Edmonds, WA) to standardize excitation time (33 ms). Images were acquired in real time and stored in a desktop computer for later analysis of mean emission intensity. Photooxidation artifact was controlled by conducting experiments in a darkened laboratory and by standardizing excitation parameters to minimize the cumulative delivered energy; residual error was corrected according to Arbogast and Reid (2). Data were collected in paired comparisons of treated vs. untreated soleri from individual animals. Muscles were mounted in separate chambers containing Krebs-Ringer solution aerated with 95% O₂ and 5% CO₂ at 37°C. Each was pretreated with 10 μM BEL, 180 nM DMSO, 1 kU/ml SOD, or buffer (control) for 20 min and loaded with DCFH. After 60-min passive incubation, fluorescence emissions were measured to assess oxidant activity. Separate experiments using a muscle-free system tested for chemical interactions between DCFH and each drug; none altered fluorescence.

Cytochrome c reduction assay. Superoxide anion activity was measured using an adaptation of the spectrophotometric technique described by McCord and Fridovich (20). In brief, cytochrome c (Sigma-Aldrich) was added to oxygenated, buffered Krebs-Ringer solution to achieve 10 μM final concentration. A spectrophotometer (Biomate 3, Thermo Electron, Rochester, NY) was used to measure absorbance at 540 nm (A₅₄₀), 550 nm (A₅₅₀), and 560 nm (A₅₆₀). The cytochrome c solution was supplemented with a superoxide-generating system (1.25 μM hypoxanthine, 20 μM xanthine oxidase) plus 10 μM BEL, 180 nM DMSO (diluent control), 1 kU/ml SOD, or nothing (buffer control). After 30 min, absorbance was remeasured at each wavelength. A₅₅₀ Values were normalized (A₅₅₀ normalized = A₅₅₀/0.5(A₅₄₀ + A₅₆₀)) and used to compute net cytochrome c reduction (in pmol) from a standard curve. In follow-up studies using preruced cytochrome c, we tested the capacity of 10 μM BEL to inhibit changes in normalised A₅₅₀ caused by addition of 250 μM hydrogen peroxide (Sigma-Aldrich) or 1 mM NOC-7 (nitric oxide donor; Calbiochem, La Jolla, CA).
measured using an electronic caliper, and the muscle was removed, blotted dry, and weighed. Cross-sectional area was calculated according to Close (4).

Data analyses. Statistical analyses were performed using commercial software (Sigma Stat, SPSS, Chicago, IL). Averaged data are reported as means ± SE. Overall differences in a single end point among two or more groups were tested by one-way ANOVA and post hoc comparison by Tukey’s test (13). For recurrent measurements, a two-way, repeated-measures ANOVA was used (13). Paired comparisons between two conditions were evaluated by Student’s paired t-test (41). Differences were considered significant at P < 0.05.

RESULTS

iPLA2 expression patterns. iPLA2 gene expression was a robust finding among widely divergent murine muscle preparations. iPLA2 mRNA levels were clearly detectable by real-time PCR in each of five striated muscles (Fig. 1). Constitutive iPLA2 mRNA levels are comparable among diaphragm, gastronemius, and EDL; relative to diaphragm, levels are lower in soleus (P < 0.05) and heart (P < 0.02). For comparison, we also screened differentiated myotubes from the stable, mouse muscle-derived C2C12 cell line and confirmed measurable levels of iPLA2 mRNA (mean iPLA2/18S RNA: 3.7 ± 1.2 (SE)). The iPLA2 mRNA content in myotubes was higher overall than in excised muscles (P < 0.05).

Immunoblot analysis identified a single major iPLA2 protein band at the expected molecular mass of ∼85 kDa in each of the excised muscles tested (Fig. 2A) and in C2C12 myotubes (data not shown). Averaged densitometry data (Fig. 2B) illustrate the similarity of iPLA2 protein contents across individual mouse muscles. We detected no differences among skeletal muscles, but iPLA2 protein content was lower in heart than gastrocnemius (P < 0.02).

iPLA2 and intracellular oxidant activity. Cytosolic oxidant activity was measured in intact muscle cells by use of the DCFH oxidation assay. Low levels of basal activity were detectable in soleus muscle fibers and in C2C12 myotubes (data not shown). The iPLA2 contribution to this signal was tested by use of BEL, a selective iPLA2 inhibitor (11).

Enzymatic iPLA2 activity in skeletal muscle is decreased by 85% by treatment with 10 μM BEL [34.1 ± 2.2 (SE) pmol·min⁻¹·mg⁻¹ basal vs. 5.0 ± 0.5 pmol·min⁻¹·mg⁻¹ treated; n = 3/group; P < 0.01]. As shown in Fig. 3A, 10 μM BEL also decreases oxidant activity in intact muscle fibers [77 ± 3 (SE) %DMSO control; P < 0.05] and myotubes [74 ± 8 (SE) %DMSO control; P < 0.05]. Similar decrements in oxidant activity were produced by selective ROS depletion. As shown in Fig. 3B, exposure to SOD (degrades superoxide anions) decreased oxidant activity in soleus fibers (P < 0.05) and in myotubes (P < 0.05).

The similarity of BEL and SOD actions does not reflect direct scavenging of superoxide anions by BEL. We tested the capacity of each compound to degrade superoxide anions generated by a superoxide-generating system in the absence of muscle cells. As shown in Fig. 4, the superoxide signal was blocked by SOD. The signal was unaffected by BEL or its diluent, DMSO, at the concentrations used. Follow-up studies using a similar protocol determined that BEL does not inhibit changes in cytochrome c redox state caused by exposure to hydrogen peroxide (P < 0.36) or a nitric oxide donor (P < 0.72). In combination, these data suggest that BEL inhibits oxidant activity via iPLA2 blockade and not by direct antioxidant actions.

iPLA2 blockade and muscle function. iPLA2 effects on contractile function were tested using excised soleus muscles pretreated with 10 μM BEL. Specific force was depressed by 15–23% across a range of submaximal stimulus frequencies (30–80 Hz; P < 0.05). There were no changes in maximal tetanic force (32.8 ± 2.0 N/cm² treated vs. 34.6 ± 1.8 N/cm² control), twitch force (4.0 ± 0.4 vs. 4.5 ± 0.4 N/cm²), time to peak twitch force (24 ± 1 vs. 23 ± 2 ms), or twitch half relaxation time (30 ± 2 vs. 33 ± 1 ms). Normalized for variations in maximal force, the relative force-frequency curve (Fig. 5A) demonstrates the contractile depression caused by iPLA2 inhibition. As with oxidant activity, SOD pretreatment mimicked the effect of iPLA2 inhibition, shifting the relative force-frequency curve rightward (Fig. 5B).

Acute fatigue characteristics were also affected by iPLA2 inhibition. During repetitive, submaximal tetanic (40 Hz) stimulation, developed force was systematically depressed in BEL-treated muscles (Fig. 6A), a difference that was evident at time 0 and throughout most of the 300-s protocol. SOD-treated muscles exhibited a similar response during acute fatigue (Fig.

![Fig. 1.](http://jap.physiology.org/) Calcium-independent phospholipase A2 (iPLA2) mRNA levels in murine striated muscle. Data depict mean iPLA2/18S values (±SE) in diaphragm (Diaph), heart, gastrocnemius (Gastroc), soleus, and extensor digitorum longus (EDL) as measured using real-time PCR; n = 3–6/group. *P < 0.05 vs. diaphragm.

![Fig. 2.](http://jap.physiology.org/) iPLA2 protein in muscle. A: representative autoradiograph depicts 85-kDa bands detected in striated muscles. B: mean protein levels in individual muscles (±SE) as measured by densitometry. *P < 0.05 vs. gastrocnemius.
As muscles fatigue, relaxation between tetanic contractions becomes incomplete such that residual force persists in the absence of electrical stimulation (37). In the present protocol, residual force increased monotonically as control muscles fatigued; the rise in residual force was unaffected by either BEL or SOD pretreatment (data not shown).

**DISCUSSION**

iPLA2 expression patterns among skeletal muscles have not been examined at either the message or protein levels. Nor has a functional role for iPLA2 been identified in this cell type. These are the first data to indicate that iPLA2 modulates oxidant activity and contractile properties of muscle. Our findings help bridge gaps among prior reports on this subject, broadening our understanding of phospholipase function in skeletal muscle.

**iPLA2 expression in striated muscle.** Fukusawa and Serrero (9) originally reported that iPLA2 activity was less in skeletal muscle and heart than in nine other rat tissues; in particular, it was two to three orders of magnitude lower than intestine. Tang et al. (35) measured iPLA2 mRNA in a mouse-rat multiple-tissue Northern blot. They detected a 3 kbp iPLA2 transcript that was abundant in muscle and barely detectable in heart. Forsell and associates (8) detected four iPLA2 transcripts of 1.8, 2.0, 3.2, and 4.2 kbp in a human multiple-tissue Northern blot. All four transcripts were detectable in skeletal muscle and in heart. More recently, Mancuso et al. (18) and Tanaka et al. (34) have also reported that skeletal muscle and heart express a membrane-associated iPLA2 with an mRNA of ∼3.4 kb. Note that iPLA2 expression by muscle was not the focus of these prior studies. Separate skeletal muscles were not evaluated individually, and protein levels were not measured.

To define iPLA2 distribution among muscles, we measured iPLA2 mRNA and protein levels in a panel of striated muscle preparations selected for adaptational divergence. Murine skeletal muscles included an endurance-adapted respiratory muscle (diaphragm), an endurance-adapted limb muscle (soleus), a sprint-adapted limb muscle (EDL), and a limb muscle of intermediate contractile and metabolic properties (gastrocnemius). Highly aerobic cardiac muscle was also included for comparison, as were differentiated myotubes from the immortalized C2C12 cell line derived from mouse skeletal muscle. iPLA2 mRNA and protein levels were detectable in all of these preparations and were of similar magnitude among the various excised muscles. This finding suggests that constitutive iPLA2 expression is a robust property of striated muscle, at least in the mouse, and that expression patterns are not strongly adaptation dependent.

**iPLA2 and oxidant activity.** Several prior reports indicate that PLA2 activity supports ROS production by skeletal muscle. Nethery and coworkers (24) studied intracellular ROS levels in isolated, perfused rat hemidiaphragms. They showed that ROS levels increase during repetitive, fatiguing contrac-
ions and that this increase is inhibited by PLA2 blockade. In a follow-up study (23), they later identified muscle mitochondria as the likely site of PLA2-dependent ROS production within muscle fibers. Extracellular ROS release by muscle fibers also appears to be modulated by PLA2. Zuo et al. (42) identified 5-lipoxygenase as the primary source of superoxide anions released from rat diaphragm fiber bundles, both under basal conditions and during heat stress. Their study suggested that PLA2 supports superoxide anion production by generating arachidonic acid for 5-lipoxygenase metabolism. Thus PLA2 is implicated as an upstream modulator of ROS production via two cellular sources, mitochondria and 5-lipoxygenase, that affect the activity of muscle-derived ROS in both intracellular and extracellular compartments.

PLA2 effects on muscle-derived ROS appear to be state and isoform dependent. Our data identify the calcium-independent iPLA2 isoform as a major determinant of ROS activity under resting conditions. This is consistent with the facts that cytosolic calcium concentration is low in resting muscle fibers (36). ROS production is increased above resting levels by conditions that elevate cytoplasmic calcium, e.g., repetitive muscle contraction (7, 19, 24, 27), heat stress (42), or direct mitochondrial exposure to calcium (23). Elevated calcium levels activate the 14-kDa calcium-dependent PLA2 isoform (cPLA2), which stimulates ROS production at supranormal rates (24, 42). Integrating these data, the calcium-independent iPLA2 isoform appears to play a housekeeping role, supporting low rates of ROS production under resting conditions, whereas the cPLA2 isoform provides a calcium-sensitive mechanism of stimulating additional ROS production when cytosolic calcium levels rise.

Our present data may underestimate iPLA2 contributions to oxidant activity. It is possible that iPLA2 inhibition by BEL was incomplete. This error, if present, is likely to be small, because the concentration we used (10 μM) exceeds the EC50 by more than threefold (11). Another potential source of underestimation is the DMSO used in diluent control studies. DMSO has antioxidant effects (12). To the extent DMSO buffered muscle-derived oxidants, BEL effects would be obscured. This would lessen the apparent contribution of iPLA2 to oxidant activity. One final limitation of our present study,
and PLA2 studies in general, is the widespread reliance on pharmacological probes to assess isoform specificity. BEL is a potent, irreversible inhibitor of iPLA2 with specificity vs. calcium-dependent PLA2 isoforms of >1,000-fold (11) and is the standard intervention for contemporary studies of iPLA2 signaling in muscle (10, 23, 24, 42) and nonmuscle cell types (3, 17, 22, 25, 32, 33). This said, stronger conclusions about the role of iPLA2 in muscle will require genetic interventions to complement existing drug studies.

iPLA2 and contractile function. iPLA2 blockade caused a drop in the forces developed by unfatigued muscle at physiological activation frequencies. This is consistent with the decrease in cytosolic oxidant activity that we observed after iPLA2 inhibition. Prior work by our laboratory (1, 14, 28, 29) and by others (6, 21, 30, 39) has shown that antioxidants decrease the force of contraction in unfatigued muscle. This phenomenon is illustrated in our present experiments by the actions of SOD, an antioxidant enzyme that selectively depletes muscle-derived superoxide anions. As with iPLA2 blockade, SOD exposure caused both cytosolic oxidant activity and force production to fall. The most straightforward interpretation of these data is that constitutive iPLA2 activity contributes to redox homeostasis in resting muscle, supporting ROS production and redox-sensitive contractile function.

Our data contribute to the evidence that ROS effects on muscle function are temperature dependent. Muscle-derived ROS activity and ROS effects on muscle function are strongly affected by temperature (2, 5, 43). To optimize resolution of these processes, the present studies were conducted at 37°C. In contrast, Nethery and associates (24) showed that intramuscular ROS levels are very low at 24°C and that iPLA2 inhibition using 25 μM BEL has no discernable effect on contractile function. Differences between our findings and those of Nethery et al. are consistent with reports that ROS activity is diminished by cooling (2, 43) and that ROS effects are less evident at subphysiological temperatures (24).

Conclusions. These findings identify iPLA2 as a constitutively expressed modulator of cytosolic oxidant activity and contractile function in skeletal muscle cells. They contribute to growing evidence that the PLA2 superfamily is a major regulator of ROS production by muscle. Pharmacological studies indicate that PLA2 effects are isoform dependent, such that iPLA2 regulates oxidant activity under basal conditions and cPLA2 boosts ROS production during metabolic stress, i.e., fatiguing exercise or hyperthermia. This model has important implications for redox-dependent signaling in muscle and highlights the importance of future experiments using genetic interventions to define the roles of iPLA2 and cPLA2.

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