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SKELETAL MUSCLE GROUP VIA PHOSPHOLIPASE A₂ (iPLA₂β): EXPRESSION AND ROLE IN FATTY ACID OXIDATION†

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Abstract

Among the phospholipases A₂ (PLA₂s) are the Group VI Ca²⁺-independent PLA₂s (iPLA₂s) and expression of multiple transcripts of iPLA₂ in skeletal muscle has been reported. In the present study, phospholipase activity and sequential ATP and calmodulin affinity column chromatography analyses reveal that skeletal muscle iPLA₂ exhibits properties characteristic of the iPLA₂β isoform. The phospholipase activity of iPLA₂β has been demonstrated to participate in signal transduction, cell proliferation, and apoptosis. We also report here that skeletal muscle from iPLA₂β-null mice, relative to wild type muscle, exhibits a reduced capacity to oxidize palmitate but not palmitoyl-CoA or acetyl-CoA in the absence of changes in fatty acid transporters CD36 and CPT1 or β-hydroxyacyl-CoA dehydrogenase activity. Recently, purified iPLA₂β was demonstrated to manifest a thioesterase activity which catalyzes hydrolysis of fatty acyl-CoAs. The liberated CoA-SH facilitates fatty acid transport into the mitochondria. In this regard, we find that fractions eluted from the ATP column and containing iPLA₂β phospholipase activity also contained acyl-CoA thioesterase activity that was inhibited by the bromoenol lactone (BEL) suicide inhibitor of iPLA₂β. We further find that acyl-CoA thioesterase activity in skeletal muscle preparations from iPLA₂β-null mice is significantly reduced, relative to WT activity. These findings suggest that the absence of acyl-CoA thioesterase activity of iPLA₂β can lead to reduced fatty acyl-CoA generation and impair fatty acid oxidation in iPLA₂β-null mice. Our findings therefore reveal a novel function of iPLA₂β, related not to its phospholipase activity but to its thioesterase activity, which contributes to optimal fatty acid oxidation in skeletal muscle.

Keywords

Affinity chromatography; iPLA₂β-null; fatty acid oxidation; acyl-CoA thioesterase activity; skeletal muscle

Phospholipases A₂ (PLA₂) are a diverse group of enzymes that catalyze hydrolysis of the *sn*-2 substituent from glycerophospholipid substrates to yield a free fatty acid and a 2-lyso-phospholipid (1). At present, the recognized PLA₂s are classified into 15 different groups that include the low MW secretory PLA₂s and the higher MW Ca²⁺-dependent and Ca²⁺-independent PLA₂s (2). Among the PLA₂s is an 84 kDa cytosolic PLA₂ that does not require Ca²⁺ for catalysis, is activated by ATP and inhibited by BEL, and is classified as Group VIA PLA₂, designated iPLA₂β (3–5). Inhibition of iPLA₂β has been reported to suppress incorporation of arachidonic acid into membrane phospholipids of macrophage-like P388D1 cells (6,7) leading to the suggestion that iPLA₂β participates in phospholipid remodeling (8,

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9). Findings in other cells, however, suggest that iPLA₂β is involved in signal transduction (10–16).

The group VIA Ca²⁺-independent phospholipase A₂ (iPLA₂β) has been cloned from several sources and is encoded by mRNA species that yield proteins (expected molecular masses of 80–88 kDa) that contain a phospholipase motif preceded by eight ankyrin repeats in the N-terminal half of the molecule (17–19). An 88 kDa iPLA₂β isoform is a product of a mRNA species that arises from an exon-skipping mechanism of alternate splicing and contains a 54 amino acid insert that interrupts the eighth ankyrin repeat (20,21). Proteolytic processing of iPLA₂β also yields truncated protein products that are constitutively active. These products arise from caspase-3-catalyzed cleavage of iPLA₂β in human promonocytic U937 cells (22) and β-cells (23), and calmodulin-dependent cleavage of iPLA₂β under *in vitro* conditions (24). Further, pancreatic islets predominantly express a catalytically active 70 kDa iPLA₂β variant that is not a product of alternate splicing (25).

Multiple reports suggest that muscle tissue expresses more than one iPLA₂ transcript. In one of the first such survey studies, Tang et al. (19) observed an iPLA₂ transcript at 3 kb in Northern analyses of a mouse and rat multiple tissue blot. Subsequently, human skeletal muscle was shown to express four iPLA₂ transcripts of 1.8, 2.0, 3.2, and 4.2 kb (20). The membrane-associated iPLA₂γ in heart and skeletal muscle are thought to be products of an iPLA₂ transcript of 3.4 kb (5,26). Recently, we observed expression of two iPLA₂ transcripts of 3.0 and 3.2 kb in several mouse tissues including skeletal muscle and pancreatic islets (27). We have previously demonstrated that iPLA₂β is expressed in the β-cell but not in the non-β-cell population of islets (28). To determine if the iPLA₂ expressed in skeletal muscle was analogous to that expressed in β-cells we examined the chromatographic profile and catalytic properties of skeletal muscle iPLA₂. Here, we report that skeletal muscle expresses the iPLA₂β isoform that manifests phospholipase and acyl-CoA thioesterase activity and participates in fatty acid oxidation.

Experimental Procedures

Materials

Materials used in these studies were obtained from the following (sources): [¹⁴C]-Palmitoyl-CoA (50 mCi/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO); (16:0/[¹⁴C]-18:2)-GPC (PLPC, 55 mCi/mmol), rainbow molecular mass standards, and enhanced chemiluminescence (ECL) reagent (Amersham, Arlington Heights, IL); SYBR Green PCR Kit (Applied Biosystems, Foster City, CA); Coomassie reagent, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) supplies, and Triton X-100 (BioRad, Hercules, CA); iPLA₂ antibody (Cayman, Ann Arbor, MI); normal goat serum, Cy3-conjugated affinipure goat anti-rabbit IgG (H+L) (Jackson Immuno Research Laboratories, West Grove, PA); pentex fraction V fatty acid-free bovine serum albumin (Miles Laboratories, Eckert, IN); RIPA buffer (Pierce, Rockford, IL); peroxidase-conjugated goat anti-rabbit IgG, T-14 iPLA₂, CPT1 and CD36 antibodies (Santa Cruz Biotech. Inc., Santa Cruz, CA); protease inhibitor cocktail, common reagents, and salts (Sigma Chemical Co., St. Louis, MO); and Immobolin-P PVDF membrane and Catch and Release V 2.0 immunoprecipitation kit (Upstate, Charlottesville, VA).

Animals and INS-1 cells

Male Sprague-Dawley rats (250 g) were obtained from Harlan Breeders (Indianapolis, IN). INS-1 β-cells were generously provided by Dr. C. Newgard (Duke University Medical Center, Durham, NC). A retroviral system was used to stably transfect INS-1 cells with a vector construct containing iPLA₂β cDNA (OE INS-1 cells), as described (4) to generate iPLA₂β

overexpressing (OE) INS-1 cells. Wild type (WT) and iPLA₂β-null mice were generously provided by Dr. John Turk (Washington University School of Medicine, St. Louis, MO).

Northern analyses of skeletal muscle iPLA₂

Total RNA from skeletal muscle was isolated after solubilization in guanidinium thiocyanate by phenol/chloroform/isoamyl alcohol extraction and isopropyl alcohol precipitation and Northern analyses were performed using the Northern Max kit (29,30). Tissue total RNA analyzed by electrophoresis was transferred to nylon membranes that were hybridized with iPLA₂ cDNA probes labeled by random priming. The iPLA₂ cDNA probe was amplified using reverse transcription-PCR (sense primer, 5'-TGTGACGTGGACAGCACTAGC; antisense primer, 5'-CCCCAGAGAAACGACTATGGA), which hybridizes to both short and long isoforms of iPLA₂ (31). This region of cDNA represents the sequence that encodes amino acid residues 307–552 of the short isoform of iPLA₂. A final stringency wash was followed by autoradiography, and the filters were then stripped and hybridized with cDNA probes to rat glyceraldehyde-3-phosphate dehydrogenase to mark RNA load (17).

Immunoblotting analyses

iPLA₂ was immunoprecipitated from skeletal muscle using the Catch and Release V 2.0 kit, according to manufacturer's instructions (Upstate, Charlottesville, VA). Briefly, skeletal muscle was homogenized in RIPA buffer (100 mg tissue/ml buffer) containing protease inhibitor cocktail (50 µl/ml) and iPLA₂ was immunoprecipitated (O/N, 4 °C) using primary antibody (from Cayman) directed against iPLA₂ (12 µg/ml lysates). Bound fraction was subsequently recovered, boiled in sample buffer, analyzed by SDS-PAGE (7.5%), and transferred onto Immobolin-P PVDF membranes. T-14 iPLA₂ primary antibody (0.0015 µg/µl) and peroxidase-conjugated goat anti-rabbit IgG (1:40,000) secondary antibody were then used for iPLA₂ immunoblotting analyses. Skeletal muscle homogenates prepared for substrate oxidation studies (described below) were used for immunoblotting analyses of CPT1 and CD36. Protein was analyzed by SDS-PAGE (8%), transferred onto PVDF membranes, and probed for CPT1 and CD36 (primary antibodies 1:200). Peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody (CPT1 1:10,000; CD36 1:5,000). GAPDH was used as loading control. Immunoreactive protein bands were visualized by ECL.

Elution of iPLA₂ by sequential chromatography

Rat skeletal muscle (12g) was sonicated (Sonics & Materials Vibra Cell, 12%, 10 sec) in homogenization buffer (10 mM HEPES, 1 mM EDTA, 340 mM sucrose, 1 mM DTT, pH 7.5) and the pooled sonicate was centrifuged (1000 × g, 10 min, 4 °C). Protein in the supernatant was precipitated overnight with the addition of ammonium sulfate (50%). The mixture was then centrifuged (20,000 × g, 30 min), and the protein pellet was resuspended in buffer A (10 mM HEPES, 1 mM EDTA, 340 mM sucrose, 1 mM DTT, 1 mM triton X-100, pH 7.5) and loaded onto an ATP-agarose affinity column (1.5 ml). Buffer A containing ATP (1 mM) was then added to the column to elute iPLA₂ and Ca²⁺-independent phospholipase activity and protein in each fraction were determined. Fractions containing activity were then combined and the concentration of calcium in the pooled sample was increased to 5 mM prior to loading onto calmodulin-Sepharose column. The void was collected and re-loaded and the column was washed with buffer A containing 0.5 mM Ca²⁺. iPLA₂ was eluted with 8 mM EGTA-containing buffer A and each fraction was assayed for Ca²⁺-independent phospholipase activity and protein content.

Skeletal muscle iPLA₂β phospholipase activity

Gastrocnemius muscle was excised from rats and 1 ml of homogenization buffer (250 mM sucrose, 40 mM Tris-HCl, 1 mM EGTA, pH 7.1 at 4 °C) containing protease inhibitor cocktail

(1:100) was added to 100 mg tissue. The muscle was homogenized by sonication (2×15 sec, 12% pulse) and the cytosol obtained, as described (23,28). Cytosolic protein concentration was determined using Coomassie reagent. Ca^{2+} -independent PLA_2 enzymatic activity was then determined in 30 μg protein aliquot of cytosol in the absence and presence of ATP (10 mM) or BEL (10 μM) by ethanolic injection (5 μl) of the substrate 1-palmitoyl-2-[^{14}C]-linoleoyl-*sn*-glycero-3-phosphocholine (5 μM) in assay buffer (40 mM Tris, pH 7.5, 5 mM EGTA) and quantitated, as described (23,25). To test the effects of BEL on activity, the sample protein was first pre-incubated (2 min, RT) with BEL before addition of substrate.

Skeletal muscle homogenate preparation for substrate oxidation

Homogenization procedure was performed as previously described (32). Briefly, ~75 mg of gastrocnemius muscle was thoroughly minced with scissors in 200 μl of homogenization buffer containing: 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, and 2 mM ATP, pH 7.4. The homogenization buffer was then brought up to yield a 20 fold (w/v) dilution. This was then transferred to a 3 ml Teflon on glass homogenization vessel. The muscle was then homogenized twice on ice at 12 passes over a 30 s time period at ~1,500 rpm. Homogenates were kept on ice for no more than 1h until oxidation experiments could be performed.

Substrate oxidation

$^{14}\text{CO}_2$ production and release from [$1\text{-}^{14}\text{C}$]-glucose, [$1\text{-}^{14}\text{C}$]-palmitate, [$1\text{-}^{14}\text{C}$]-acetyl CoA, and [$1\text{-}^{14}\text{C}$]-palmitoyl-CoA were used to assess oxidation as described (33), with some variation. Briefly, palmitate and palmitoyl-CoA (100 μM) were bound to 0.5% BSA (final concentration) and acetyl-CoA and glucose were added directly to the reaction buffer. Each substrate was brought up in reaction buffer containing: 100 mM sucrose, 10 mM Tris-HCl, 10 mM KPO_4 , 100 mM KCl, 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mM *L*-carnitine, 0.1 mM malate, 2 mM ATP, 0.05 mM coenzyme A, and 1 mM dithiothreitol, pH 7.4. Aliquots (80 μl) of the diluted muscle homogenates were plated in triplicate in a 100 well trapping device. Reaction buffer (325 μl) was placed in the bottom of each well. In addition, 1N NaOH (400 μl) was placed in a 500 μl microcentrifuge tube without the cap, which was also placed in each well to allow CO_2 trapping. The trapping device was then sealed with parafilm, then a siliconized rubber gasket, and finally the lid of the trapping device and allowed to incubate in a shaking incubator at 37 °C for 1h. Reactions were terminated by the addition of 100 μl of 70% perchloric acid via injection ports in the trapping device lid. The trapping device was placed back into the shaking incubator and released $^{14}\text{CO}_2$ was trapped for 1h at room temperature. CO_2 released during substrate oxidation and trapped in NaOH was quantitated by liquid scintillation spectrometry and expressed as CO_2 produced/mg protein.

β -HAD activity

Total β -hydroxyacyl-CoA dehydrogenase activity was measured as previously described (34) with some variation. Briefly, muscle homogenate prepared as described above was freeze-fractured 3 times in liquid nitrogen and allowed to thaw. The reactions were started by the addition of 100 μM acetoacetyl-CoA and the absorbance was measured at 340 nm over a 5 min period.

Acyl-CoA thioesterase activity assay

To identify $\text{iPLA}_2\beta$ -associated thioesterase activity, hydrolysis of [$1\text{-}^{14}\text{C}$]-palmitoyl-CoA by $\text{iPLA}_2\beta$ eluted from the ATP affinity column or in skeletal muscle cytosol prepared from WT and $\text{iPLA}_2\beta$ -null mice was determined. Fractions eluted from the ATP affinity column containing phospholipase activity were pooled and an aliquot (containing 1 μg protein) was pre-incubated without or with BEL (10 μM , 2 min, room temperature) and acyl-CoA thioesterase activity was assayed in the presence of 0.5–5 μM substrate. Activity in skeletal

muscle cytosol (containing 30 μg protein) was assayed in the presence of 0.5–10 μM substrate. The reaction (in a total volume of 200 μl) was started by ethanolic injection of the substrate [$1\text{-}^{14}\text{C}$]-palmitoyl-CoA in assay buffer (40 mM Tris, pH 7.5, 5 mM EGTA). Following an incubation period of 2 min, the reaction was stopped by addition of butanol (100 μl). The mixture was then centrifuged and hydrolysed [^{14}C]-fatty acid contained in an aliquot of the butanol layer was separated by TLC and quantitated by liquid scintillation spectrometry.

Statistical analyses

Data were converted to mean \pm SEM values and the Students' t-test was applied to determine significant differences between two samples ($p < 0.05$). Statistical difference ($p < 0.05$) between WT and iPLA $_2\beta$ -null thioesterase activity was determined by determining area under curve (AUC).

RESULTS

Skeletal muscle expresses iPLA $_2$ mRNA and protein

Skeletal expression of iPLA $_2$ was identified by Northern, immunoblotting, and enzymatic activity analyses. As shown in Figure 1, skeletal muscle expressed a transcript of $\sim 3\text{kb}$, an iPLA $_2$ -immunoreactive band with an apparent molecular mass of 85 kDa, and Ca^{2+} -independent phospholipase activity that was stimulated by ATP and inhibited by BEL. These properties are characteristic of iPLA $_2\beta$ expressed in insulinoma cells, pancreatic islets, and heart (28,35–38).

Elution of rat skeletal muscle iPLA $_2$ by sequential ATP and calmodulin affinity chromatography

To further establish the identity of skeletal muscle iPLA $_2$ isoform, sequential ATP and calmodulin affinity column chromatography, a scheme used previously in the purification of iPLA $_2\beta$ (39), was used to elute skeletal muscle iPLA $_2$. As illustrated in Figure 2A, fractions eluted from the ATP column with the addition of 1 mM ATP contained Ca^{2+} -independent phospholipase activity that was associated with an immunoreactive protein band that migrated with apparent mass of 85 kDa on SDS-PAGE analyses (Figure 2A *inset*). The phospholipase activity-containing fractions were then pooled, and the [Ca^{2+}] in the pool was increased to 5 mM to facilitate binding to calmodulin-Sepharose. Subsequent addition of EGTA promoted recovery of Ca^{2+} -independent phospholipase activity (Figure 2B) that was associated with an immunoreactive band that migrated with an apparent mass of 85 kDa (Fig. 2B *inset*).

Two additional bands at ca. 100 and 60 kDa (not shown) were evident in fractions collected from both the ATP and calmodulin columns. However, mass spectrometry analyses revealed that neither of the bands contained iPLA $_2\beta$ and that the higher band corresponded to glycogen phosphorylase and the lower band to precursor of albumin. Collectively, the findings presented in Figure 1 and Figure 2 indicate that skeletal muscle express an iPLA $_2$ that is similar to the β -isoform of iPLA $_2\beta$ expressed in insulinoma cells, pancreatic islets, and heart (28,35–38).

Substrate oxidation in skeletal muscle

In view of the low phospholipase A $_2$ activity in skeletal muscle, other potential catalytic activities of iPLA $_2\beta$ in skeletal muscle were considered. Recently, it was suggested that iPLA $_2\beta$ manifests an acyl-CoA thioesterase activity under *in vitro* conditions and that saturated acyl-CoA substrates (14–16C) are its preferred substrates (40). Because fatty acyl-CoAs are important substrates of mitochondrial β -oxidation, we examined whether skeletal muscle fatty acid oxidation differed in WT and iPLA $_2\beta$ -null mice. The mice were generated and genotyped by Southern analyses, as described earlier (29). Subsequently, Northern analyses confirmed

the presence of iPLA₂β message in skeletal muscle from WT mice and its absence in skeletal muscle from the iPLA₂β-null mice (29). To verify that the WT mouse skeletal muscle expressed analogous protein and catalytic activity, analyses were performed in gastrocnemius muscle. As shown in Figure 3, an immunoprecipitation protocol used to identify rat skeletal muscle iPLA₂β yielded an iPLA₂-immunoreactive band in WT muscle with an apparent molecular mass of 85 kDa. Cytosolic preparations from the WT skeletal muscle were found to express a Ca²⁺-independent phospholipase activity that was stimulated by ATP and inhibited by BEL. In contrast, muscle from iPLA₂β-null mice expressed background phospholipase activity that was not stimutable by ATP. The analyses in mice therefore, collectively, indicated that iPLA₂β is expressed in skeletal muscle of WT but not iPLA₂β-null mice.

To examine whether iPLA₂β contributes to substrate oxidation, homogenates were prepared from gastrocnemius muscle isolated from WT and iPLA₂β-null mice and ¹⁴CO₂ production and release from [1-¹⁴C]-glucose, [1-¹⁴C]-palmitate, [1-¹⁴C]-acetyl-CoA, and [1-¹⁴C]-palmitoyl-CoA were determined. As shown in Figure 4A, glucose oxidation was not affected by the absence of iPLA₂β. Unexpectedly, palmitate oxidation was reduced by nearly 40% in the iPLA₂β-null muscle (Figure 4B), relative to wild type muscle, while oxidation of acetyl-CoA and palmitoyl-CoA were similar in the WT and iPLA₂β-null groups. These findings suggest that the absence of iPLA₂β does not interfere with the β-oxidation pathway or the Krebs's cycle but may impair the processing of palmitate prior to its entry into the β-oxidation pathway.

Fatty acid transport and β-hydroxyacyl-CoA dehydrogenase (β-HAD)

Fatty acid transport across the plasma membrane and the inner mitochondrial membrane occurs via facilitated diffusion requiring CD36 and carnitine palmitoyltransferase 1 (CPT1). We therefore examined whether the abundance of CD36 or CPT1 is altered in iPLA₂β-null mice. As shown in Figures 5A and B, expression levels of CD36 and CPT1 were similar in WT and iPLA₂β-null skeletal muscle. Next, we measured the activity of β-HAD which catalyzes dehydrogenation of *l*-β-hydroxyacyl-CoA to β-ketoacyl-CoA in the β-oxidation pathway. This step yields an NADH, which donates its electrons to the respiratory chain to generate ATP. β-HAD activity was also found to be similar in the WT and iPLA₂β-null skeletal muscle (Figure 5C). Consistent with the oxidation results, these findings indicate that the expression and/or activity of key components of the fatty acid oxidation pathway required to deliver an acetyl-CoA residue to the Krebs's cycle are normal in the iPLA₂β-null skeletal muscle. However, these findings support the possibility that the defect in muscle palmitate oxidation in the absence of iPLA₂β is most likely due to reduced availability of the appropriate substrate to these components.

Skeletal muscle iPLA₂β expresses acyl-CoA thioesterase activity that is reduced in the iPLA₂β-null mice

During periods of elevated fatty acid oxidation the majority of mitochondrial CoA-SH is bound to long-chain free fatty acids at the outer mitochondrial membrane (41). Mitochondrial thioesterase activity (also known as acyl-CoA thioesterase) catalyzes the hydrolysis of fatty acyl-CoAs to free fatty acid and CoA-SH and the liberated CoA-SH supports fatty acid transport and subsequent oxidation (42). In view of the recent demonstration that iPLA₂β manifests an acyl-CoA thioesterase-like activity (40), we predicted that in its absence there is a decrease in the availability of CoA-SH to support optimal fatty acid oxidation. To examine the contribution of iPLA₂β to the needed acyl-CoA thioesterase activity during fatty acid oxidation in skeletal muscle, generation of radiolabeled fatty acid from hydrolysis of [1-¹⁴C]-palmitoyl-CoA was determined by two approaches. Because the skeletal muscle iPLA₂β was demonstrated to be similar to the one in pancreatic islets, β-cell iPLA₂β overexpressed in INS-1 cells was eluted from the ATP affinity column (Figure 6A). As described previously (25),

pancreatic islets and insulinoma cells express catalytically active 85 and 70 kDa iPLA₂ β isoforms and iPLA₂ β -immunoreactive bands at these apparent molecular masses are evident in the ATP column eluants (Figure 6A, **inset**). The fractions containing iPLA₂ β phospholipase activity were pooled and used to assess acyl-CoA thioesterase activity of iPLA₂ β in the absence and presence of BEL. As seen in Figure 6B, a [substrate]-dependent acyl-CoA thioesterase activity was evident in the pooled fraction in the absence of BEL. However, in the presence of BEL there was a dramatic reduction in thioesterase activity, raising the possibility that the skeletal muscle iPLA₂ β also manifests such activity. We therefore compared thioesterase activity in skeletal muscle prepared from WT and iPLA₂ β -null mice. We previously reported that skeletal muscle from WT, but not from iPLA₂ β -null mice, expresses iPLA₂ β message. As shown in Figure 7, WT expressed an acyl-CoA thioesterase activity that was nearly linear over the range of [1-¹⁴C]-palmitoyl-CoA concentrations tested. Muscle from iPLA₂ β -null mice, not unexpectedly, also expressed an acyl-CoA thioesterase activity. However, the acyl-CoA thioesterase activity in the absence of iPLA₂ β was significantly reduced, relative to WT activity, as the substrate concentration was increased from 0.5 to 10 μ M (AUC: WT, 10547 \pm 942 vs. KO, 7319 \pm 482, p = 0.0133). Calculation of protein yields (μ g protein/mg wet weight) revealed no significant difference between the two groups (WT, 88 \pm 10 and KO, 90 \pm 12, p = 0.8723, n =12 in each group), suggesting that general changes in skeletal muscle protein content do not contribute to the observed decrement in thioesterase activity in the iPLA₂ β -null muscle. Taken together, these two approaches indicate that an acyl-CoA thioesterase activity is manifested by skeletal muscle iPLA₂ β raising the possibility that such activity is necessary for optimal fatty acid oxidation.

Discussion

In view of reports suggesting expression of multiple transcripts of muscle iPLA₂ in humans and rodents, in the present study we examined the chromatographic profile and catalytic properties of skeletal muscle iPLA₂. Our findings reveal that rodent skeletal muscle (a) contains an iPLA₂ transcript of ~3 kb, (b) expresses iPLA₂-immunoreactive protein with an apparent molecular mass of 85 kDa, (c) manifests iPLA₂ phospholipase activity that is stimulated by ATP and inhibited by BEL, and (d) expresses iPLA₂ that exhibits affinity for ATP and calmodulin during sequential chromatography. These properties are analogous to those of iPLA₂ β expressed in islets, β -cells, and cardiac muscle (28,35–38) and suggest that skeletal muscle expresses an 85 kDa isoform of iPLA₂ β protein.

We previously reported that INS-1 insulinoma cells and pancreatic islets express an additional 70 kDa iPLA₂ β -immunoreactive protein that is catalytically active and not generated by a mechanism of alternate splicing of iPLA₂ transcript (25). Sequential chromatography of the skeletal protein revealed three protein bands that migrated with apparent molecular masses of ca. 100 kDa, 85 kDa, and 60 kDa. However, the phospholipase activity of skeletal muscle iPLA₂ β appears to be manifested by the iPLA₂ β -immunoreactive 85 kDa isoform. This is supported by the findings that only the iPLA₂ β -immunoreactive band that eluted with an apparent molecular mass of 85 kDa correlated with phospholipase activity elution and mass spectrometry analyses revealing that the ~100 kDa and 60 kDa protein bands corresponded to glycogen phosphorylase and precursor of albumin, respectively.

Though the specific phospholipase activity of skeletal muscle iPLA₂ β appears to be low, it has been suggested that iPLA₂ phospholipase activity can influence skeletal muscle contractile function by modulating cytosolic oxidant activity (43). In view of the absence of a prominent Ca²⁺-independent phospholipase A₂ activity in the skeletal muscle, it is of interest to note that an acyl-CoA thioesterase activity was recently attributed to iPLA₂ β (40). That study demonstrated a preferential hydrolysis of 14–16C acyl-CoAs by iPLA₂ β . Site-directed mutagenesis of the phospholipase catalytic serine (GTS⁴⁶⁵TG) abolished the phospholipase as

well as the acyl-CoA thioesterase activity of iPLA₂β. Pretreatment with BEL also significantly inhibited both catalytic activities. These observations taken together with our findings that palmitate but not acetyl-CoA or palmitoyl-CoA oxidation is reduced in the absence of changes in fatty acid transport proteins CD36 and CPT1 or β-HAD activity in the skeletal muscle from iPLA₂β-null mice, raise the possibility that acyl-CoA thioesterase activity of iPLA₂β might be important in fatty acid oxidation in skeletal muscle.

Thioesterase activity is critical to support fatty acid oxidation because it results in the liberation of CoA-SH from fatty acyl-CoAs. CoA-SH is required to facilitate fatty acid transport into the mitochondria, the β-oxidation pathway step catalyzed by β-ketothiolase dehydrogenase, and the Krebs' cycle reaction catalyzed by α-ketoglutarate dehydrogenase. Hence, the possibility that acyl-CoA thioesterase activity of iPLA₂β is required for optimal fatty acid oxidation was first examined using iPLA₂β eluted from the ATP affinity column. Incubation of an aliquot of the eluant pool containing phospholipase activity with [1-¹⁴C]-palmitoyl-CoA resulted in hydrolysis of the substrate that was inhibited by the addition of BEL. This suggested that the measured acyl-CoA thioesterase activity is most likely manifested by iPLA₂β. To overcome the possibility of non-specific effects of BEL or presence of non- iPLA₂β proteins in the partially-purified preparation from the ATP column, acyl-CoA thioesterase activity was next determined in skeletal muscle from WT and iPLA₂β-null mice. Consistent with the earlier demonstration of iPLA₂β message in skeletal muscle of WT but not from iPLA₂β-null mice (29), catalytic activity characteristic of iPLA₂β was not evident in skeletal muscle of the iPLA₂β-null mice.

As expected, both WT and iPLA₂β-null preparations expressed significant acyl-CoA thioesterase activity. However, AUC analyses revealed that the activity in the iPLA₂β-null group, relative to the WT group, was significantly diminished as the concentration of the [1-¹⁴C]-palmitoyl-CoA substrate was increased from 0.5 to 10 μM. These findings therefore are the first demonstration of expression of acyl-CoA thioesterase activity by skeletal muscle iPLA₂β. In summary, our findings indicate that skeletal muscle expresses an 85 kDa iPLA₂β isoform that manifests both phospholipase and acyl-CoA thioesterase activities. Further, they suggest that the acyl-CoA thioesterase activity of iPLA₂β could be an important contributor for optimal fatty acid oxidation by skeletal muscle.

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The abbreviations used are

AUC, area under curve; BEL, bromoenol lactone suicide inhibitor of iPLA₂β; β-HAD, β-hydroxyacyl-CoA dehydrogenase; CPT1, carnitine palmitoyltransferase 1; cPLA₂, group IV cytosolic phospholipase A₂; iPLA₂β, β-isoform of group VIA calcium-independent phospholipase A₂; OE, iPLA₂β-overexpressing cells; PLA₂, phospholipase A₂; SEM, standard error of the mean..

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Evidence for iPLA₂ Expression in Skeletal Muscle

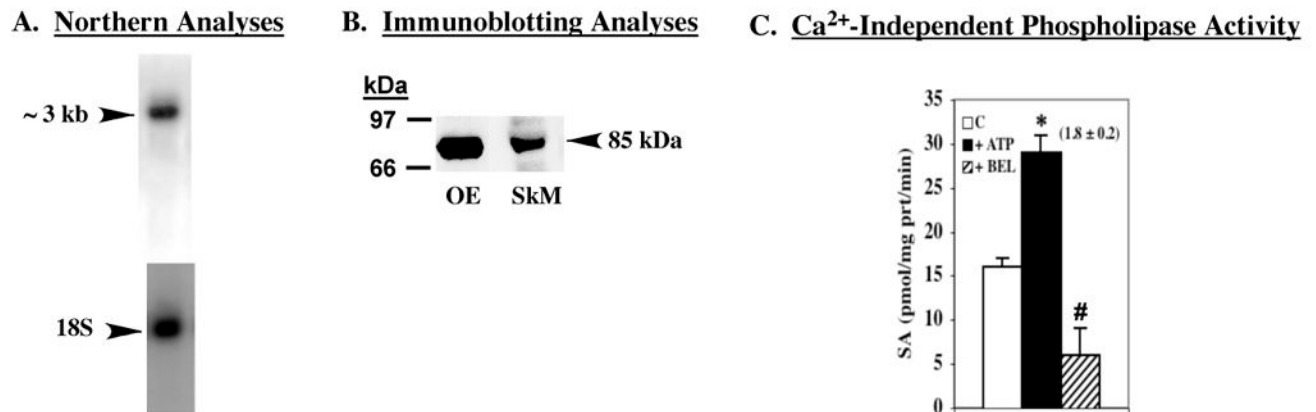


FIGURE 1. Expression of iPLA₂β message, protein, and phospholipase catalytic activity in rat skeletal muscle

A. Northern analyses. B. Immunoblotting analyses. iPLA₂ was immunoprecipitated from skeletal muscle lysates and the immunoprecipitated proteins were analysed by SDS-PAGE and transferred onto Immobolin-P PVDF membrane. The electroblot was then probed with antibodies against iPLA₂β (T-14) and immunoreactive protein was visualized by enhanced chemiluminescence (ECL). C. Phospholipase catalytic activity. An aliquot (30 μg protein) of cytosol prepared from rat gastrocnemius muscle was used to determine Ca²⁺-independent phospholipase activity of iPLA₂β in the absence and presence of ATP (1 mM) or BEL (10 μM) and the mean specific activities ± SEMs are presented (n = 6). Value presented in parenthesis over the ATP bar represents fold-stimulation of activity, relative to control. (*#Significantly different from control activity, p < 0.005 and p < 0.0001, respectively).

Elution Profile of Skeletal Muscle iPLA₂

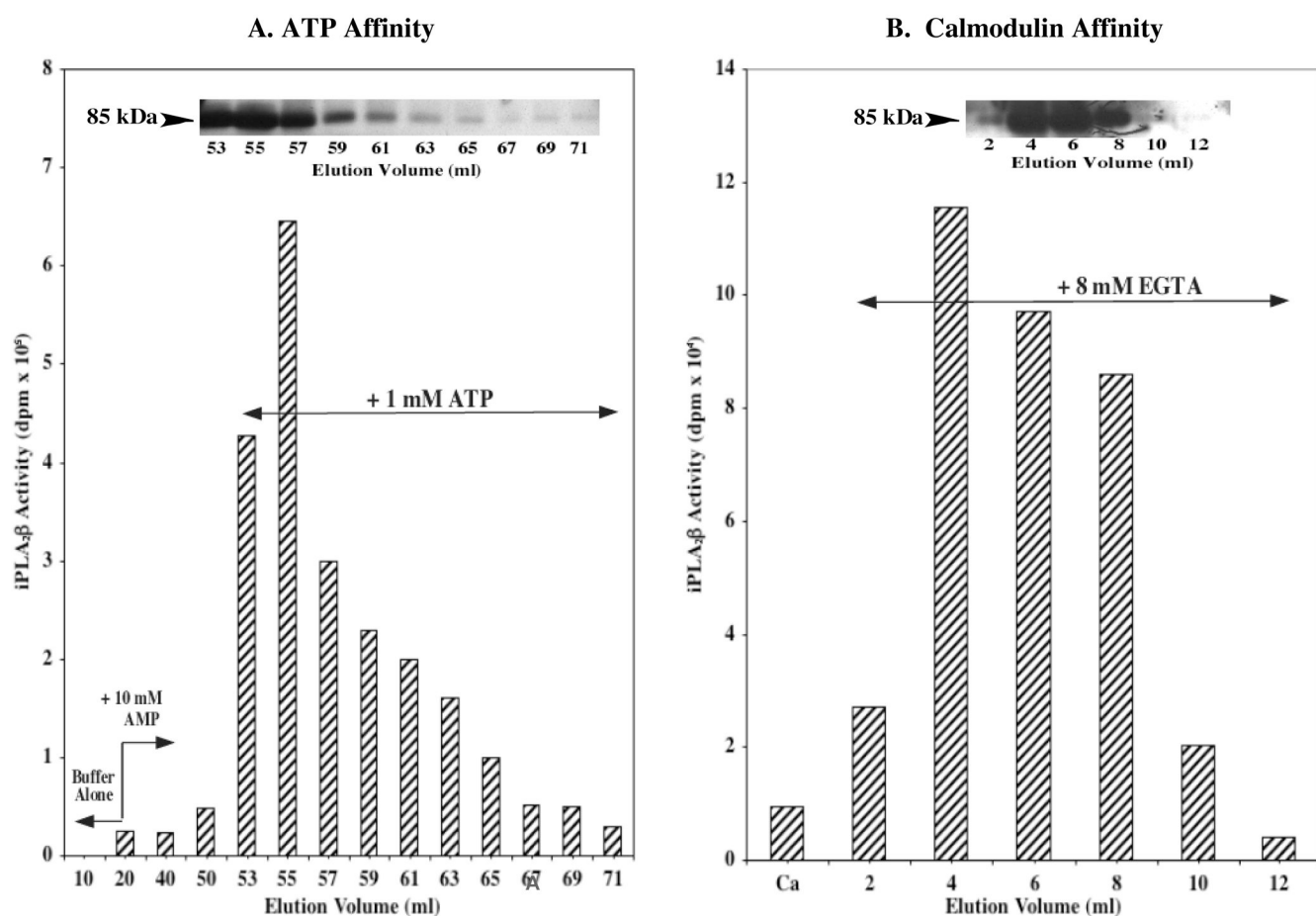


FIGURE 2. ATP and calmodulin affinity column chromatography profiles of iPLA₂β elution from skeletal muscle

Skeletal muscle protein was prepared as described in Methods and loaded onto an ATP-agarose column. The column was washed with buffer alone and then with buffer containing AMP (10 mM) prior to elution of iPLA₂β with buffer containing ATP (1 mM). Fractions containing Ca²⁺-independent phospholipase activity were pooled, supplemented with CaCl₂, and loaded onto a calmodulin-Sepharose column. The column was washed with buffer and Ca²⁺-independent phospholipase activity was subsequently eluted with buffer containing EGTA (8 mM). A. ATP-Agarose affinity chromatography. B. Calmodulin-Sepharose affinity chromatography. (*Insets*, iPLA₂β immunoblotting analyses). (Ca, 0.5 mM Ca²⁺).

iPLA₂ Phospholipase Activity and Protein in Mouse Skeletal Muscle

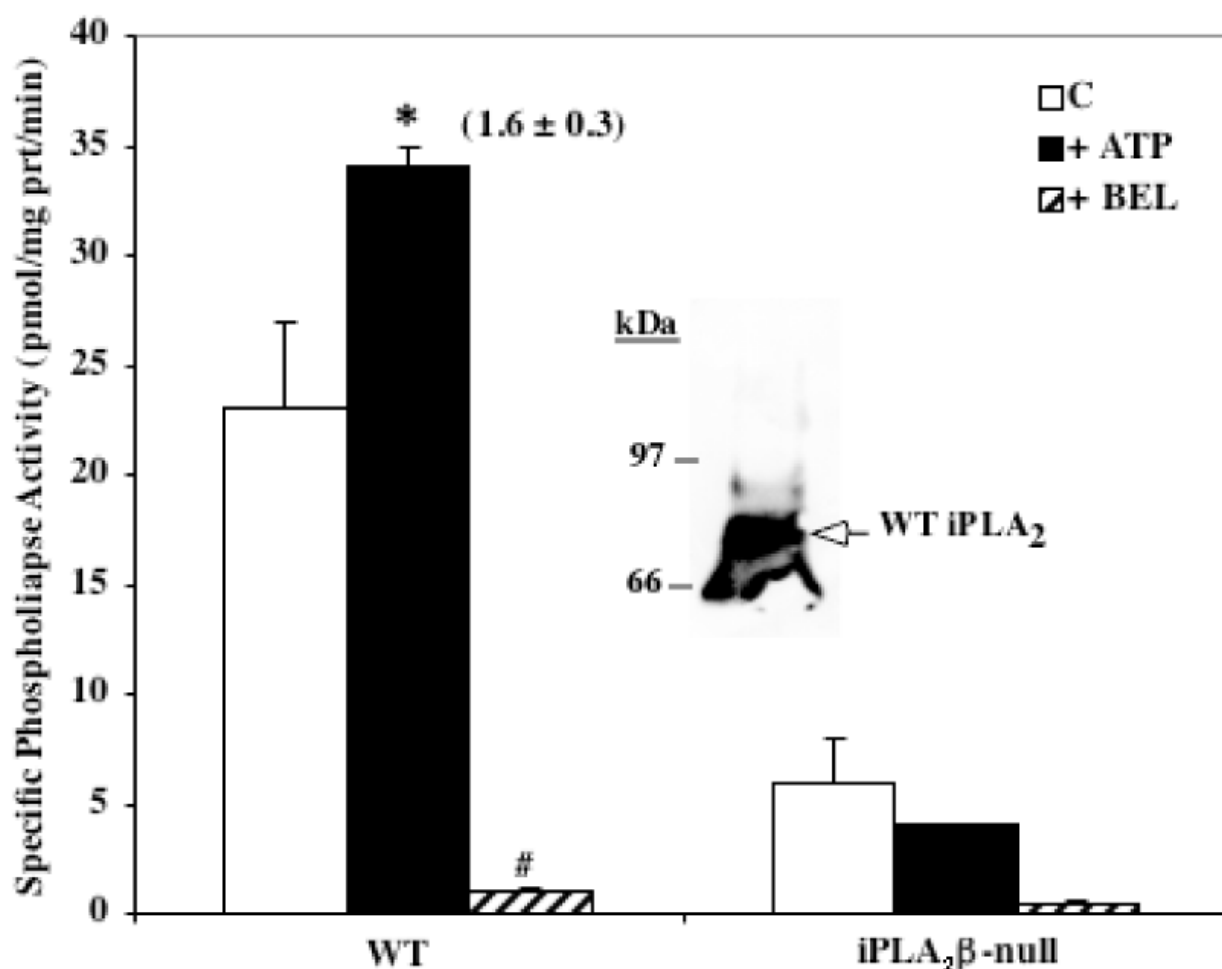


FIGURE 3. Expression of iPLA₂β protein in WT mouse and phospholipase catalytic activity in WT and iPLA₂β-null mice skeletal muscle

iPLA₂ was immunoprecipitated from mouse skeletal muscle and processed as in Figure 1. An aliquot (30 μg protein) of cytosol prepared from mouse gastrocnemius muscle was used to determine Ca²⁺-independent phospholipase activity of iPLA₂β in the absence (Control) and presence of ATP (1 mM) or BEL (10 μM) and the mean specific activities ± SEMs are presented (n = 6). Value presented in parenthesis over the ATP bar represents fold-stimulation of activity, relative to control. (*Significantly different from control activity, p < 0.05 and p < 0.0001, respectively).

Comparison of Substrate Oxidation in Skeletal Muscle From WT and iPLA₂β-Null Mice

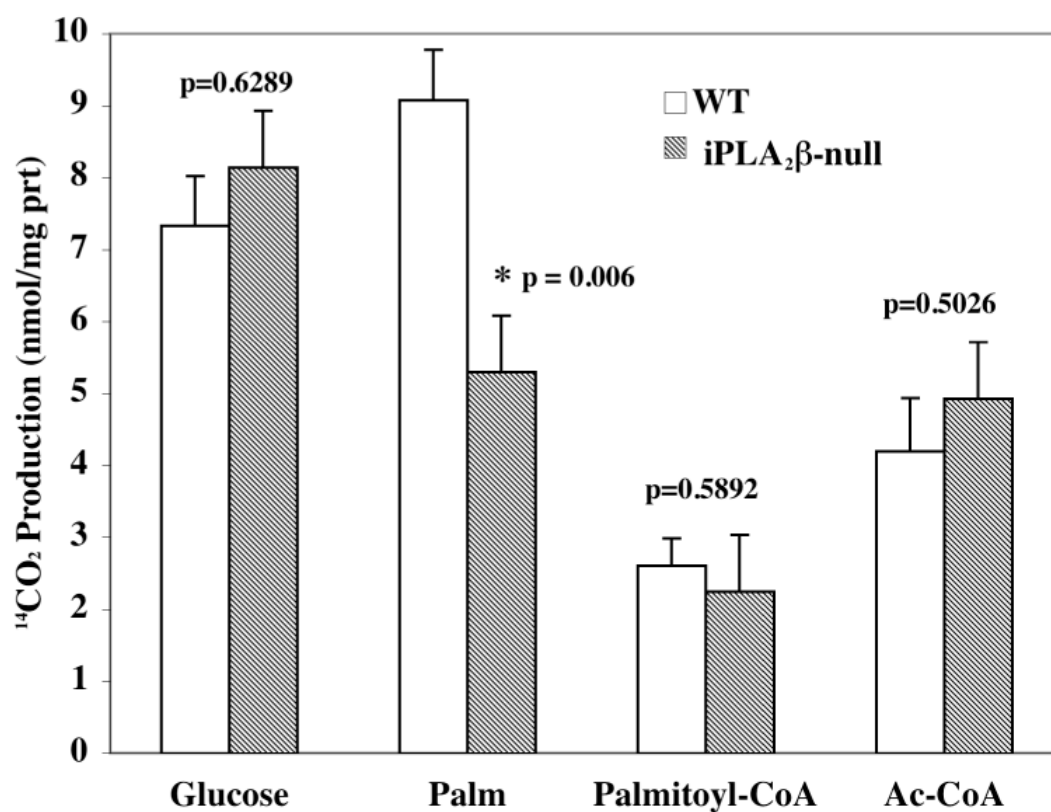


FIGURE 4. Substrate oxidation in skeletal muscle of WT and iPLA₂β-null mice

A. Glucose oxidation. B. Palmitate, palmitoyl-CoA, and acetyl CoA oxidation. Oxidation was measured as ¹⁴CO₂ production from [¹⁴C]-labeled substrates in skeletal muscle homogenates prepared from gastrocnemius muscle. Results are mean ± SEM (n = 6-18 in each group). (*iPLA₂β-null group significantly different from WT group.)

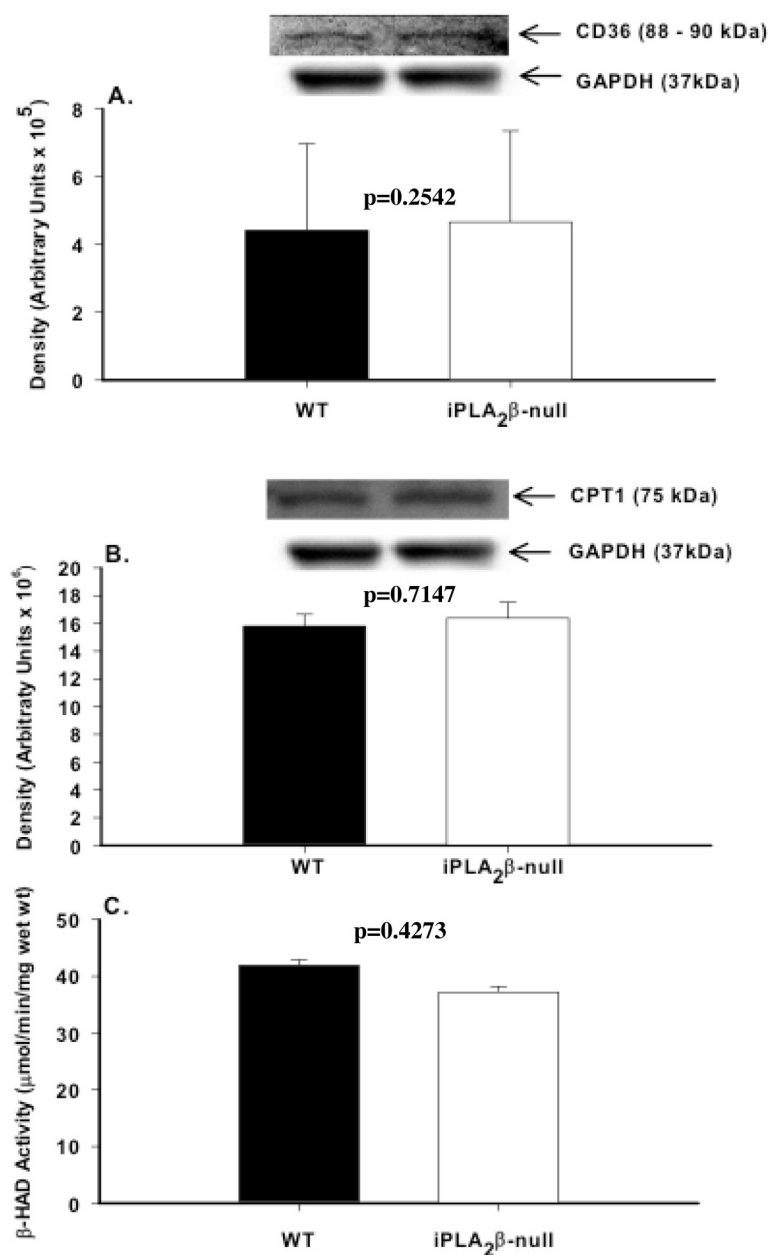
SKELETAL MUSCLE CD36, CPT1, and β -HAD

FIGURE 5. CPT-1 and CD36 protein expression and β -HAD activity in skeletal muscle of WT and iPLA₂ β -null mice

Skeletal muscle homogenates were prepared from gastrocnemius and processed for immunoblotting analyses of (A) CPT-1 and (B) CD36. Each panel shows quantified data and each bar represents the mean \pm SEM (n = 4 in each group) of the respective protein. Inserts are immunoblots for each protein and corresponding GAPDH control. (C) β -HAD activity. β -hydroxyacyl-CoA dehydrogenase activity prepared from gastrocnemius skeletal muscle homogenates. Data presented are means \pm SEMs of β -HAD activity (n = 4 in each group).

Phospholipase and Acyl-CoA Thioesterase Activities Associated with iPLA₂ β Eluted From ATP Affinity Chromatography

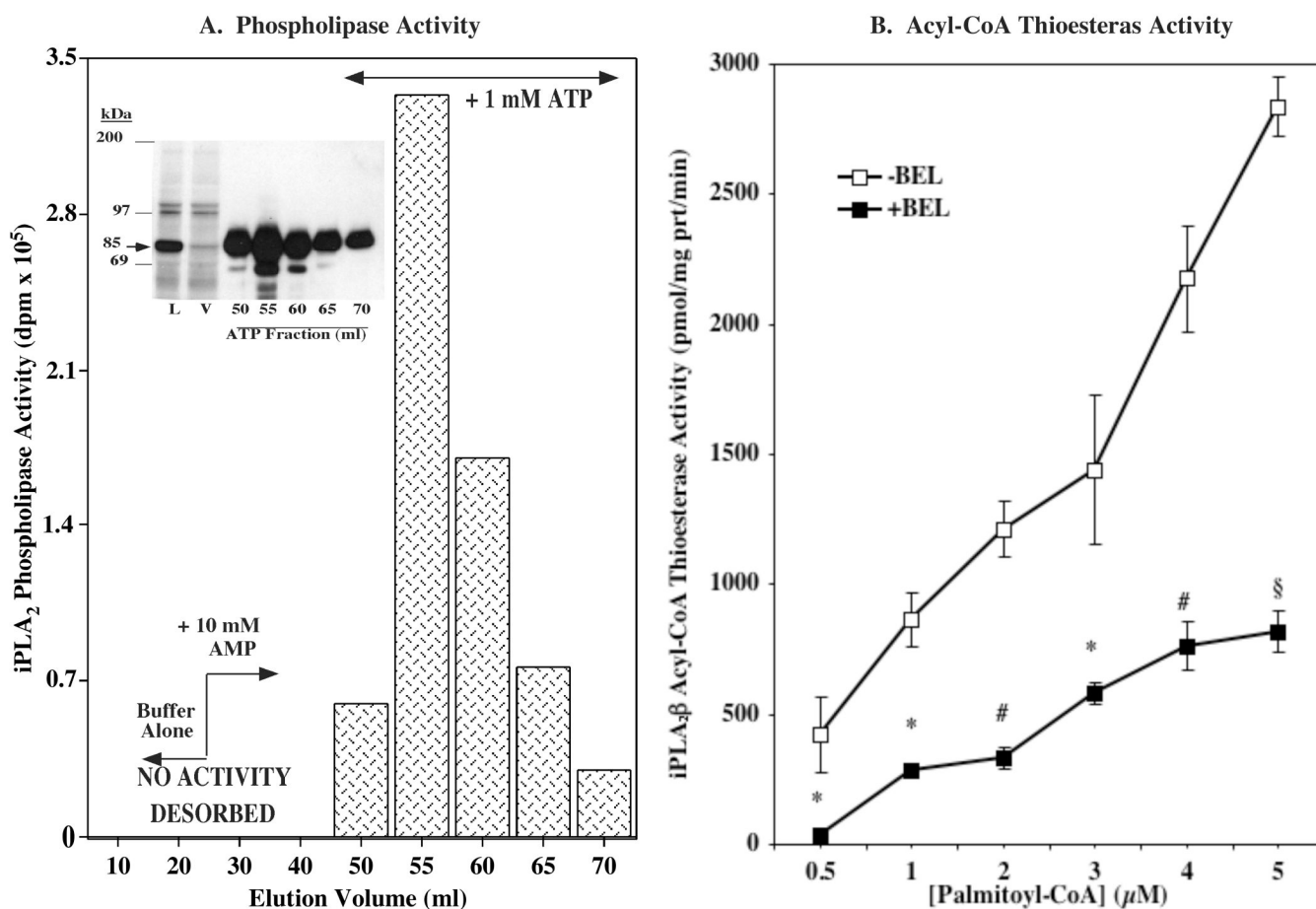


FIGURE 6. iPLA₂ β -associated phospholipase and acyl-CoA thioesterase activity

A. Phospholipase activity. Partially-purified iPLA₂ β was prepared from iPLA₂ β -overexpressing INS-1 cells (30 \times T225 flasks), as described (25). Ca²⁺-independent phospholipase activity of iPLA₂ β was determined in eluants from the ATP affinity column using [¹⁴C]-PLPC as the substrate. (Inset, immunoblotting analyses of iPLA₂ β in ATP column eluants). B. Acyl-CoA thioesterase activity. Fractions containing phospholipase activity were pooled, protein concentration determined, and acyl-CoA thioesterase specific activity was measured in 1 μ g protein aliquots using [¹⁴C]-palmitoyl-CoA as substrate in the absence or presence of BEL (10 μ M). (*, #, \$BEL-treated group significantly different from control group, $p < 0.05$, $p < 0.01$, and $p < 0.005$, respectively, $n=3$).

Skeletal Muscle iPLA₂ β -Associated Thioesterase Activity

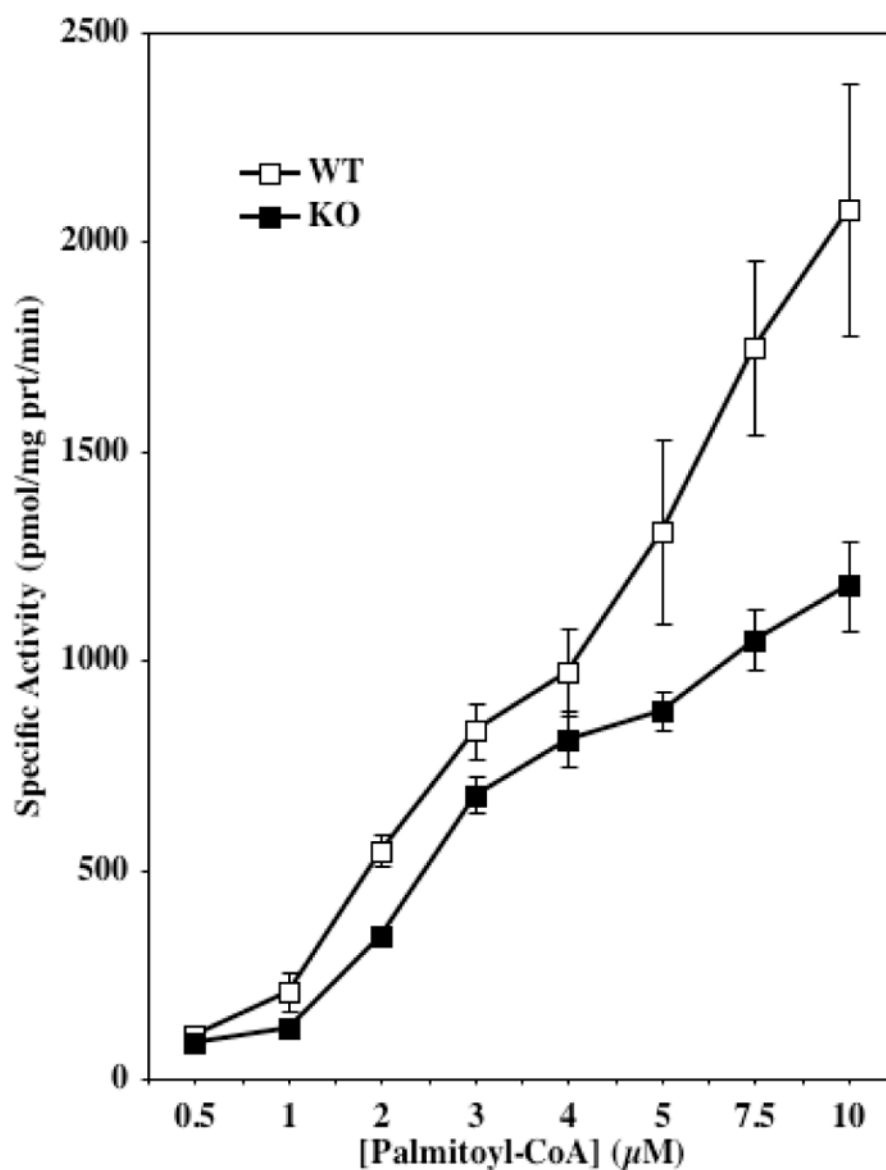


Figure 7. Acyl-CoA thioesterase activity manifested by skeletal muscle iPLA₂ β

Cytosol was prepared from muscle isolated from WT and iPLA₂ β -null mice and acyl-CoA thioesterase activity was measured in 30 μg protein aliquots. The mean \pm SEMs of WT and KO muscle acyl-CoA thioesterase specific activity in the presence of [¹⁴C]-palmitoyl-CoA (0.50–10 μM) substrate are presented ($n = 8$ in each group). iPLA₂ β -null activity significantly ($p = 0.0133$) reduced, relative to WT activity, as reflected by AUC analyses.