Role of Phospholipase A$_2$ in the Induction of Drip Loss in Porcine Muscle

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The role of phospholipase A$_2$ in the induction of drip loss from pig muscle has been investigated. In samples from porcine *M. longissimus dorsi*, total PLA$_2$ activity as well as mRNA and protein levels of the group VIA iPLA$_2$ (iPLA$_2$-VIA) increased during the initial 4 h post-mortem period. Morphological studies of porcine muscle showed that at 4 h post-mortem, gaps had formed between muscle fibers and that the sarcolemma membrane borders appeared blurred. At the same time iPLA$_2$-VIA protein levels were increased inside muscle fibers and at the sarcolemma. iPLA$_2$-VIA mRNA abundance in samples from different breeds of pigs with variations in drip loss revealed no clear correlation between drip loss level and iPLA$_2$-VIA expression. Together, these data indicate that during the post-mortem period, iPLA$_2$-VIA expression and activity is increased at the muscle fiber membranes. PLA$_2$ activity may affect membrane permeability and consequently the progression of drip formation in porcine muscle.

KEYWORDS: Water-holding capacity; phospholipase A$_2$; pig muscle; bromoenol lactone; iPLA$_2$-VIA; C2C12 myotubes; iPLA$_2$-VIB

INTRODUCTION

The ability of meat to retain water, known as water-holding capacity (WHC), is an important economical and quality trait for the meat industry. It has been estimated that drip loss from porcine *M. longissimus dorsi* (LD) may vary from 1% to 15% dependent on preslaughter stress and the genetic background of the pig (*1*).

It is widely accepted that the early progress in post-mortem processes is critical for the final WHC of the meat (see (*1*)). The slaughtering process, including transportation, stalling, stunning of the pig, and subsequent handling of the carcass, is associated with massive hormonal stimulation, metabolic stress, and anoxic/ischemic conditions in the muscle (*2, 3*). At the cellular level oxygen deprivation is accompanied by cellular acidification, an increased production of reactive oxygen species, and cell swelling due to an increase in the cellular content of Na$^+$, inorganic phosphates, and metabolites (*2, 4*). At some point when the intracellular accumulation of osmotically active solutes exceeds the regulatory capability of the cell, the ion homeostasis and membrane integrity cannot be maintained and outflow of cellular constituents will proceed. Prerigor cell swelling of

porcine LD has been shown to take place within the first 2−4 h post-mortem (*5*). This initial cell swelling is followed by a secondary cell shrinkage during which membrane structures disintegrate, the myofilament lattice contract due to rigor, and water is expelled from the myofilament space into the extramyofibrillar compartment (*5*) from where it presumably is lost as drip. The drip contains ions and organic osmolytes as well as a significant amount of protein, presumably reflecting the loss of cellular constituents (*3*).

Although incompletely understood, a number of proposals as to which basic structural elements and biochemical reactions affect the post-mortem rearrangement of myofibrillar water in porcine muscle have been put forward: (i) variation in thin and thick filament spacing within the sarcomere caused by acidification and temperature changes, (ii) contraction-induced water loss, i.e., water once expelled from the myofibrils is squeezed from the space between the myofibrils to the extracellular compartment due to shrinkage and contraction of the mechanically interconnected myofibrils and sarcolemma, (iii) the extent of "drip channel" formation as water expelled from muscle fibers separates into two different spaces either between individual fibers or between large fiber bundles, (iv) an osmotic effect involving a cell swelling mediated release of osmolytes and water from the extramyofibrillar space which contribute to the cell water loss, and, finally, (v) a general loss of membrane integrity (*1, 3*).

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According to experiments performed in C2C12 myotube cultures under conditions which simulate the situation in the muscle following slaughter, cell swelling and activation of phospholipase A_2 (PLA_2) are initial upstream events which lead to increased membrane permeability (*3*). Membrane permeability is affected by the physical properties of the membrane bilayer which, in part, is regulated by PLA_2-mediated *sn-2* deacylation and CoA-dependent acyltransferase-mediated reacylation of membrane phospholipids (*6*). Under steady-state conditions reacylation dominates deacylation, maintaining a low cellular concentration of free fatty acids and lysophospholipids. However, during cellular stress, such as ischemia, PLA_2 activity may be increased to a level which exceeds the capacity for reacylation, resulting in an increased level of free fatty acids and lysophospholipids (*6*). High levels of lysophospholipids, in particular lysophosphatidylcholine (LPC), in the membrane may result in membrane destabilization and consequently membrane leakage. The fatty acid, arachidonic acid (AA), once made available, may act as a substrate for eicosanoid synthesis and initiate a wide array of cellular processes, including organic osmolyte release (*9–12*). PLA_2 activity might also, in coordination with acyl transacylases, act in a protective manner by replacing toxic oxidized fatty acids with native fatty acids, thus restoring membrane function (*7, 8*).

The PLA_2 superfamily is divided into the low molecular weight, secretory Ca^{2+}-dependent PLA_2s ($sPLA_2$), the high molecular weight, intracellular Ca^{2+}-dependent PLA_2s ($cPLA_2$), the Ca^{2+}-independent PLA_2s ($iPLA_2$), and the platelet-activating factor acylhydrolases (PAH-AH) (*13*). Two members of the group VI $iPLA_2$ s ($iPLA_2$-VIA and $iPLA_2$-VIB) have been cloned in mammalian cells (*13*). $iPLA_2$-VIA has been proposed to be involved in a number of cellular processes including membrane remodeling (*17*), cell volume regulation (*18*), and cell death (*16, 19, 20*), whereas $iPLA_2$-VIB is reported to be involved in organelle membrane remodeling and protection against oxidative stress (*21, 22*). Ischemia/hypoxia has been associated with increases in $iPLA_2$ activity in, e.g., heart (*8*), proximal turbules (*14*), human umbilical vein endochelial cells (*15*), and PC12 culture cells (*16*).

$iPLA_2$s may play important roles in the stress response in porcine muscle during the post-mortem phase. Recently we have shown that post-mortem-like conditions (oxygen and glucose deprivation) in C2C12 myotubes induce a time-dependent increase in $iPLA_2$-VIA activity which is associated with changes in the expression and subcellular localization of the $iPLA_2$-VIA protein (Poulsen, K. A.; Pedersen, S. F.; Kolko, M.; Lambert, I. H. Manuscript in preparation). Here we investigate the role of PLA_2 in the induction of drip loss from DLY (Danish Duroc × (Danish Landrace × Yorkshire) pork. We demonstrate that PLA_2 activity and $iPLA_2$-VIA mRNA and protein levels are increased in porcine muscle during the initial post-mortem period. Furthermore, the time for peak PLA_2 activity and expression correlates roughly with the onset of the water redistribution phase previously demonstrated by Bertram and co-workers (*5*).

MATERIALS AND METHODS

Animal Production, Slaughter, and Sample Collection. Six female pigs of DLY cross breed were reared until an average weight of 99 ± 10 kg and transported to the experimental slaughter plant (200 m) at the Danish Institute of Agricultural Sciences, Research Centre Foulum. The pigs were stunned by 80% CO_2 for 3 min, exsanguinated, scalded at 62 °C for 3 min, cleaned, and eviscerated within 30 min. At 75 min post-mortem the carcasses were placed in a chilling room at 4 °C. Biopsies were taken from LD at the time of exsanguinations (time 0)

and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24, 36, and 48 h post-mortem, frozen in liquid nitrogen, and kept at −80 °C until analysis.

Cell Culture. The C2C12 myoblast cell line was isolated and cultured as previously described (*3*). In brief, myoblast were seeded in culture flasks (75 cm^2) at a density of approximately 6000 cells/cm^2. The myoblasts were grown in 10 mL Dulbecco-modified Eagles medium (DMEM, Invitrogen Life Technologies, Denmark) supplemented with 10% FCS, 100 IU/mL penicillin, 100 μg/mL streptomycin sulfate, 20 μg/mL gentamicin, and 3 μg/mL amphotericin B (Invitrogen Life Technologies, Denmark). Cells were kept in an atmosphere of 95% air, 5% CO_2 at 37 °C. Only C2C12 myoblasts from passages 3–8 were used for experiments.

Reagents and Media. Unless otherwise indicated, reagents were of the highest available grade and obtained from Sigma Aldrich (St Louis, MO). Tris-buffered saline (TBS) contained 150 mM NaCl, 10 mM Tris-HCl, 1 mM $MgCl_2$, and 1 mM EGTA at pH 7.4.

Phospholipase A_2 Activity. Protein for activity measurements was isolated from biopsies taken from LD of DLY pigs or C2C12 cell cultures. For isolation of protein from porcine LD, 200 μL of lysis buffer containing 50 mM HEPES, 1 mM EDTA, 1 mM Na-ortho-vanadate, and protease inhibitors 1:100 (v/v) containing 4-(2-amino-ethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma Aldrich, St. Louis, MO) was added to 50–100 mg of sample. The samples were homogenized 3 × 20 s on ice using an ultrathorax homogenizer (Polytron PT 1200). C2C12 myoblast cultures were washed in PBS, lysed in 500 μL of lysis buffer (same as above), scraped off using a cell scraper, and transferred to Eppendorf tubes. All samples were sonicated 2 × 10 s followed by centrifuging at 10.000*g* at 4 °C for 10 min. The supernatants were subsequently spun through 30 kDa Microcon cutoff filters (Millipore, Copenhagen, Denmark) for 12 min at 14.000*g* to remove low molecular weight protein including $sPLA_2$s. PLA_2 activity was measured using the PLA_2 substrate arachidonoyl thio-phosphatidylcholine and the protocol recommended by the manufacturer (Cayman Chem., Ann Arbor, MI). Arachidonoyl thio-phosphatidylcholine was dissolved in assay buffer (80 mM HEPES, 150 mM NaCl, 10 mM $CaCl_2$, 4 mM Triton X-100, 30% glycerol, and 1 mg/mL BSA) to a final concentration of 1.5 mM. For the pH experiments MES and Bicine/tricine were substituted for HEPES to buffer at pH 5.5–6.0 and 8.0, respectively. Aliquots of 10 μL of lysate containing 80–100 μg of protein were mixed with 5 μL of assay buffer in 96 well microtiter plates. The reaction was initiated by addition of 200 μL of substrate to each well and terminated after 60 min by addition of 10 μL of 25 mM 5,5′-dinitrobis(2-dinitrobenzoic azid (DTNB) plus 475 mM EGTA in 0.5 M Tris-HCl (pH 8.0). The absorbance was measured at 405 nm in a plate reader (BMG LabTechnologies, Offenburg, Germany). PLA_2 activity was expressed in nmol (or μmol)·mg protein^{-1}·min^{-1} determined from the extinction coefficient of DNTB and the protein content in the supernatants using Lambert–Beer's law. Bromoenol lactone (BEL)-sensitive $iPLA_2$ activity was estimated as the total activity minus the activity in the presence of 10 μM BEL.

Western Blotting. Protein for Western blotting was prepared as described above for activity measurements using a lysis buffer containing 20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM Na-ortho-vanadate, and protease inhibitors 1:100 (v/v) containing AEBSF, pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma)). Equal amounts of protein were separated by SDS-PAGE on precast NuPAGE 10% Bis-Tris Gels (Invitrogen, Copenhagen, Denmark) and transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat dry milk in TBS for 1 h and incubated with primary antibody overnight ($iPLA_2$-VIA 1:500, Cayman Chem, Ann Arbor, MI). After incubation, membranes were washed in TBS and incubated with Alkaline Phosphatase conjugated anti-rabbit or anti-mouse secondary antibodies (1:600, Jackson Immuno Research Europe, Newmarked, Suffolk, U.K.). Immunospecific staining was developed directly on the membranes using the alkaline phosphatase substrate BCIP/NBT (KPL, Gaithersburg, MD).
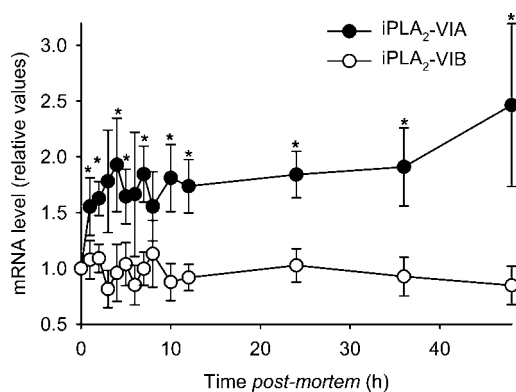
Immunohistochemistry. Samples taken from LD of DLY pigs at the time of exsanguinations (time 0) and 4 h post-mortem were fixed in formalin, embedded in paraffin blocks, and cut in 4 μm sections. Sections were deparaffinized, boiled 20 min in TE buffer, pH = 9.5,

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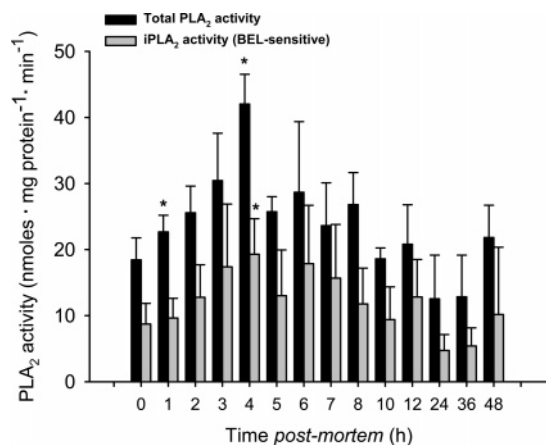
Figure 1. PLA$_2$ activity in DLY pig muscle post-mortem. Total PLA$_2$ (black bars) and iPLA$_2$ (gray bars) activity in porcine LD 0–48 h post-mortem. iPLA$_2$ activity was determined as the difference between total PLA$_2$ activity minus the activity in the presence of 10 μM of the iPLA$_2$ inhibitor BEL. Values in nmol·mg protein^{-1}·min^{-1} are mean values ± SEM from samples from three pigs. The asterisk (*) indicates significance ($p < 0.05$) relative to exsanguination time (0 h controls).

Figure 2. Post-mortem progress in pig iPLA$_2$-VIA and VIB mRNA levels. mRNA levels were estimated by quantitative real-time PCR as the relative iPLA$_2$-VIA or VIB mRNA level adjusted for the average level of β-actin and GAPDH. Data are presented as mean ± SEM for samples from six DLY pigs. The asterisk (*) indicates significance ($p < 0.05$) relative to time 0 h controls. Open circles: iPLA$_2$-VIB. Closed circles: iPLA$_2$-VIA.

for antigen retrieval, and incubated with 3% H$_2$O$_2$ for 8 min at room temperature to inhibit endogenous peroxidase activity. Sections were exposed to primary antibody (iPLA$_2$-VIA 1:200, Cayman Chem, Ann Arbor, MI), washed twice with PBS, and exposed to biotinylated anti-rabbit IgG followed by incubation with a dilution of streptavidin peroxidase complex reagent and visualized by use of AEC chromogen (DAKO Inc., Copenhagen, Denmark) according to the manufacturer's instructions. Finally, sections were counterstained with haematoxylin. Immunostained sections were examined by light microscopy.

Real-Time RT-PCR. RNA was extracted from biopsies using a trireagent extraction procedure as described by Chomczynski and co-workers (*23*−*25*). Purified RNA (1 μg) was reverse transcribed with oligo-dT primers and Superscript II RNase H reverse transcriptase kit (Invitrogen, Copenhagen, Denmark). Reverse-transcribed material (1 μL) was amplified with TaqMan Universal PCR Master Mix (Applied Biosystems, Stockholm, Sweden). The genes encoding pig iPLA$_2$-VIA or iPLA$_2$-VIB have not been cloned; however, homology search in the Pig Genome Project database (NCBI) and the Tigr gene index using the mouse miPLA$_2$-VIA and miPLA$_2$-VIB sequences as templates identified considerable portions of iPLA$_2$-VIA sequence in the pig genome (90−95% homology), a pig EST (BE031346) with 90% homology to mouse iPLA$_2$-VIA, and a pig EST (AJ663091) with 91% homology to mouse iPLA$_2$-VIB that subsequently were used as templates for primer and probe design. Primers and probes were designed using Primer Express 2.0 software (Applied Biosystems, Stockholm, Sweden), and probe, forward, or reverse primer was designed to anneal to an exon boundary. Exon structures reported for mice were used. The sequences of probes and forward and reverse primers, respectively, were as follows: iPLA$_2$-VIA (5′-CGGCATC-CAGTACTTCAGACTGAACCCC-3′,5′-CAGTGTTGCACGGATC-CAGAT-3′,5′-CATTGACCTCATCCAGCATGA-3′) and iPLA$_2$-VIB (5′-TCAAGATGGAGGTCTGCTTCTGAATAACCCT-3′,5′-AATAT-GCCTTGGGAAATGATCTTC-3′,5′-CATTTACACTCGTGCAT-TGCTAGTG-3′), GAPDH (5′-CGCCTGGTCACCAGGGCTGCT-3′,5′-GTCGGAGTGAACGGATTTGG-3′,5′-CAATGTCCACTTTGCCA-GAGTTAA-3′), and β-actin (5′-CTGTATGCCTCTGGCCGCACCA-3′,5′-ACCCAGATCATGTTCGAGACCTT-3′,5′-TCACCGGAGTC-CATCACGAT-3′). Amplicon length was tested by standard RT-PCR analysis on 2% agarose gels (data not shown). Probes were labeled with FAM in the 5′ end and TAMRA in the 3′ end. For PCR, 40 cycles at 95 °C for 15 s and 60 °C for 60 s were applied to amplify the PCR products. A selected sample was diluted serially and analyzed in triplicate to test linearity and efficiency of the PCR amplifications. Furthermore, absence of amplification in control wells with either water or genomic DNA was used as negative controls. All samples were

analyzed in duplicates using the ABI 7900HT detection system (Applied Biosystems, Stockholm, Sweden).

The signal for mRNA expression was normalized to the abundance of the reference gene (average of β-actin and GAPDH) and the relative expression ratio calculated using the equation $(E_{\text{target}})^{\Delta C(t)^{\text{target(control−treated)}}} / (E_{\text{ref}})^{\Delta C(t)^{\text{ref(control−treated)}}}$, where E_{target} and E_{ref} are the PCR amplification efficiencies for the target and reference genes and $\Delta C(t)^{\text{target}}$ and $\Delta C(t)^{\text{ref}}$ are the change in $C(t)$ values for target and reference gene transcripts (see (*26*)).

Statistics. Data are presented either as mean ± standard error of the mean (SEM) of three or more independent experiments unless otherwise indicated. Statistical significance was estimated by two-sided Student's *t*-test with 0.05 as the level of significance as indicated.

RESULTS

PLA$_2$ Activity Post-mortem. To estimate PLA$_2$ activity in pig muscle during the post-mortem progression, biopsies were taken from LD of DLY pigs immediately following slaughter and at intervals during 48 h post-mortem. From **Figure 1** it is seen that PLA$_2$ activity, measured at pH 7.4 was 19 nmol·mg protein^{-1}·min^{-1} at the time of exsanguinations and increased gradually to a maximal activity at 42 nmol·mg protein^{-1}·min^{-1} at 4 h post-mortem. PLA$_2$ activity subsequently slowly decreased with time to below exsanguination levels at 36 h post-mortem. The iPLA$_2$ activity, estimated as the BEL-sensitive fraction of the total PLA$_2$ activity, exhibited the same pattern with the activity increasing from 9 nmol·mg protein^{-1}·min^{-1} at the time of slaughter to 19 nmol·mg protein^{-1}·min^{-1} 4 h post-mortem (**Figure 1**).

iPLA$_2$ mRNA and Protein Levels Post-mortem. As iPLA$_2$ activity was increased in porcine DLY muscle post-mortem, we investigated whether it involved changes in mRNA and protein levels for either group VIA or VIB iPLA$_2$ during the 48 h post-mortem period using quantitative real-time PCR (qPCR) and Western blotting. As seen from **Figure 2**, the mRNA abundance of iPLA$_2$-VIB was unaltered throughout the post-mortem period whereas the level of iPLA$_2$-VIA was up-regulated about 2-fold within 4 h and remained elevated throughout the measured 48 h post-mortem period. Western blotting, using a commercially available antibody raised against iPLA$_2$-VIA, verified the expression of the 85 kDa iPLA$_2$-VIA protein in porcine muscle and showed a gradual increase in abundance within the first 6 h post-mortem (**Figure 3**).

Immunohistochemistry of Post-mortem Samples− Localization of iPLA$_2$-VIA. Samples, cut from LD at the time

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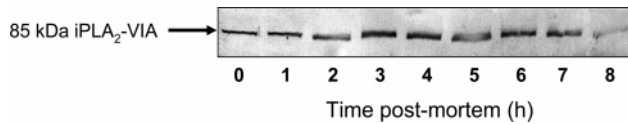
Figure 3. Post-mortem changes in iPLA$_2$-VIA protein levels. Representative Western blot showing recognition of the 85 kDa iPLA$_2$-VIA protein from DLY pigs and the time-dependent increase in protein level post-mortem. The blot is representative of three separate sets of post-mortem samples tested.
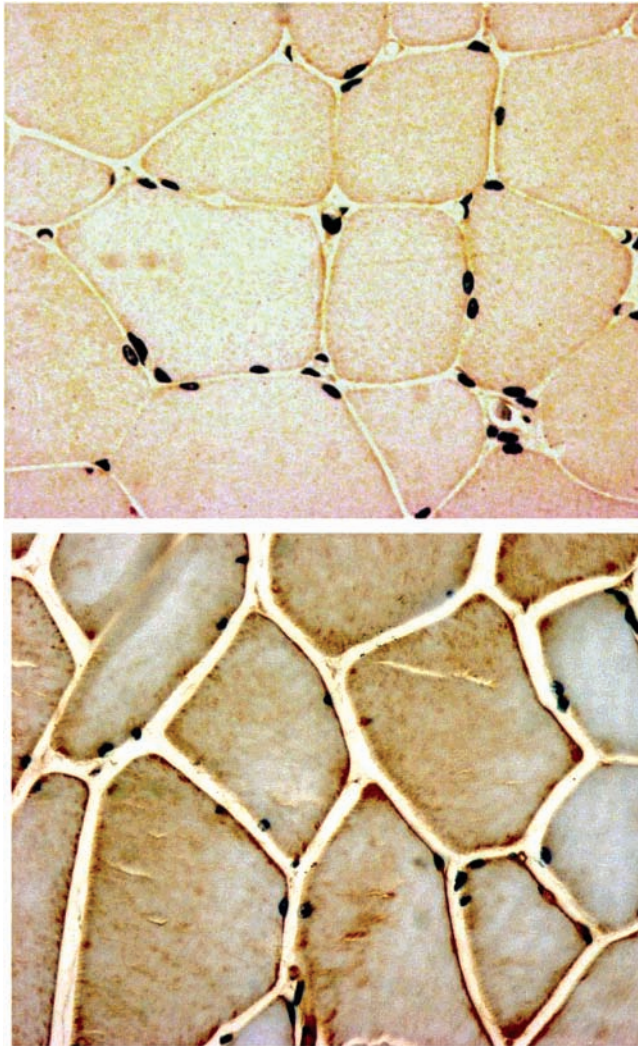
Figure 4. Immunohistochemical examination of iPLA$_2$-VIA localization in pig LD. Sections (4 μm) of paraffin-imbedded muscle samples from DLY pigs (0 h post-mortem (top) and 4 h post-mortem (bottom)) were stained for iPLA$_2$-VIA (red) and counterstained with haematoxylin (blue).

of exsanguinations and 4 h post-mortem, were used for immunohistochemistry. Marked differences were consistently observed between 0 and 4 h post-mortem sections (**Figure 4**). At the time of exsanguinations muscle fibers were closely ordered with well-defined borders resembling the sarcolemma. iPLA$_2$-VIA staining was moderate and dispersed throughout the muscle fibers and at the sarcolemma membrane borders, suggesting cytoplasmic and sarcolemma localization. At 4 h post-mortem, significant morphological changes have occurred. Gaps between individual muscle fibers have opened and the membrane borders appeared less defined and irregular. iPLA$_2$-VIA staining increased inside muscle fibers and at the membrane surface.
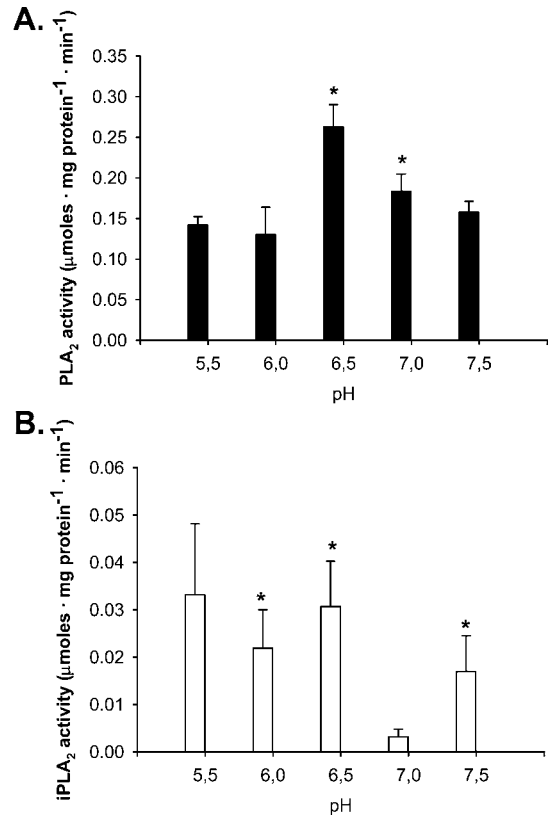
Figure 5. Effect of pH on PLA$_2$ activity in myotubes. Total PLA$_2$ activity (A) and BEL-sensitive iPLA$_2$ activity (B) in mouse C2C12 myotubes was estimated at pH 5.5–7.5 in 0.5 intervals in the presence and absence of 10 μM BEL. The number of experiments was 4, and data are displayed as mean $\pm$ SEM. The asterisk (*) indicates significance ($p < 0.05$) relative to pH 7.5 in panel A and relative to pH 7 in panel B.

pH Sensitivity of Skeletal Muscle PLA$_2$. During the post-mortem period, a gradual acidification of the muscle tissue takes place (*27*). To investigate the effect of pH changes on PLA$_2$ activity, the pH dependence of the skeletal muscle PLA$_2$ activity in the pH 5.5−7.5 range was estimated on protein isolated from C2C12 myoblasts (see Materials and Methods). Total PLA$_2$ activity was significantly increased at pH 6.5−7.0 compared to pH 7.5 with highest activity at pH = 6.5 (**Figure 5A**). iPLA$_2$ activity was lowest at physiological pH 7 and significantly increased at both higher pH (7.5) and lower pH (6.0−6-5) (**Figure 5B**).

iPLA$_2$-VIA Expression−Relation to Drip Loss. In a recent study, Young and co-workers (*27*) found that Landrace and Duroc pigs had different levels of drip loss and that 5 days creatine supplementation before slaughter affected the drip loss differently in the two breeds. To investigate a possible link between iPLA$_2$-VIA and drip loss and explore the possible use of iPLA$_2$-VIA as a marker for high/low drip loss from pork, we measured the mRNA expression of iPLA$_2$-VIA in samples from control and creatine-supplemented pigs. **Figure 6A** shows the drip loss (data reproduced from (*27*)) and basal iPLA$_2$-VIA mRNA level in Landrace and Duroc pigs. As seen, drip loss was about 20% higher in Landrace pigs compared to Duroc pigs, whereas iPLA$_2$-VIA expression was 20% lower in Landrace compared to Duroc pigs. When supplemented with creatine 12.5, 25, and 50 g/d for 5 days drip loss was modestly increased in Landrace pigs, whereas drip loss was significantly reduced in Duroc pigs (**Figure 6B**, data reproduced from (*27*)). iPLA$_2$-VIA mRNA was significantly decreased in Landrace pigs

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A.

B.

C.
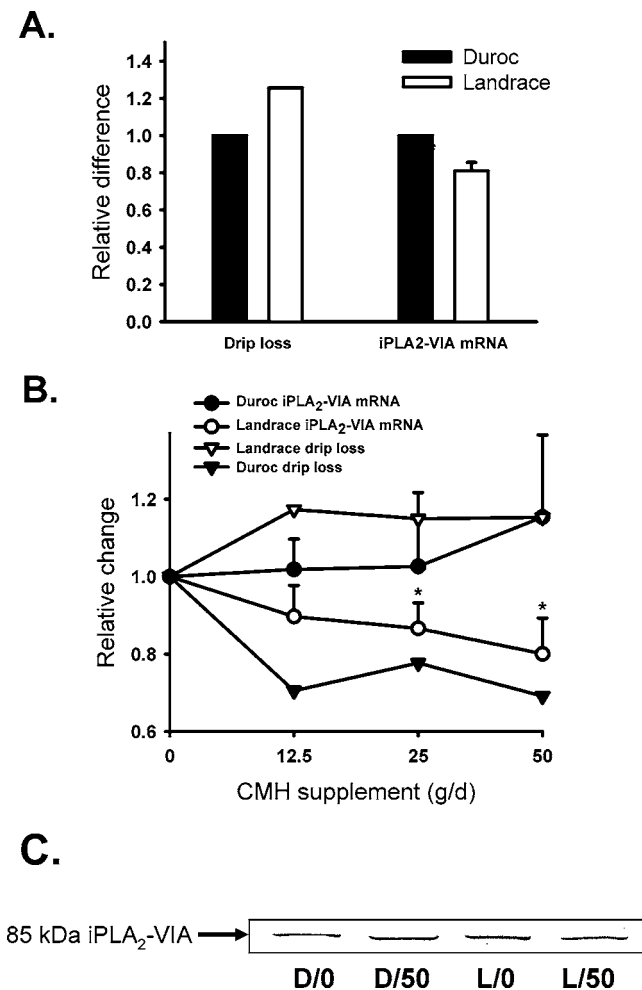
Figure 6. iPLA$_2$-VIA expression levels in relation to changes in drip loss from LD of Duroc and Landrace pigs. (**A**) Relative amount of drip loss (reproduced from (*27*)) and iPLA$_2$-VIA mRNA expression level in Landrace compared to Duroc pigs. (**B**) Effect of creatine supplementation (CMH (g/d)) on drip loss (reproduced from (*27*)) and iPLA$_2$-VIA mRNA expression in Duroc and landrace LD. Results are average ± SEM of samples from 10 pigs of each breed. The asterisk (*) indicates significant difference (*p* < 0.05) relative to time 0 h controls. (**C**) Representative Western blot (*n* = 5) showing iPLA$_2$-VIA protein levels in Duroc (D/0) and Landrace (L/0) LD and the effect of supplementation with 50 g/d creatine (D/50 and L/50).

but moderately increased in supplemented Duroc Pigs (**Figure 6B**). At the protein level, there were no measurable differences in the level of iPLA$_2$-VIA protein between breeds or after supplementation with 50 g/d of creatine (**Figure 6C**). Thus, a tendency toward an inverse correlation between drip loss and iPLA$_2$-VIA mRNA levels is evident; however, this is not clearly manifested at the protein level. In addition, no significant changes in protein expression of the AA-metabolizing enzymes COX-1, COX-2, and 5-LO were detected between pig breeds or following supplementation (data not shown). Thus, iPLA$_2$-VIA, COX, or 5-LO expression levels are apparently not a determining factor for drip loss generation; however, expression levels may not reflect the true enzymatic activity.

DISCUSSION

The water-holding capacity (WHC) of meat, i.e., its ability to retain water, is reported to have an impact on the appearance of the pork and on the consumer's perception of its quality as well as influencing processing yield (*3, 28*). The post-mortem

condition in muscle fibers is characterized by hormonal stimulation and ischemic-induced hypoxia following slaughter. Hypoxia is associated with increases in intracellular [Ca^{2+}] and [Na$^+$], ATP-depletion, acidification, oxidative stress, and activation of proteases and phospholipases (*4*).

Earlier studies performed on mouse C2C12 and primary porcine myotubes have indicated that activation of PLA$_2$ is an initial upstream event that leads to modulation of the permeability of the sarcolemmal membrane toward osmolytes (*2, 3*). This work implies that osmotic cell swelling as a result of metabolite accumulation in porcine muscle induces release of the organic osmolyte taurine and osmotically obliged water as part of a volume restoring process. Taurine release involves sequential activation of a PLA$_2$, mobilization of AA, followed by oxidation via the 5-lipoxygenase system to secondary messengers (*3*). Taurine is found in the drip loss from porcine LD (*3*), and taurine release from porcine myotube cultures following osmotic cell swelling and during anoxia has likewise been demonstrated (*2*). In a recent study we demonstrated that simulation of the post-mortem situation in C2C12 myotubes induced a specific, time-dependent increase in iPLA$_2$-VIA activity which was accompanied by an increase in iPLA$_2$-VIA protein and changes in its subcellular localization (Poulsen, K. A.; Pedersen, S. F.; Kolko, M.; Lambert, I. H. Manuscript in preparation).

Bertram and co-workers previously demonstrated that porcine LD swell within the first 4 h post-mortem most likely due to osmotic water uptake from the extramyofibrillar compartment caused by osmolyte and metabolite accumulation (*5*). During this initial phase the membrane structures appeared to be almost intact. After 3−4 h, the initial cell swelling was followed by a phase where destabilization and disruption of membranes occurred and water was expelled from the myofibrillar space to the extramyofibrillar compartment probably as a result of longitudinal myofilament contraction and lateral myofilament shrinkage (*5*). The intracellular water, once it appears in the extracellular space, presumably forms the drip. As seen from **Figure 1**, total PLA$_2$ activity as well as iPLA$_2$ activity increased in porcine DLY LD within the initial 4 h post-mortem. Total PLA$_2$ activity increased by approximately 24 nmol·mg protein^{-1}·min^{-1} and iPLA$_2$ activity by 11 nmol. Peak PLA$_2$ activity was measured 4 h post-mortem and coincided with the time for disruption of the plasma membrane and onset of the water redistribution phase. The increase in iPLA$_2$ activity was found to be associated with increased mRNA and protein levels of the porcine iPLA$_2$-VIA. In contrast, the mRNA level of porcine iPLA$_2$-VIB remained constant throughout the 48 h post-mortem period, indicating that a selective activation and up-regulation of iPLA$_2$-VIA takes place. This is further supported by the morphological studies (**Figure 4**) which indicate that iPLA$_2$-VIA accumulate in the cytoplasm and in particular at the sarcolemma in what appears to be a punctuate, domain-like pattern. At 4 h post-mortem, gaps have been formed between muscle fibers and the membrane borders appear blurred, probably the result of membrane disruption, cell shrinkage, and a concomitant accumulation of water in the extramyofibrillar space. Together, these data indicate that iPLA$_2$-VIA activity could affect drip formation in post-mortem porcine muscle.

PLA$_2$ activity (**Figure 1**) was estimated at pH 7.4, which resembles the pH of an unpertubated muscle. However, a significant acidification takes place during the post-mortem progression. The pH in LD of Duroc and Landrace pigs drops to 6.55 and 6.48, respectively, at the time of slaughter, decreasing to 6.24 and 6.19 within 2 h post-mortem (*27*). From

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Figure 5 it is estimated that both total PLA$_2$ and iPLA$_2$ activity in C2C12 myotubes has a pH optimum of approximately 6.5. Thus, taking the initial acidification into consideration it is assumed that the PLA$_2$ activity within the first 0−4 h post-mortem, obtained at pH 7.4, is an underestimation of the actual PLA$_2$ activity in porcine LD.

Apart from its role in regulating initial osmolyte liberation from myotubes (*3*), activation of PLA$_2$s might affect drip loss in a more direct manner. The rate and degree of membrane disruption most likely depends on the integrity and fluidity of the phospholipid membrane. Stress-induced loss of phospholipids and elevated LPC levels may decrease membrane integrity and/or function. Activation of iPLA$_2$-VIA has been associated with break down of membrane phospholipids during ischemia-induced hypoxia, oxidative stress, and the cell death process (*16, 20, 29−31*). Conversely, activation of PLA$_2$s may also play protective roles against oxidative injury by release of damaging oxidized fatty acid from phospholipids (*32*). High oxidative stress, which is experienced in pig muscle at the time of slaughter and during the initial post-mortem phase, results in lipoperoxidation, a harmful process affecting membrane structure and function (*7*). Peroxidation predominantly occurs at mono- and polyunsaturated fatty aids that are esterified at the *sn-2* position of phospholipids and may alter the structure of the acyl chain (*32*). Repair of damaged phospholipids may be regarded as a special type of phospholipid remodeling that requires a PLA$_2$ with a broad substrate specificity, i.e., no headgroup or fatty acid preference, making the PLA$_2$ capable of cleaving different species of phospholipids not only with intact acetyl side chains but possibly also those containing short and medium length peroxidized acyl chains. iPLA$_2$-VIA has a broad substrate selectivity as well as *sn-1*, *sn-2,* and lysophospholipase activity (*33, 34*) and plays a role in membrane/lipid bilayer remodeling (*35*), thus making it a candidate for membrane repair functions. Recently it was suggested that iPLA$_2$-VIA is involved in mitochondrial membrane remodeling and repair under physiological conditions and that it protects mitochondria from oxidative damage during apoptosis induced by staurosporine (*36*). Thus, iPLA$_2$-VIA activity may have dual roles in cell injury: a phospholipid repair function which maintains membrane stability and a severe hydrolytic function which, maybe when cell death is inevitable, accelerates membrane breakdown. To further explore a possible damaging or protective function of iPLA$_2$-VIA during the post-mortem progression we measured mRNA and protein levels for iPLA$_2$-VIA in different pig breeds with variations in drip loss (*27*). Drip loss from Landrace pigs are about 20% larger compared to Duroc pigs, and supplementation with creatine 12.5, 25, and 50 g/d for 5 days before slaughter increases drip loss in Landrace pigs, whereas supplementation reduces drip loss from Duroc pigs. From **Figure 6A−C** it is seen that there is a tendency to increased levels of iPLA$_2$-VIA mRNA with decreased drip loss and visa versa, whereas at the protein level no clear correlation can be established. These data may indicate that iPLA$_2$-VIA is at the membrane for repair purposes and that increased iPLA$_2$-VIA activity may delay, but not prevent, membrane disruption. On the other hand, as the knock-down and overexpression data from C2C12 myotubes indicated that iPLA$_2$-VIA activation has a cell damaging effect under post-mortem-like conditions (Poulsen, K. A.; Pedersen, S. F.; Kolko, M.; Lambert, I. H. Manuscript in preparation), it is likely that increased presence of iPLA$_2$-VIA at the membrane, instead of repair, may facilitate membrane destruction. Although iPLA$_2$-VIA may have a role in determining the final amount of drip generated, variation in

the iPLA$_2$-VIA mRNA and protein expression level appears not to affect drip loss significantly and to be too small to be used as a parameter for breed selection.

In conclusion, PLA$_2$ activation and presumably iPLA$_2$-VIA may act at two levels in the muscle post-mortem, i.e., as previously suggested (*3*) indirectly during the initial phase attempting to control cell volume by mobilization of lipid second messengers from phospholipids, resulting in subsequent release of osmolytes and osmotically obliged cell water, and more directly by modulation of the phospholipid composition of the cellular membranes rendering them less or more susceptive to rupture. The notable timing between iPLA$_2$-VIA activity, mRNA and protein regulation, iPLA$_2$-VIA localization, and the physical loss of muscle membrane barrier in porcine muscle post-mortem points to a significant role for this enzyme in the generation of drip loss. Although a weak inverse correlation between drip loss and iPLA$_2$-VIA mRNA levels seems to exist, the damaging or protective consequence of iPLA$_2$-VIA activation in post-mortem porcine muscle remains to be established.

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