**Lysosomal Phospholipase A2 Activity in Pig Aqueous Humor**

**Akira Abe, Miki Hiraoka, Shuichiro Inatomi, Ikuyo Ogburo and Hiroshi Ogburo**

**PURPOSE.** The present study was conducted to determine lysosomal phospholipase A2 (LPLA2) activity in the aqueous humor (AH) and to identify the possible sources of the LPLA2 found in the AH.

**METHODS.** To detect LPLA2 activity in pig AH and ocular tissues, liposomes consisting of 1,2-dioleoylphosphatidylglycerol/N-acetylsphingosine were used as substrates in an activity assay under acidic conditions. The reaction products were separated by thin-layer chromatography. To identify the LPLA2 in pig AH, the AH was analyzed by Western immunoblot analysis with an anti-LPLA2 antibody. Distribution of the LPLA2 in pig ocular tissues was studied by determining its activity in individual tissue extracts.

**RESULTS.** LPLA2 activity was detected in the AH obtained from pig eyes. Consistent with the known properties of LPLA2, the activity was heat-labile and undetectable at neutral pH. The immunoblot of pig AH showed the anti-LPLA2 antibody–reactive protein band. In addition, the specific activity of the enzyme, when normalized to volume, was higher in pig AH than in pig serum. Individual tissue extracts obtained from pig ocular tissues showed different specific activity of LPLA2. In particular, the extract prepared from the trabecular meshwork provided the highest specific activity.

**Conclusions.** The present findings suggest that the phospholipase A2 activity found in pig AH under acidic conditions is due to LPLA2 and that it originates from ocular tissues surrounding the anterior chamber as well as plasma. (Invest Ophthalmol Vis Sci. 2012;53:152–156) DOI:10.1167/iovs.11-7891

In the normal eye, the aqueous humor (AH) must be constantly cleaned to maintain normal aqueous outflow. Failure to eliminate aged or defective cells, cell particles, and foreign materials in the AH may reduce optical clarity, interfere with aqueous circulation, and result in abnormal aqueous outflow and an increase in intraocular pressure (IOP). Abnormal IOP evokes optical and refractive disorders of the eye and in some cases is associated with ocular diseases such as glaucoma.

In the anterior segment, the corneal endothelium, ciliary body epithelium, and trabecular meshwork play a role in the degradation of phospplipid-associated debris contained in the AH. However, there has been no publication reporting the presence of LPLA2 in the AH or the ocular tissues surrounding the anterior chamber, although other lysosomal enzymes such as proteases, glycosidases, phosphatases, and lipases have been investigated.

In the present study, the LPLA2 activity in pig AH and ocular tissues was investigated with an LPLA2-specific assay system, which includes liposomes consisting of 1,2-dioleoylphosphatidylglycerol/N-acetylsphingosine (NAS) under acidic conditions. In addition, a rabbit polyclonal antibody against human LPLA2 was used to identify the LPLA2 protein in the AH.

**Materials and Methods**

**Reagents**

1,2-Dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DOPG), NAS, and N-octeylsphingosine were obtained from Avanti Polar Lipids Corp. (Alabaster, AL); the BCA protein assay reagent was from Thermo Fisher Scientific KK (Tokyo, Japan); HPTLC silica gel plates, 10 × 20 cm, were from Merck (Darmstadt, Germany); the protein A, 1-mL column (Hi-Trap) was from Pharmacia (Uppsala, Sweden); the POD immunostain (RAM Trap) was from Pharmacia (Uppsala, Sweden); the POD immunostain was from Wako Pure Chemical Industries (Osaka, Japan); anti-

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Submitted for publication May 17, 2011; revised August 22 and November 8, 2011; accepted November 24, 2011.

Disclosure: A. Abe, None; M. Hiraoka, None; S. Inatomi, None; I. Ogburo, None; H. Ogburo, None

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In the anterior segment, the corneal endothelium, ciliary body epithelium, and trabecular meshwork show phagocytic activity and various lysosomal enzymatic activities. In particular, trabecular meshwork cells are known to be actively phagocytic and to digest ingested particles such as zymosan, suggesting that the lysosomal enzyme in the cells is responsible for the removal of the debris that collects in the aqueous flow system.

A decade ago, a novel enzyme was discovered in the lysosomal fraction of MDCK cells with dual enzyme activity, transacylase and phospholipase A2, in acidic conditions. The enzyme was purified, cloned, sequenced, and expressed, and named lysosomal phospholipase A2 (LPLA2). The enzyme purified from calf brain is a water-soluble glycoprotein consisting of a single polypeptide chain with a molecular weight of 45 kDa with Ca\(^{2+}\)-independent phospholipase A2 activity at an optimal pH of 4.5. LPLA2 belongs to the \(\alpha\)-hydroxylase superfamily, has 49% protein sequence identity to lecithin cholesterol acyltransferase, and is now classified as phospholipase A2 group XV. In mouse and rat, LPLA2 is highly expressed in phagocytic cells such as macrophages. As shown in alveolar macrophages, LPLA2 is secreted similar to other lysosomal enzymes. LPLA2-null mice showed that LPLA2 is involved in the digestion of intracellular and exogenously presented glycerophospholipids in alveolar macrophages. In addition, macrophages from LPLA2-null mice failed to digest engulfed apoptotic bodies, indicating that this impairment is due to the absence of LPLA2. Thus, LPLA2 activity may be necessary for the catabolism of glycerophospholipids taken in the phagocytic cells.
LAM-1 mouse monoclonal antibody was from Santa Cruz Biotechnolog-
y (Santa Cruz, CA); anti-mouse immunoglobulins goat polyclonal
antibody, HPR-conjugate, was from Dako (Kyoto, Japan); and anti-
rabbit IgG goat polyclonal antibody, HPR-conjugate, was from MP
Biomedicals (Tokyo, Japan). Rabbit polyclonal antibody against human
LPLA2 was the generous gift of Takeda Pharmaceutical Company, Ltd.
(Osaka, Japan).

Collection of Pig AH

Fresh pig eyeballs purchased from an abattoir were immediately
cooled and processed by the common practice of cooling the eyes on
ice. The AH was collected by piercing the cornea with a 26-gauge
needle and withdrawing the solution. The solution was subsequently
centrifuged at 20,000g for 10 minutes at 4°C. The resultant supernatant
was used for the enzyme assay.

Lactate Dehydrogenase Assay

The reaction velocity was determined by a decrease in absorbance at
340 nm, resulting from the oxidation of NADH. One unit (U) is defined
as the oxidation of NADH of 1 micromole/min at 25°C. The reaction
mixture, containing 125 mM Tris-HCl (pH 7.4), 0.22 mM NADH, and 1
mM sodium pyruvate in a total volume of 1 mL was preincubated for
4 minutes at 25°C. The reaction was initiated by the addition of 25 µL
of pig AH.

Transacylase Activity of LPLA2

Preparation of Liposomes. DOPG and NAS were mixed in a
glass tube and dried in a stream of nitrogen gas. The dried lipid mixture
was dispersed into 50 mM sodium citrate (pH 4.5) by a probe-type
sonicator for 8 minutes in an ice-water bath.

Transacylase Assay. The reaction mixture consisted of 48 mM
sodium citrate (pH 4.5), 10 µg/mL BSA, 38 µM NAS incorporated into
127 µM DOPG liposomes, and pig AH serum or ocular tissue extract in
a total volume of 500 µL. The reaction was initiated by adding the
tissue and cell homogenates and plasma under acidic con-
ditions is specifically catalyzed by LPLA2 enzymatic activity.22 In the measure-
ment, DOPG and NAS, which form anionic liposomes, were
use as an acyl group donor and an acyl group acceptor,
respectively (Fig. 1A). In the present study, we applied the
same assay method to pig AH and ocular tissue extracts.

When the AH was incubated with liposomes consisting of
DOPG/NAS for 60 minutes at pH 4.5, two reaction products
were produced (Fig. 1B). One reaction product corresponded to
acylated NAS on TLC and was alkaline-unstable (Fig. 4), which is 70-fold
higher than that of pig serum. The formation of acylated NAS
in the AH is due to LPLA2.

RESULTS

Transacylase (LPLA2) Activity in Pig AH

Recently, a method for detecting LPLA2 activity in extracellular
fluid of humans was established by measuring the transacylase activity of LPLA2 under acidic conditions.22 In the measure-
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Lactate Dehydrogenase in Pig AH

Lactate dehydrogenase (LDH) is a cytosolic enzyme and a
useful marker to assess cell membrane damage. To assess pig
ocular tissue damage, the lactate LDH activity of pig AH was
examined. The activity was 10.3 ± 3.11 U/L (mean ± SD; n = 4), which is a much lower value than that (115 U/L) reported
by Wurster et al.26

LPLA2 Activity in Pig Serum and Ocular Tissues

In the transacylase activity assay, human plasma and serum
induce formation of insoluble aggregates in the presence of
DOPG/NAS liposomes under acidic conditions.22 By contrast,
pig AH did not induce aggregate formation under the same
conditions. This difference is possibly due to the lower protein
congestion in the AH. The protein concentration of pig AH
was 1.06 ± 0.14 mg/mL (mean ± SD; n = 4), which is 70-fold
lower than that of pig serum. The formation of acylated NAS

Preparation of the Tissue Extract from Pig
Ocular Tissues

For the preparation of pig ocular tissue homogenates, each tissue was
washed with cold phosphate-buffered saline (PBS), weighed, and ho-
genized in a Potter-Elvehjem-type homogenizer with cold 0.25 M
sucrese, 10 mM HEPES (pH 7.4) and 1 mM EDTA, to obtain a 10% homogenate. The homogenate was sonicated five times in a probe-type
sonicator for 10 seconds at 0°C and centrifuged for 10 minutes at
20,000g at 4°C. The resultant supernatant was used as the tissue
extract in the LPLA2 activity assay.

Western Immunoblot Assay

To remove the IgG in pig AH, which cross-reacts with a goat polyclonal
antibody against rabbit IgG, 2.8 mL of pig AH was applied to a
prepacked protein A column (HiTrap Protein A, 1 mL) that had been
pre-equilibrated with 20 mM sodium phosphate (pH 7.4) and washed
with 5 mL of the same buffer in a cold chamber. The unbound effluent
was collected in one tube and divided into four glass tubes (13 × 100
mm). Each effluent was precipitated by the method of Bensadoun and
Weinstein.23 The resultant pellet was dissolved with 30 µL of loading
buffer consisting of 125 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol,
2% 2-mercaptoethanol, and 10 µg/mL bromophenol blue in addition to 1.5 µL of 2 M Tris for SDS-polyacrylamide gel electrophoresis. Proteins
were separated on a 12% acrylamide gel and transferred to a polyvi-
nylidene difluoride membrane (biotin Gel Transfer System; Invitrogen,
Carlsbad, CA). The membrane was incubated with or without the
rabbit polyclonal antibody against human LPLA2. The antigen-antibody
complex on the membrane was visualized with an anti-rabbit IgG
HRP-conjugated goat antibody using the POD immunoblast set, includ-
ing NADH, nitrotetraizolium blue, and hydrogen peroxide.

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increased linearly with time and AH concentration (Figs. 2). In addition, the LPLA2 activity in a given volume of pig AH was significantly higher than that observed in the same volume of pig serum (Fig. 3A).

To compare the difference between the specific activities of LPLA2 in pig ocular tissues, we examined the supernatant prepared from ocular tissue homogenates centrifuged at 20,000g. As shown in Figure 3B, LPLA2 activity was detected in all supernatants tested, except the lens. The supernatants obtained from the homogenates of the iris/ciliary body mixture and the choroid showed relatively higher LPLA2 specific activity than the supernatants obtained from other ocular tissues such as the cornea, sclera, lens, and retina. The highest specific activity of LPLA2 was found in the preparation obtained from the trabecular meshwork.

**DISCUSSION**

In the present study, we used the LPLA2-specific assay method and Western immunoblot analysis to show that the phospholipase A2 activity found in pig AH under acidic conditions is due to LPLA2 (Fig. 1).

To confirm that LPLA2 is not due to the rupture of cells, but that it is specifically secreted, a nonlysosomal enzyme activity and the protein level of other lysosomal protein were investigated. LDH is a cytosolic enzyme and a useful marker for assessing cell membrane damage. The LDH activity of pig AH was 10.3 ± 3.1 U/L (mean ± SD; n = 4), which is about one tenth of that reported by Wurster et al. They pointed out that the high value of LDH activity in their pig AH may be due to the special mode of killing the animal (electrocution). Also, the...
LDH activities in the AHs of six other mammalian species (no electrocution) ranged from 3.6 U/L (dog) to 8.5 U/L (rabbit). The value of our pig AH is close to that of those mammalians. Furthermore, we did not detect LAMP-1 in pig AH by Western immunoblot analysis (data not shown). LAMP-1 is a lysosomal membrane-associated glycoprotein. Taken together, these results show that the LPLA2 activity found in the AH in this study was not due to the damage or injury of ocular tissues and cells. At this stage, the source of the LPLA2 found in the AH is still unknown. Although the proteins found in AH are thought to come from plasma, there are some differences in the protein content and component between AH and plasma. Actually, the LPLA2 activity detected in a specific volume of pig AH was significantly higher than that of pig serum (Fig. 3A). In addition, the activity of LPLA2 was found in the extracts prepared from most ocular tissues, except for the lens (Fig. 3B). A similar distribution of lysosomal enzyme was reported for three lysosomal glycosidase activities in hog serum, AH, and ocular tissues. As shown Figure 3B, the iris/ciliary body mixture, trabecular meshwork, and choroid had a higher specific activity of LPLA2 than other tissues. In particular, the trabecular meshwork provided the highest specific activity. When the ciliary body epithelium and trabecular meshwork cells are challenged by phagocytic stimuli, the release of their lysosomal
enzyme into the extracellular space is enhanced.\textsuperscript{2,21} Taken together, the present findings indicate that the LPLA2 activity found in the AH may originate from plasma and ocular tissues surrounding the anterior chamber, in particular, the ciliary body and trabecular meshwork.

As shown in Figure 1 and 2, LPLA2 is active at acidic pH. Therefore, under physiological conditions, the degradation of glycerophospholipids and glycerophospholipid-associated materials ingested from the AH to phagocytic cells with high LPLA2 activity such as the trabecular meshwork cells is thought to be caused by LPLA2 in their phagosomes fused with lysosomes. Interestingly, it has been reported that LPLA2 shows an esterase activity against a small ester molecule over the wide range of pH.\textsuperscript{24,28} Thus, the LPLA2 found in the AH may contribute to the degradation of some small ester molecules in the AH.

A balance between the AH production at the ciliary body and the AH drainage through the trabecular meshwork is important to keep the normal IOP. A disorder or dysfunction of the trabecular meshwork may block the aqueous outflow system from the anterior chamber to Schlemm’s canal and induce an abnormal IOP, causing a certain type of glaucoma. The secretion and uptake of lysosomal phospholipase A2 by aleveolar macrophages. J Biol Chem. 1998;273:8467–8474.


Acknowledgments

The authors thank James A. Shayman for helpful suggestions and editorial review of this article and Robert Kelly for kindly reading and providing careful advice on the manuscript.

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