RESEARCH REPORTS
Biological

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Expression of Resolvin D1 Biosynthetic Pathways in Salivary Epithelium

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APPENDIX

MATERIALS & METHODS

Description of Age-matched C57BL/6 and NOD/ShiLtJ Mice

Female C57BL/6 and NOD/ShiLtJ Sjögren's syndrome (SS) mouse models were utilized as controls and experimental animals, respectively. The NOD/ShiLtJ mouse strain displays an autoimmune exocrinopathy similar to that of SS (Cha *et al*., 2002a,b). For the studies outlined here, we used mice at the preclinical phase (3-6 wks) and at disease onset (20 wks) (Yamano *et al*., 1999). Mouse submandibular glands (mSMG) were removed and used for frozen sections, tissue lysates, and cell dispersion.

Frozen Sections

mSMG and human minor salivary glands (hMSG) histological sections (10 μm) were prepared at The University at Buffalo, Histological Services, Department of Pathology and Anatomical Sciences. Sections were used for grading and focus score, as well as for the confocal analyses described below.

Grade and Focus Score

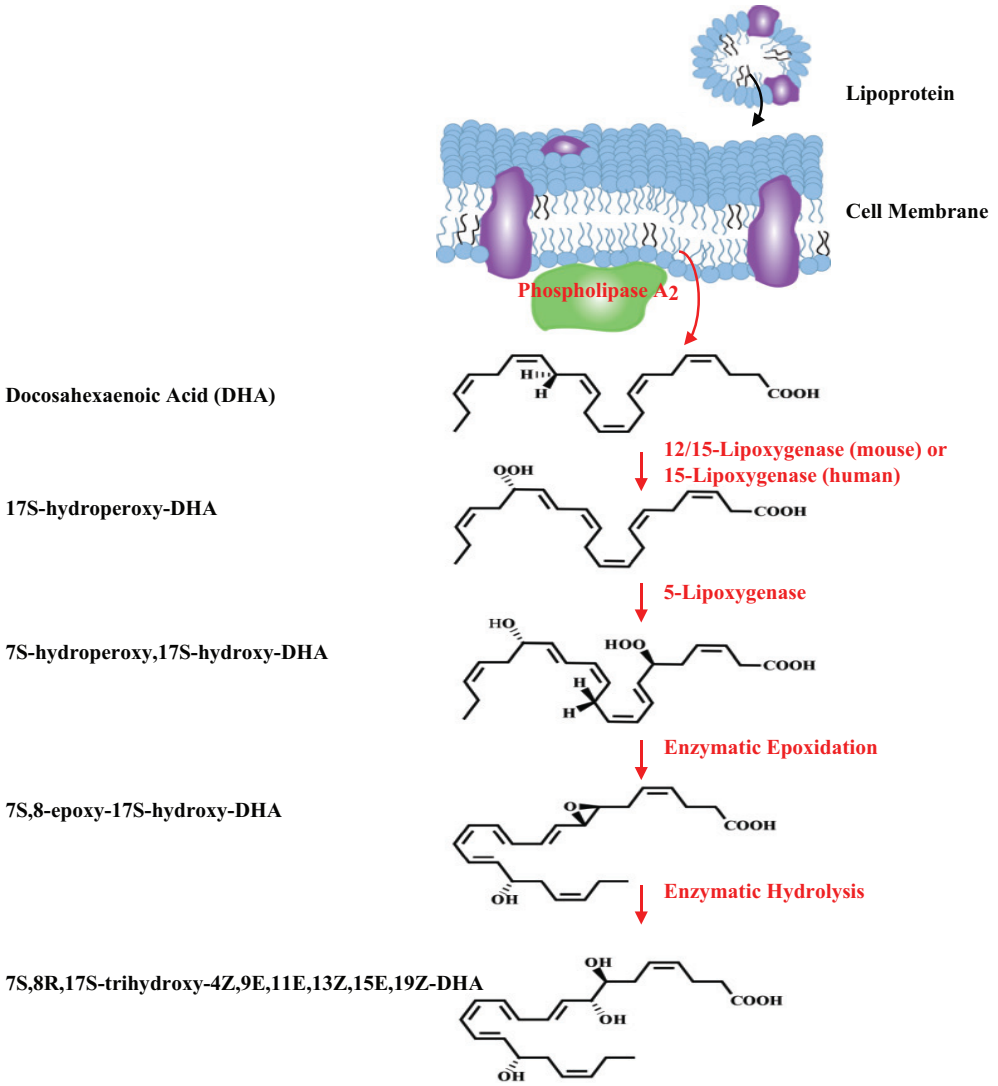
hMSG sections were stained with hematoxylin & eosin, mounted, and examined for lymphocytic infiltration by means of an Olympus BH-2 microscope (Olympus Optical Company, LTD., Tokyo, Japan). Grading of hMSG histological sections was performed as described by Chisholm and Mason (1968): 0, absent; 1, slight infiltrate; 2, moderate infiltrate of less than one focus/4 mm^2; 3, one focus *per* 4 mm^2; and 4, more than one focus *per* 4 mm^2. Focus score was performed as described previously (Greenspan *et al.*, 1974), where number of foci is (1-12) *per* 4 mm^2. For our studies, we used two groups as follows: (a) a non-SS hMSG group in which we used 2 specimens with microscopic features consistent with those of normal salivary glands (*i.e*., grade 0 and focus score 0) and 2 specimens with microscopic features consistent with those of mild chronic sialadenitis (*i.e.*, grade 3 and focus score 1); and (b) a SS human hMSG group in which we used 4 hMSG with microscopic features consistent with those of SS salivary glands (*i.e*., grade 4 and focus score 2-9).

Whole-tissue Lysates

mSMG from C57BL/6 and NOD/ShiLtJ (at 5 and 20 wks of age) underwent lysis in 600 μL of 2× Laemmli buffer [120 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 1 mM dithiothreitol, and 0.002% (w/v) bromophenol blue], sonicated for 60 sec with a Sonic Dismembrator (model 120; microtip; amplification 70%; Fisher Scientific, Pittsburgh, PA, USA), and boiled for 5 min. These lysates were used for Western blot analyses of $cPLA_2$, $iPLA_2$, 12/15-LOX, and 5-LOX.

Cell Dispersion

Freshly isolated mSMG cells from C57BL/6 mice (at 3-6 and 20 wks of age) and NOD/ShiLtJ (at 5 and 20 wks of age) were prepared as described in our previous studies (Odusanwo *et al*., 2012). Briefly, glands were minced in dispersion medium consisting of Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (1:1) (Hyclone, Logan, UT, USA) and 0.2 mM $CaCl_2$, 1% (wt/vol) bovine serum albumin (BSA), 50 units/mL collagenase (Worthington Biochemical, Freehold, NJ, USA), and 400 units/mL hyaluronidase at 37°C for 30 min with aeration (95% air-5% CO_2). Cell aggregates in dispersion medium were suspended by pipetting at 20 and 30 min. The dispersed cell aggregates were washed with enzyme-free assay buffer (120 mM NaCl, 4 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, and 15 mM N-2-hydroxyethy lpiperazine-N′-2-ethanesulfonic acid [HEPES], pH 7.4) containing 1% (wt/vol) BSA and filtered through a nylon mesh. Cells were washed again in DMEM/Ham's F12 (1:1) containing 2.5% (v/v) fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and the following supplements: 0.1 μM retinoic acid, 80 ng/mL epidermal growth factor, 2 nM triiodothyronine, 5 mM glutamine, 0.4 μg/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, and freshly added 100 μg/mL Normocin™ (InvivoGen, San Diego, CA, USA). mSMG freshly isolated cells were centrifuged at 1,000 rpm, and washed twice at room temperature for 5 min each.

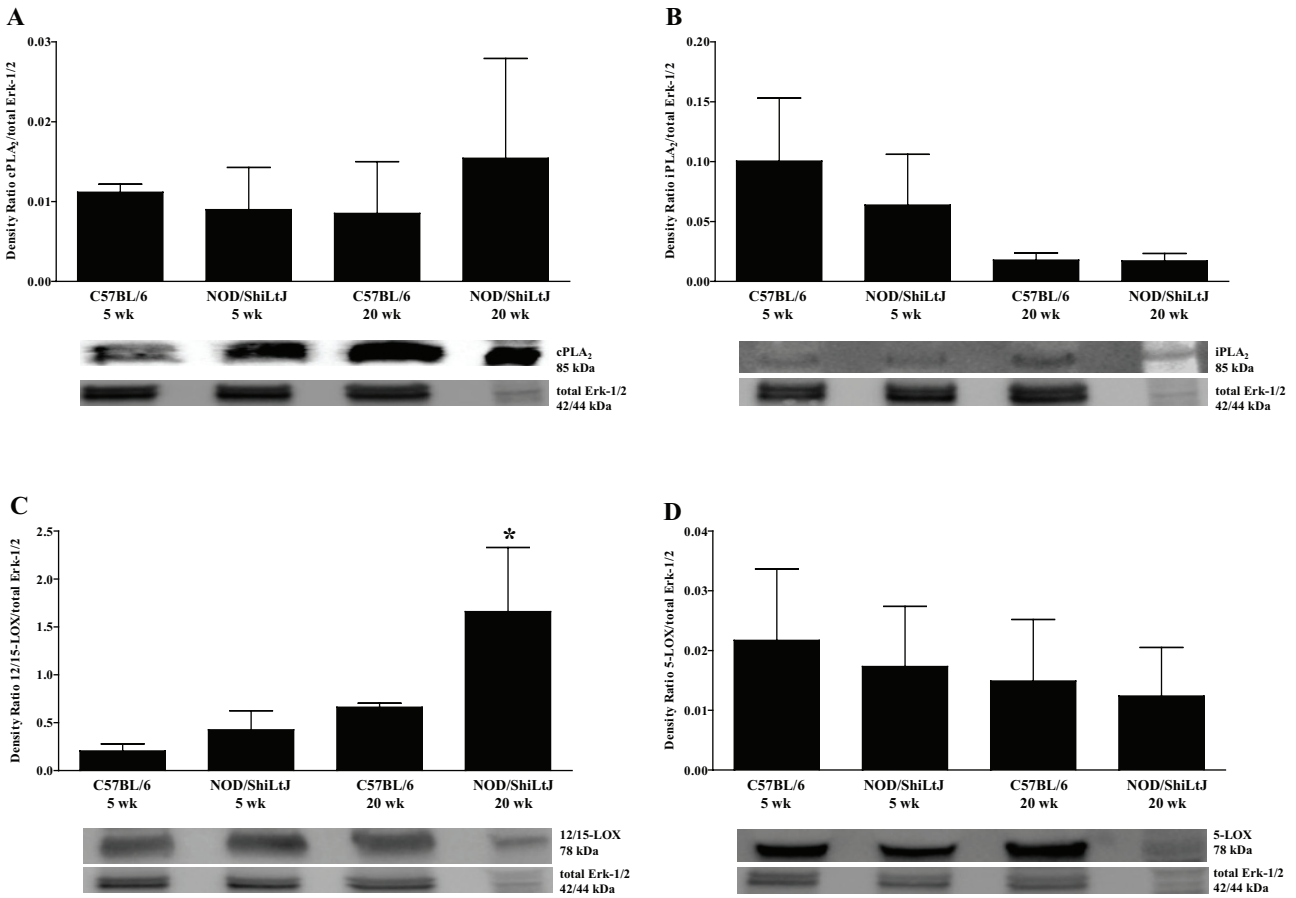


Appendix Figure 1. Resolvin D1 (RvD1) biosynthetic pathway in salivary glands. Docosahexaenoic acid (DHA) (black tails) is released from the cell membrane by the action of PLA_2 *via* a membrane-dependent mechanism. Alternatively, free DHA is released from lipoproteins in its free form *via* an exudate-dependent mechanism. DHA is then oxygenated *via* 12/15-LOX or 15-LOX type-1 and converted into 17S-hydroxy-DHA. Furthermore, 17S-hydroxy-DHA is oxygenated *via* 5-LOX into 7S-hydroperoxy-17S-hydroxy-DHA, followed by enzymatic epoxidation into 7S,8-epoxy-17S-hydroxy-DHA and converted into 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-DHA (Resolvin D1). [For complete Resolvin D1 stereochemical assignment, see Sun *et al.* (2007)].

Cells were cultured at 37°C in a humidified atmosphere of 95% air-5% CO_2 and used for qPCR and Western blot analyses of biosynthetic enzymes (*e.g.*, $cPLA_2$, $iPLA_2$, 12/15-LOX, and 5-LOX) as well as resolvin D1 (RvD1) detection at specified time-points.

qPCR Analysis

Total RNA from mSMG cells was extracted by means of an RNeasy tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA quantity was assessed with a BioTek Epoch microplate spectrophotometer and corresponding software Gen 5 Ver. 1.11.5 (BioTek, Winooski, VT, USA) and diluted to 30 ng/μL in RNA-free water. Then, RNA purity was assessed by measurement of A260/A280 in a BioTek Epoch microplate spectrophotometer. Total RNA (30 ng) was reverse-transcribed to cDNA with the RT^2 First Strand kit (Qiagen). A customized PrimePCR assay was used to detect RvD1 biosynthetic machinery gene expression as recommended by the manufacturer (Bio-Rad, Hercules, CA, USA). The cycling protocol was performed as follows: denaturing at 95°C for 2 min, followed by 40 cycles of denaturing at 95°C for 5 sec and annealing/extension at 60°C for 30 sec. A melt curve at 65°C to 95°C for 5 sec/step (increments of 0.5°C) followed the cycling protocol. Gene expression was normalized against glyceraldehyde 3-phosphate dehydrogenase and β-actin with CFX



Appendix Figure 2. Resolvin D1 (RvD1) biosynthetic machinery expression in mouse submandibular glands (mSMG) with and without Sjögren's syndrome. C57BL/6 and NOD/ShiLtJ mSMG underwent lysis. Then, protein expression of **(A)** $cPLA_2$, **(B)** $iPLA_2$, **(C)** 12/15-LOX, **(D)** 5-LOX, and total Erk-1/2 was detected by Western blot analyses as described in Appendix Materials & Methods. Data are expressed as the means ± SEM of results from 3 or more experiments, where $*p < .05$ represents a significant difference from C57BL/6 mice. The *t* test analyses resulted in the following values: (A) 5 wk *vs.* 5 wk $t(4) = 0.2796$, $p = .8289$, and 20 wk *vs.* 20 wk $t(4) = 0.1923$, $p = .7688$; (B) 5 wk *vs.* 5 wk $t(4) = 1.040$, $p = .2390$, and 20 wk *vs.* 20 wk $t(4) = 1.490$, $*p = .0081$; (C) 5 wk *vs.* 5 wk $t(4) = .4039$, $p = .0719$, and 20 wk *vs.* 20 wk $t(4) = 0.4912$, $p = .4250$; and (D) 5 wk *vs.* 5 wk $t(4) = 0.5450$, $p = .7865$, and 20 wk *vs.* 20 wk $t(4) = 0.0711$, $p = .9750$. Linear contrast enhancements of the images were made for aesthetic purposes, and scoring was performed on the original Western blot images.

manager software (Bio-Rad). Positive PCR controls and reverse transcription controls were used in this study. The array results were confirmed by real-time PCR using gene-specific primer pairs in triplicates.

Western Blot Analyses

The following antibodies were prepared in 3% BSA: rabbit anti-cytosolic phospholipase A_2 ($cPLA_2$) (1:200, Cell Signaling, Danvers, MA, USA), rabbit anti-calcium independent phospholipase A_2 ($iPLA_2$) (1:200, Cayman Chemical Company, Ann Arbor, MI, USA), rabbit anti-12/15-lipoxygenase (LOX) (1:200, Abcam, Cambridge, MA, USA), and rabbit anti-5-LOX (1:200, Cayman Chemical Company). Peroxidase-linked goat anti-rabbit IgG antibody (1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for $cPLA_2$, $iPLA_2$, 12/15-LOX, and 5-LOX detection. For signal normalization, membranes were treated with stripping buffer (Pierce Biotechnology, Rockford, IL, USA) and re-probed with rabbit total anti-extracellular signal-regulated kinases 1/2 (total Erk-1/2, 1:500; Santa Cruz Biotechnology), followed by incubation with a peroxidase-linked goat anti-rabbit IgG antibody (1:5,000, Santa Cruz Biotechnology). The membranes were treated with Clarity™ chemiluminescence detection reagent (Bio-Rad). Protein bands were then visualized and quantitated in a ChemiDoc® MP/Image Lab v4.1 system (Bio-Rad).

Confocal Microscopy Analyses

The following primary antibodies were prepared in 5% goat serum: rabbit anti-$cPLA_2$ (1:50, Cell Signaling), rabbit anti-$iPLA_2$, rabbit anti-5-LOX, and rabbit anti-12/15-LOX (1:50, all from Cayman Chemical Company). Sheep anti-15-LOX type-1 (1:50, Cayman Chemical Company) was diluted with 5%

donkey serum. The following secondary antibodies were utilized: AlexaFluor 488-conjugated goat anti-rabbit or AlexaFluor 488-conjugated donkey anti-goat (1:200). Tissues were stained with either Hoechst nuclear stain (1:20,000, Sigma Aldrich, St. Louis, MO, USA) or Propidium Iodide Nucleic Acid Stain (Invitrogen, Carlsbad, CA, USA). All images were captured with a Carl Zeiss 510 confocal microscope and analyzed with ZEN software (black edition; Carl Zeiss, Thornwood, NY, USA). Exposure times of secondary antibody controls (negatives) were matched to their corresponding experimental images.

RESULTS

RvD1 Biosynthetic Machinery Expression in Mouse Submandibular Glands

To determine whether inflammatory conditions (*i.e.,* SS) alter the expression of enzymes involved in RvD1 biosynthesis, we determined the levels of the RvD1 biosynthetic machinery in mSMG tissue lysates from age-matched C57BL/6 and NOD/ShiLtJ mice. We found that $cPLA_2$, $iPLA_2$, 12/15-LOX, and 5-LOX are expressed in salivary gland tissue lysates. We also compared expression levels of these enzymes in C57BL/6 and NOD/ShiLtJ mice. As shown in Appendix Figs. 2A, 2B, and 2D, no significant differences in expression of $cPLA_2$, $iPLA_2$, and 5-LOX were observed in these mice. However, 12/15-LOX protein expression levels in SMG from NOD/ShiLtJ mice increased significantly ($p = .0081$) as compared with those in C57BL/6 mice at the same age (Appendix Fig. 2C).

DISCUSSION

Our studies indicate that the machinery involved in RvD1 production is expressed in salivary glands. Although this is the first study of RvD1 biosynthetic machinery in salivary glands, the enzymes and substrates involved in RvD1 synthesis have been widely studied in other systems. For instance, PLA_2 family members are expressed in mammalian cells (Nicolas *et al.*, 1997). These enzymes are mostly localized as cytosolic and membrane-bound forms (Nakamura *et al*., 2010). Although PLA_2 family members have been implicated in the promotion of inflammation through mobilization of lipid mediators, the identity of PLA_2 subtypes acting upstream of anti-inflammatory lipid mediators remains unknown. The Murakami laboratory reported that secreted PLA_2 ($sPLA_2$) is preferentially expressed in dendritic cells and macrophages, and displays pro-resolving functions (Miki *et al.*, 2013). This group also provided evidence that a granule-associated $sPLA_2$ facilitates the maturation of mast cells by activating lipid mediator circuits (Taketomi *et al.*, 2013). The enzyme 12/15-LOX is the murine ortholog of human 15-LOX type-1 and oxygenates both free and phospholipid-bound polyunsaturated fatty acids (Kuhn and Thiele, 1999). 12/15-LOX is expressed in mouse epidermis (McDonnell *et al*., 2001), macrophages (Kriska *et al*., 2012), and platelets (Chen *et al.*, 1994). 15-LOX type-1 is primarily expressed in leukocytes and bronchial epithelial cells (Chanez *et al.*, 2002; Vachier *et al.*, 2005; Gulliksson *et al*., 2007). 5-LOX is mainly present in mature leukocytes including granulocytes, monocytes/macrophages, mast cells, and B-lymphocytes (Steinhilber, 1999).

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