## MATERIALS AND METHODS

**Sources of genetically modified mice and description of dietary intervention studies**. All animal studies were performed with the approval of the UTHSCSA Institutional Animal Care and Use Committee. $LDLR^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME). $iPLA_2\beta^{-/-}$ mice ($iPLA_2\beta$-KO), and their wild type littermates were generated by mating heterozygotes and maintained as previously described.[1-3] For dietary intervention studies, male $LDLR^{-/-}$ mice 8 wk of age were housed in colony cages and maintained on a 12-h light/12-h dark cycle. After one week on a standard chow maintenance diet (MD, 7% soybean oil, AIN-93G, F3156, BioServ), mice were randomized into two groups and fed either MD (control mice) or a Western-style high-fat diet (HFD; 21% milk fat and 0.15% cholesterol, AIN-76A, F5540, BioServ) for 8, 16, or 24 weeks. Evening body weights and blood glucose levels were monitored biweekly and at the end of the study using a Contour®meter (Bayer). Plasma was prepared from whole blood drawn by cardiac puncture at the time of animal sacrifice. Plasma cholesterol and triglyceride levels were determined using enzymatic assay kits (Wako Chemicals). Epididymal fat pads were isolated and weighed. HFD-fed $LDLR^{-/-}$ mice eventually developed hyperglycemia and hyperlipidemia (Table).

**Quantitative morphometric analyses of atherosclerotic lesions.** Heart and aortic arch were processed as described previously to prepare aortic sinus sections for staining and histological analyses.[4-7] Tissues were frozen in OCT compound (Tissue-Tek) and cut into sections (10 μm thickness) that were stained with Oil-Red O to visualize neutral lipids and counterstained with hematoxylin. Atherosclerotic lesion areas were determined in in 10 serial sections at 80 μm intervals that represented the entire length of each aortic sinus and quantified with Image-Pro Plus 6.0 software as previously described.[3, 5, 8, 9] Macrophages in atherosclerotic lesions were identified by immunohistochemical staining with a primary antibody against CD68 and quantified with Image-Pro Plus 6.0 software.

**Adenoviral vectors to drive overexpression of $iPLA_2\beta$ and Nox4**. An adenoviral construct encoding $iPLA_2\beta$ was prepared with a ViraPower adenovirus expression system (Invitrogen) according to the manufacturer's instructions.[10] Briefly, cDNA that encodes the 84-kDa $iPLA_2\beta$ was subcloned into the pENTR directional TOPO cloning vector.[11] After sequence verification, the $iPLA_2\beta$ cDNA was transferred into pAd/CMV/V5-DEST vector with the Gateway system using LR clonase (Invitrogen). Positive clones were confirmed by sequencing. The clones were linearized using PacI (New England Biolabs) and then transfected into 293A cells with Lipofectamine 2000 using Opti-MEM medium. Virus was prepared and amplified with the ViraPower adenoviral expression system (Invitrogen), and viral titers were determined by plaque-forming assays with 293A cells. An aliquot of viral suspension was used to infect mouse peritoneal macrophages, and $iPLA_2$ activity was assayed 3 days after infection. As a control, pAd/CMV/V5-GW/lacZ vector (Invitrogen) was transfected into 293A cells to produce lacZ-bearing adenovirus that did not contain the $iPLA_2\beta$ coding sequence. Adenovirus encoding wild type Nox4 (AdWTNox4) was generously provided by Dr. B. J. Goldstein (Merck, Sharp, & Dohme Corp.) Experiments *ex vivo* to examine whether overexpression of $iPLA_2\beta$ or Nox4 reversed effects of the inhibitors BEL and GKT137831, respectively, involved preincubating macrophages with the appropriate inhibitor, followed by removal of the medium and replacing it with fresh medium without inhibitor, rinsing the cells once with PBS (37°C), and then transfection with the adenoviral vectors described above.

**Isolation and culture of mouse peritoneal macrophages**. Resident peritoneal macrophages from male $LDLR^{-/-}$ mice fed MD or HFD, and from male C57BL/6J mice (age 16 wk) fed standard chow were harvested by lavage[4, 5] and cultured (45 min) in medium supplemented with 15% fetal bovine serum (FBS) in petri dishes that had been coated with heat-inactivated FBS by overnight incubation. Non-adherent cells were removed by washing,

and macrophages, which remained attached, were cultured overnight in medium containing 15% FBS. Adherent cells were detached by incubation in $Ca^{2+}$ and $Mg^{2+}$-free PBS containing 0.02% EDTA. Peritoneal macrophage recoveries of 90% were routinely obtained, and all recovered cells were viable, as demonstrated by trypan blue exclusion. *Ex vivo* experiments with the isolated macrophages were performed at a measured cell density of 2 x $10^5$ cells/mL in RPMI 1640 medium containing 5 mM D-glucose (normal glucose, NG) or 30 mM D-glucose (high glucose, HG) without or with human LDL (100 µg/mL) for 8, 16, or 24 hr as specified in the figure legends. The designation "MS" denotes culture in medium that contained both 30 mM D-glucose and 100 µg/mL LDL.

**LDL isolation***.* LDL was purchased from Biomedical Technologies (Stoughton, MA) or prepared from pooled plasma from healthy blood donors by KBr-gradient ultracentrifugation and gel-filtration chromatography, followed by sterilization by filtration, and characterized by described procedures.[4]

**Extraction and quantification of macrophage 2-lysophosphatidic acid (LPA)**. LPA was extracted from macrophages essentially as described with minor modifications [12]. In brief, isolated macrophages were homogenized in normal saline (0.9% NaCl, 200 µl, ice-cold) containing o-vanadate (100 mM) and EDTA (1 mM). Homogenates were placed in glass tubes (13 × 100 mm), mixed with acetone (1 mL), vortex-mixed, and centrifuged (1300 × g, 5 min). The resultant pellet was washed (0.5 mL acetone, twice), and concentrated to dryness under nitrogen. The residue was reconstituted in chloroform (0.1 mL), methanol (0.2 mL), and water (0.08 mL), and the mixture was centrifuged (1300 × g, 5 min). Supernatant was mixed with chloroform (0.2 mL), aqueous KCl (5%, 0.2 mL), and aqueous ammonia (28%, 0.001 ml ), and the mixture was again centrifuged (1300 × g, 5 min). The supernatant was washed with chloroform/methanol (17/3, v/v, 0.4 mL, four times), and then monoisotopic $[^{68}Zn]^{2+}$-Phos-tag (10 nmol) and chloroform/methanol (17:3, v/v, 0.4 mL)) were added to the supernatant (that consisted of the water/methanol phase). The mixture was vortex-mixed and centrifuged, and the lower (chloroform) phase was collected. The residual water/methanol phase was extracted again, and the combined chloroform phases were concentrated to dryness under nitrogen. The residue was reconstituted (50 µL, methanol containing 0.1% aqueous ammonia) and stored at -20°C until the time of analysis. Quantification of LPA content was performed with an Echelon LPA competitive ELISA kit (K-2800S, Echelon, Salt Lake City, UT) according to the manufacturer's instructions.

**$iPLA_2$ enzymatic activity assay**. $iPLA_2$ enzymatic activity was measured by a commercial $iPLA_2$ assay kit (Cayman, Ann Arbor, MI) under $Ca^{2+}$-free conditions as described [13-15]. Briefly, at the end of the incubation intervals under conditions described in the figure legends, macrophages were detached from the plates with a cell scraper in buffer (500 µL, 50 mM HEPES, pH 7.4, 1 mM EDTA) and disrupted by sonication (on ice, Branson Sonifier 450, duty cycle 30%, speed 30 s, interval 1 min, 6–10 times). The resultant homogenates were centrifuged (10,000 × g, 15 min, 4°C), and the protein concentration of the supernatant was determined. $iPLA_2$ activity was measured by incubating (1 hr, 24°C) an aliquot of supernatant with substrate (arachidonoyl thio-phosphatidylcholine) in $Ca^{2+}$--free buffer (300 mM NaCl, 0.5% Triton X-100, 60% glycerol, 4 mM EGTA, 10 mM HEPES, pH 7.4, with 2 mg/ml bovine serum albumin). Reactions were terminated by adding 5,5′-dithiobis(nitrobenzoic acid. After 5 min, absorbance was determined (414 nm) with a standard plate reader and expressed per mg of protein as a measure of $iPLA_2$ activity. As described,[8, 16] a BEL-insensitive background $iPLA_2$-independent component of basal lipase activity was determined in control samples when specific $iPLA_2$ activity was inhibited by BEL pretreatment (25 µM, 30 min, 37 °C). This background activity was subtracted to achieve a measure of BEL-sensitive $iPLA_2$ activity.

**Measurements of ROS and $H_2O_2$.** Intracellular ROS ($O^{2-}$ and $H_2O_2$) production was measured in cells loaded DCFH-DA (Invitrogen, 10 μM, 1 hr), with a redox-sensitive indicator that preferentially reacts with $H_2O_2$ and other ROS [4]. Intracellular ROS production (reflected by DCF fluorescence) was measured by fluorescence-activated cell sorting (FACS Calibur System, Becton Dickinson). Measurement of intracellular $H_2O_2$ was performed with Abnova Hydrogen Peroxide Assay kits (KA0801) or the Amplex Red assay kit from Invitrogen (A22188) according to the manufacturer's instructions.

**Measurement of NADPH oxidase activity**. Macrophages were washed in PBS (ice-cold, five times), detached from the plates, and centrifuged (800 rpm, 10 min, 4°C). Cellular pellets were resuspended in lysis buffer [20 mmol/l $KH_2PO_4$, pH 7.0, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 0.5 μg/ml leupeptin], and the suspensions were homogenized (300 strokes, Dounce apparatus, on ice). Assays were initiated by adding aliquots (20 μg protein) of cellular homogenates to phosphate buffer (50 nM, pH 7.0, 1 mmol/l EGTA, 150 mmol/l sucrose, 5 μmol/l lucigenin) containing NADPH (100 μM). Photon emission was measured (20 to 30 s intervals, 10 min) in a luminometer and expressed as relative light units (RLU). There was no measurable activity in the absence of NADPH. A buffer blank was measured and subtracted from each reading. Blank values represented less than 5% of signal obtained from cellular activity. Superoxide production was expressed as RLU/(mg protein)/min. Protein content was measured using a Bio-Rad protein assay reagent.

**Macrophage migration in response to Monocyte Chemoattractant Protein-1 (MCP-1)**. Isolated mouse peritoneal macrophages were preincubated in medium supplemented with 5 mM D-glucose (NG) or 30 mM D-glucose (HG) without or with LDL (100 μg/mL), and medium was then removed and replaced with fresh medium without or with various test agents for the intervals indicated in the figure legends. At the end of the incubation intervals, the cells were placed in the upper wells of a 48-well modified Boyden chamber (NeuroProbe). Medium in the lower wells contained MCP-1 (2 nM, R&D Systems). A polyvinylpyrrolidone-free polycarbonate filter membrane with 5 μm pores was placed between the upper and lower chambers, and incubations were performed (90 min, 37°C, 5% $CO_2$). The membrane was then washed, and cells were removed from its upper side. Cells that had transmigrated through the filter to reach the lower side of the membrane were stained (Diff-Quik® Set, Dade Behring, Newark, DE) and counted (five separate high power fields, 400X magnification, light microscope).

**Western blotting analyses**. The protein contents of cellular homogenates prepared in RIPA lysis buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM $Na_3VO_4$, 1 mM PMSF, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1% NP-40) were determined by Bio-Rad protein assay, and aliquots were analyzed by SDS-PAGE. Gel protein bands were electroblotted onto polyvinylidene fluoride microporous membranes (Bio-Rad) that were then blocked (5% low-fat milk, Tris-buffered saline) and probed with primary antibodies (1:1000 dilutions) directed against target proteins specified in the figure legends that included a Nox 4 monoclonal antibody (Epitomics) and an $iPLA_2\beta$ polyclonal antibody (Cayman Chemical, Ann Arbor, MI). Appropriate HRP-conjugated secondary antibodies were then added (1:3000), and bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using NIH Image/ImageJ software.

Knockdown of LPA receptor expression with **siRNA.** Peritoneal macrophages were transiently transfected with siRNA oligonucleotides by using Lipofectamine 2000 (Invitrogen). Two or three different siRNAs against each target were tested. The most effective siRNAs for LPA1 (GAAAUGAGCGCCACCUUUA), LPA3 (CAGCAGGAGTTACCTTGTT), and non-silencing RNA (NS, D-001810-10) were obtained from Dharmacon (Lafayette, CO). The primary rabbit polyclonal IgG antibodies against LPA receptors 1 and 3 were purchased from Millipore.

**Phospholipase D activity assay.** PLD activity was measured by synthesis of [$^3$H]-phosphatidylethanol (PEth). Macrophages were seeded in 6-well-plates. To label phospholipids, cells were incubated (24 hr) in serum-free medium containing [$^3$H]-glycerol (1 µCi per mL). Subsequently, the cells were washed and incubated medium containing 5 mM glucose or 30 mM glucose and 100 µg/mL LDL. PLD inhibitors were added 30 min prior to addition of 30 mM glucose and LDL. After incubation (16 hr), phospholipids were extracted from cells and analyzed by thin layer chromatography. Spots corresponding to phosphatidylcholine, phosphatidic acid, and PEth were scraped from the plates separately, and their [$^3$H] content was determined by liquid scintillation counting.

**Statistical analyses**. Data were analyzed using ANOVA (SPSS 17.0) and subjected to parametric or nonparametric *post hoc* analyses. Multiple comparisons were performed by using the Least Significant Difference method. Linear correlation tests were performed with SigmaPlot version 12. Data are presented as mean ± SEM of at least 3 independent experiments unless otherwise indicated, and results were considered significant for $p < 0.05$.

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