Materials and Methods

Animals—Animal protocols were approved by the Animal Care and Use Committee of Indiana University and the procedures followed were in accordance with institutional guidelines. C57BL/6, *ApoE*$^{-/-}$ (ApoEKO: B6129-ApoE<tm1Unc>/J, stain#002052), Cadherin 5 Cre (B6.Cg-Tg(Cdh5-cre)7Mila/J, strain#006137) and P2Y$_2$R$^{-/-}$ (B6.129P2-P2ry2tm1Bhk/J, strain #009132) mice were purchased from Jackson laboratory. All of these mice are on a C57BL/6 background and were maintained on this background. P2Y$_2$R$^{-/-}$mice were bred to the *ApoE*$^{-/-}$ background to generate *ApoE*$^{-/-}$/P2Y$_2$R$^{-/-}$ mice. P2Y$_2$R$^{fl/fl}$ mice were generated by Ozgene using C57Bl/6 ES cells. LoxP sites were inserted into intron 2 and exon 3 of the P2Y$_2$R gene as shown in figure 1. Exon 3 includes the entire coding region of P2Y$_2$R gene (Fig. 1). The insertion of the 3' LoxP site into the 3' untranslated region of the P2Y$_2$R gene facilitated Cre mediated deletion of the entire P2Y$_2$R coding region. Correctly targeted C57BL/6 ES cells were identified by southern blotting. Targeted ES cells were injected into blastocysts from albino C57BL/6-Tyr$^{c\text{-}Brd}$/NCr mice to generate chimeric mice. The chimeras were mated to C57BL/6 females with white pups indicating germline transmission of the floxed alleles. This was confirmed by southern blotting and PCR. The Neo cassette was removed by crossing the targeted mice with FLPe mice (Fig. 1) to generate the final P2Y$_2$R flox mice (Fig. 1). P2Y$_2$R flox mice were routinely genotyped by PCR using F1 and R1 primers indicated on figure 1 that distinguish the WT and floxed loci. These mice were crossed with Cad5cre$^{-/+}$ mice to generate the final experimental ($_{EC}$P2Y$_2$R-KO: P2Y2R$^{fl/fl}$ Cad5cre$^{-/+}$) and control (P2Y$_2$R flox: P2Y$_2$R$^{fl/fl}$ Cad5cre$^{-/-}$) mice. For some experiments both P2Y$_2$R$^{fl/fl}$ and Cad5cre$^{-/+}$ mice were crossed onto an ApoE$^{-/-}$ background. All Cre recombinase mice were on a C57BL/6 background. All animals were fed a standard chow diet. Only males were used in experimental groups.

Cell isolation—Endothelial cell and vascular SMC were isolated as previously described (1-2).

Transthoracic echocardiography—Transthoracic echocardiography was performed using a Visual Sonics Vevo 770 ultrasonograph with a 30-MHz transducer as detailed previously (3). For acquisition of two-dimensional guided M-mode images at the tips of papillary muscles and Doppler studies, mice were sedated by facemask administration of 1% isoflurane, hair removed, and maintained on a heated platform. Blood velocities across the mitral, aortic and pulmonary valves were measured using Doppler pulse wave imaging angling the probe to obtain a nearly parallel orientation to the blood flow. End diastolic and systolic left ventricular (LV) diameter as well as anterior and posterior wall (AW and PW respectively) thickness were measured on line from M-mode images obtained in a parasternal long axis view using the leading edge-to-leading edge convention. All parameters were measured over at least three consecutive cardiac cycles and averaged. Left ventricular fractional shortening were calculated as [(LV diameter$_{diastole}$ –LV diameter$_{systole}$)/LV diameter$_{diastole}$] x 100; ejection fraction [(7.0/(2.4 + LV diameter$_{diastole}$)(LV diameter$_{diastole}$)^3 - (7.0/(2.4 + LV diameter$_{systole}$)(LV diameter$_{systole}$)^3/(7.0/(2.4 + LV diameter$_{diastole}$)(LV diameter$_{diastole}$)^3 x 100 and LV mass was calculated by using the formula [1.05 x ((Posterior Wall $_{diastole}$+Anterior Wall$_{diastole}$+LV diameter$_{diastole}$)^3 – (LV diameter$_{diastole}$)^3)]. Right ventricular wall

thicknesses were measured as above from m-mode images obtained in a right parasternal long-axis window. Pulmonary artery (PA) diameter was measured just distal to the pulmonary valve. Care was taken to measure PA diameter during the widest part of ejection cycle. Heart rate was determined from at least three consecutive intervals from the pulse wave Doppler tracings of the LV outflow tract. Ejection time was measured from the same outflow track tracings from the onset of flow to the end of flow. Isovolumic relaxation time was measured as the time from the closing of the aortic valve to the opening of the mitral valve from pulse wave Doppler tracings of the LV outflow tract and mitral inflow region. The same person obtained all images and measures.

Acute *in vivo* mouse blood pressure measurements—Mice were anesthetized with urethane (1g/kg), and allowed to breathe freely. The right common carotid artery was isolated and a 1.2 Fr high-fidelity pressure catheter (Scisense, Transonic) was inserted and advanced to the aorta. The pressure tracing was recorded and analyzed on commercially available software (Notocord, Croissy Sur Seine, France).

Chronic telemetric mouse blood pressure measurements—A Data Science (DSI, St Paul, MN) implantable blood pressure monitor-radio transmitter was used to monitor unanaesthetized blood pressure. The Data Science blood pressure catheter tip was placed into the left internal carotid artery. The catheter was passed subcutaneously, and the radio transmitter was inserted into a subcutaneous pocket formed by blunt dissection between the front and hind limb. The mouse was recovered for 7 days prior to any measurements. Blood pressure and heart rates were then monitored for 2 consecutive days. Data was measured during an active awake period (high) and during an inactive, asleep period (low) and at least 10 minutes of data was averaged during these time periods (4-5). Systolic blood pressure (SBP) was taken as the peak, diastolic (DBP) as the minimum and mean was calculated as mean BP = DBP + 1/3(SBP-DBP).

Vasomotor studies—Male mice (5 month old) were anesthetized and sacrificed for the aortic arch procurement. The mouse aortas free from the connective tissue were cut into about 2-3 mm rings. The aortic rings were individually mounted on a wire myograph holder and then placed into a tissue bath containing Krebs solution (131.5 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 25 $NaHCO_3$, and 10 glucose). The tissue baths were preheated to 37 ˚C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 to a pH of 7.4. The optimal pre-load of the tested aortic arch rings was determined as described elsewhere (6-7); and it was usually between 0.4 and 0.6 g. The reported force values were normalized to corresponding maximum KCl-induced tension values. 70 mM KCl-induced contractions were routinely elicited to confirm the rings' ability to contract. All of the experiments were performed in the presence of 10 μM indomethacin to prevent the endothelium dependent vasoreactivity due to endogenous prostanoids.

Monocyte transmigration assay—Isolation of mouse monocytes from whole blood and transmigration assays were performed as previously described (8-9).

Analysis of plasma cholesterol and triglycerides

Blood was drawn from the abdominal aorta after 25 weeks plasma was isolated by centrifugation at 10 000*g* for 10 minutes and maintained at 4°C. Concentrations of total cholesterol and triglycerides were determined in total plasma with an enzymatic assay as previously described (10).

Quantification of atherosclerosis in the aorta
Aortas of 25 weeks old mice were collected between the subclavian branch and the iliac bifurcation. Aortas were fixed in 60% isopropanol for 5 minutes, and stained with 3% Oil Red O for 15 minutes. The MetaMorph software was used to measure total surface covered by atherosclerotic lesions and the ratio of atherosclerotic area to the total area was calculated.

Quantification of lesion sizes in the aortic sinus—Mouse hearts were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were made and discarded until the valve cups became visible. At that point, 5-μm sections at 50-μm intervals were stained with Masson's trichrome for lesion area measurement detection of collagen fibers. The lesion area was measured with image analysis (MetaMorph software, Universal Imaging Corp, Downingtown, Pa).

Immunohistochemistry and Western blot analysis—Immunohistochemical staining was performed by the labeled streptavidin biotin method. A rabbit polyclonal antibody against human smooth muscle α-actin was used to detect smooth muscle cells (Sigma, 1/1000 dilution). Macrophages were identified by immunostaining with a rat anti-mouse Mac-3 monoclonal antibody (M3/84, 550292, BD PharMingen, San Diego, CA; 1/100 dilution). Rabbit polyclonal anti-$P2Y_2$ receptor antibodies were raised against a keyhole limpet hemocyanin-conjugated peptide (NRTVRKDLSVSSDD) corresponding to amino acids 342–355 of the mouse $P2Y_2R$ amino acid sequence (NM_01302347) as previously described and validated (10). This antibody (1/100 dilution) was used to detect $P2Y_2R$ on cross sections of aortic samples.
Western blot analysis was carried out on EC and SMC lysates using the $P2Y_2R$ as primary antibody (1/1000 dilution).

Vascular permeability assay—Vascular barrier function was examined using the Evans blue assay (11).

Measurement of NO release—Plasma nitrite and nitrate levels (NOx) were measured as nitrite using the Griess reaction as previously described (12). NO release from the aortic explants was measured by determining the production of nitrite using a NOx analyzer as previously described (13).

Ex vivo migration assay—Thoracic aortas were collected from 5 month-old control and $_{EC}P2Y_2R$-KO mice, and the inner media was dissected from the adventitia but endothelial layers left intact. The tissue was then chopped in into 1-mm^2 pieces using a McIlwain tissue chopper (Brinkman) and were then placed into each 25-cm^2 tissue culture flask in presence of DMEM as previously described (14). Explants were examined daily for evidence of any cell migration onto the plastic culture surface and

were counted as positive if ≥1 cell was observed. As described in reference 14, this method precludes any involvement of proliferating cells outside the explants since migration of only 1 cell was required for an explant to be counted as positive.

Gel-based zymography—Aortic arch segments were obtained from control and $_{EC}P2Y_2R/ApoE^{-/-}KO$ mice at week 25. Tissue was rinsed in PBS, snap frozen in liquid nitrogen and pulverized using a homogenizer and suspended in lysis buffer. Protein concentration was subsequently assayed by BCA. Experimental samples were separated using a 10% zymography gel (Invitrogen) to document cleavage of gelatin by MMP-2 and MMP-9. After electrophoresis the proteins in the gel were renatured and developed. The gel was then stained with Coomassie Blue and destained until clear. The gelatino-lytic activity was apparent as clear bands against a dark background. The relative molecular weight of each band was determined by comparison of the bands against validated recombinant mouse MMP-2 Western blot standards protein (R&D Systems). Gel image bands strength was quantified using Thermo Scientific myImageAnalysis Software v2.0.

Quantitative Real-Time PCR—Excised aortic arch segments were washed with PBS and immediately snap-frozen and pulverized for total-RNA was isolation using with an RNeasy mini kit (Qiagen, Valencia, CA). Total RNA was reverse transcribed according to the manufacturer's protocol (Invitrogen) and 2 µl of cDNA were amplified by real-time PCR with 1X TaqMan universal PCR Master mix (Applied Biosystems, Foster City, CA, USA) in 96-well plates on an ABI 7700 Sequence Detector. The following assay on Demand kits obtained from ThermoFisher were used: MMP-2, Mm00439508; MMP-9, Mm00600163; TIMP-1, Mm00441818; TIMP-2, Mm00441825.

Statistical analysis—Data in this study are expressed as means ± SEM. Differences in data between the groups were compared with Student's paired 2-tailed t test or 1-way ANOVA where appropriate. A p value less than 0.05 was considered statistically significant.

References

1. Kreisel D, Krupnick AS, Szeto WY, Popma SH, Sankaran D, Krasinskas AM, Amin KM, Rosengard BR. A simple method for culturing mouse vascular endothelium. *J Immunol Methods.* 2001; 254: 31–45.
2. Yu N., Erb L., Shivaji R., Weisman G. A., Seye C. I. Binding of the P2Y2 nucleotide receptor to filamin A regulates migration of vascular smooth muscle cells. Circ. Res. 2008; 102:581-588Harris SP, Bartley CR, Hacker TA, McDonald KS, Douglas PS, Greaser ML, Powers PA and Moss RL. Hypertrophic cardiomyopathy in cardiac myosin binding protein-C knockout mice. *Circ Res* 90: 594-601, 2002.
3. Kramer K, Kinter L, Brockway BP, Voss HP, Remie R and Van Zutphen BL. The use of radiotelemetry in small laboratory animals: recent advances. *Contemp Top Lab Anim Sci* 40: 8-16, 2001.

4. Kramer K, Voss HP, Grimbergen JA, Mills PA, Huetteman D, Zwiers L and Brockway B. Telemetric monitoring of blood pressure in freely moving mice: a preliminary study. *Lab Anim* 34: 272-280, 2000.
5. Mills PA, Huetteman DA, Brockway BP, Zwiers LM, Gelsema AJ, Schwartz RS and Kramer K. A new method for measurement of blood pressure, heart rate, and activity in the mouse by radiotelemetry. *J Appl Physiol* 88: 1537-1544, 2000.
6. Chakraborty S, Berwick ZC, Bartlett PJ, Kumar S, Thomas AP, Sturek M, Tune JD, Obukhov AG. Bromoenol lactone inhibits voltage-gated Ca2+ and transient receptor potential canonical channels. J Pharmacol Exp Ther. 2011; 339(2):329-340.
7. Hiett SC, Owen MK, Li W, Chen X, Riley A, Noblet J, Flores S, Sturek M, Tune JD, Obukhov AG. Mechanisms underlying capsaicin effects in canine coronary artery: implications for coronary spasm. Cardiovasc Res. 2014; 103(4):607-18.
8. Seye, CI, Agca Y, Agca C, Derbigny W. $P2Y_2$ receptor-mediated lymphotoxin-α (LTA) secretion regulates intercellular cell adhesion molecule (ICAM)-1 expression in vascular smooth muscle cells. J. Biol. Chem. 2012; 287: 10535-10543.
9. Sun C, Wu MH, Guo M, Day ML, Lee ES, Yuan SY. ADAM15 regulates endothelial permeabilityand neutrophil migration via Src/ERK1/2signalling. Cardiovasc Res. 2010; 87(2): 348-55.
10. Shaomin Qian; April Hoggatt; Yava L. Jones-Hall; Carl F. Ware; Paul Herring; Cheikh I. Seye. Deletion of $P2Y_2$ Receptor Reveals a Role for Lymphotoxin-α in Fatty Streak Formation. 2016. Vasc. Pharmacol. 2016; 85:11-20.
11. Radu M, Chernoff J. An in vivo assay to test blood vessel permeability. J Vis Exp. 2013; (73): 50062.
12. Yamashita T, Kawashima S, Ohashi Y, Ozaki M, Ueyama T, Ishida T, Inoue N, Hirata K, Akita H, Yokoyama M. Resistance to endotoxin shock in transgenic mice overexpressing endothelial nitric oxide synthase. Circulation. 2000;101:931–937.
13. Münzel T, Sayegh H, Freeman BA, Tarpey MM, Harrison DG. Evidence for enhanced vascular superoxide anion production in nitrate tolerance: a novel mechanism underlying tolerance and cross tolerance. J Clin Invest. 1995;95:187–194.
14. Kenagy RD, Vergel S, Mattsson E, Bendeck M, Reidy MA, Clowes AW. The role of plasminogen, plasminogen activators, and matrix metalloproteinases in primate arterial smooth muscle cell migration. *Arterioscler Thromb Vasc Biol.* 1996;**16**:1373–1382.