Supplementary Material

Material and Methods

Vascular aortic smooth muscle cells (VSMC). Mouse aortas were isolated and placed under cell culture coverslips (Nalge Nunc International, Rochester, NY) on tissue culture plates in Dulbecco's Modified Eagles Medium / Ham's F12 (Gibco BRL, Grand Island, NY) supplemented with 20% fetal bovine serum. Explants of smooth muscle cells were allowed to grow for 7 days. Cells were then routinely passaged and used at passage 3-4. Cells were serum-starved overnight before use. Protein and cell culture media were collected 12 hrs after LPS 5µg/ml treatments.

Vascular endothelial cells (EC).

The lung lobes were carefully dissected out from any visible bronchi and mediastinal connective tissue. Organs were washed in 50 ml of DMEM containing 20% FCS (DMEM-20%) to remove erythrocytes, minced finely with scissors, and digested in 15 ml of collagenase (180 to 200 U/ml) at 37°C for 45 minutes. The digested tissue was then mechanically dissociated by titurating, filtered through a 70-µm disposable cell strainer (Becton Dickinson Labware, Bedford, MA) and centrifuged at 400 x *g* for 10 minutes at 4°C. The cell pellet was resuspended in cold DPBS and incubated with PECAM-1-coated beads (Sheep anti-rat-IgG Dynal beads M-450) at room temperature for 10 minutes with end-over-end rotation. A magnetic separator was used to recover the bead-bound cells. The recovered cells were washed with DMEM-20%, suspended in 10 ml of complete culture medium (DMEM containing 20% FCS, supplemented with 100 µg/ml porcine heparin, 100 µg/ml endothelial cell growth stimulant, nonessential amino acids, sodium pyruvate, L-glutamine, and antibiotics, at standard concentrations),

and then plated in a single gelatin-coated 75-cm^2 tissue culture flask. After overnight incubation, the nonadherent cells were removed, the adherent cells washed with Hanks' balanced salt solution, and 10 ml of fresh complete media was added. Cultures were fed routinely on alternate days with fresh complete culture medium. When the cells reached 70 to 80% confluence, they were detached with warm trypsin-EDTA to generate a single cell suspension. The cells were pelleted and then resuspended in 2 ml of DPBS and sorted for a second time using ICAM-2-coated beads. The bead-bound cells were washed and plated in complete culture medium and passaged further at a 1:2 ratio. Confluent monolayers were used at passages 1 to 3 for this study. Cells were serum-starved overnight before use. Protein and cell culture media were collected 12 hrs after LPS 5μg/ml treatments.

Mass spectrometic analysis of prostanoids and their metabolites. Briefly, smooth muscle cell and endothelial cell production of PGE_2, PGD_2, TxA_2, and PGI_2 was determined by quantitation of PGE_2, PGD_2, TxA_2, and 6-keto $PGF_{1\alpha}$ in cell culture supernatants, respectively, and normalized with protein. Urine was collected over a 24 hour period before and 1 week after the mice were fed a high sodium diet (8% NaCl; diet no. 5008; Harlan Teklad). Urinary prostanoid metabolites were measured by mass spectrometry as described previously (1). Briefly, systemic production of PGE_2, PGD_2, TxA_2, and PGI_2 was determined by quantitation of their major urinary metabolites [7-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid (PGE-M), 11,15-dioxo-9α-hydroxy-, 2,3,4,5-tetranorprostan-1,20-dioic acid (tetranor PGD-M), 2,3-dinor TxB_2 (Tx-M), and 2,3-dinor 6-keto $PGF_{1\alpha}$ (PGI-M)] in 24 hr collections respectively and normalized with creatinine.

Western blot analysis of protein expression. Cell extracts from isolated VSMCs and ECs were blotted with goat-anti-mouse COX-1 antibody or rabbit-anti-mouse COX-2 antibody (Cayman Chemical, Ann Arbor, MI). Protein levels were normalized by β-actin (Sigma).

Immunostaining

LPS-stimulated primary ECs were stained for platelet-endothelial cell adhesion molecule-1 (PECAM-1, Green, X400) and COX-2 (Red, X400), and nuclei were counterstained with DAPI (blue, X400). LPS-stimulated primary VSMCs were stained for α- VSM Actin (Green, X400) and COX-2 (Red, X400), and nuclei were counterstained with DAPI (blue, X400).

Real-time PCR analysis of gene expression Aortas from mice challenged for one week with a high-salt diet (8% NaCl; diet no. 5008; Harlan Teklad) were dissected and the tissues were homogenized using a rotor-stator homogenizer. Total RNAs, prepared using TRIzol reagent (Invitrogen) and RNeasy columns (Qiagen, Valencia, CA), were reverse-transcribed into cDNA by Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). TaqMan Gene Expression Assays (Applied Biosystems) for COX-2 (Mm00478374_m1), eNOS (Mm00516004_m1) were performed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Results were normalized with 18s rRNA (Hs99999901_s1).

References and notes

1. Y. Cheng, M. Wang, Y. Yu, J. Lawson, C.D. Funk, G. A. Fitzgerald. Cyclooxygenases, microsomal prostaglandin E synthase-1, and cardiovascular function. *J Clin Invest.* **116**, 1391-1399 (2006).

Figure legends

Fig. S1. (**A**) **Prostanoid profiles in supernatants of cultured unstimulated and LPS stimulated VSMC.** PGE_2 and PGI_2 were determined by mass spectrometry quantification of PGE_2 and 6-keto $PGF_{1\alpha}$. All data were normalized with total proteins (n=6; **P*<0.01). (**B**) **Prostanoid profiles in supernatants of cultured unstimulated and LPS stimulated EC.** All data were normalized with total proteins (n=6; **P*<0.01).

Fig. S2. Profiles of urinary prostanoid metabolites. Twenty-four–hour urine was collected from WT, EC COX-2 KO, VSMC COX-2 KO and EC/VSMC dKO mice. Biosynthesis of PGE_2, TxA_2 and PGD_2 was assessed by quantification of their major urinary metabolites: PGE-M (**A** and **B**), 2,3-dinor-TxB_2 (TxM) (**C**) and tetranor PGD-M (**D**), respectively. All urine data were normalized with creatinine. A significant decrease in urinary PGEM was observed in male dKOs as compared to controls. No alteration of TxM and PGDM were observed in EC COX-2 KO, VSMC COX-2 KO and EC/VSMC dKO. (8-10 weeks old, n=16-22).

Fig. S1

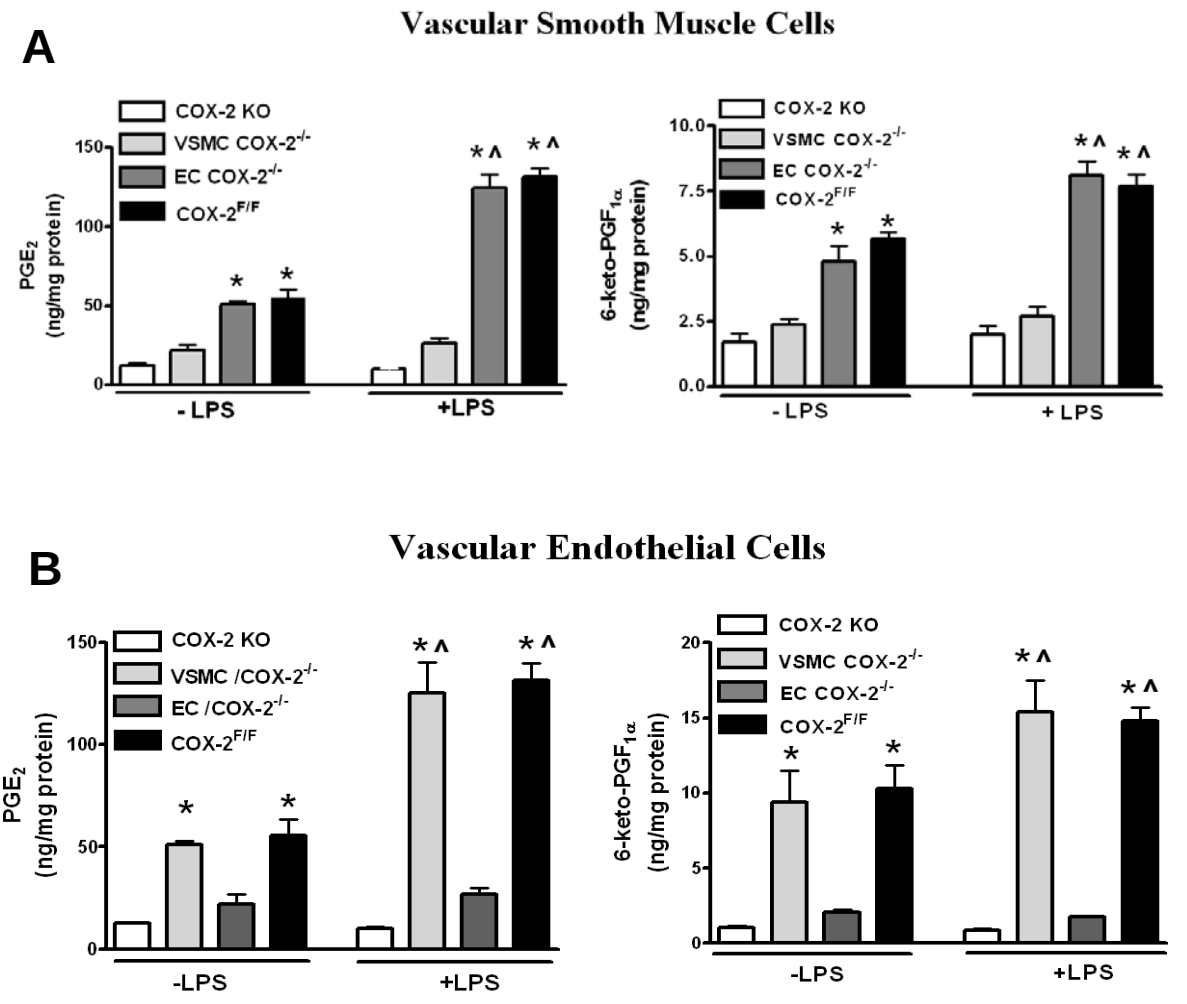
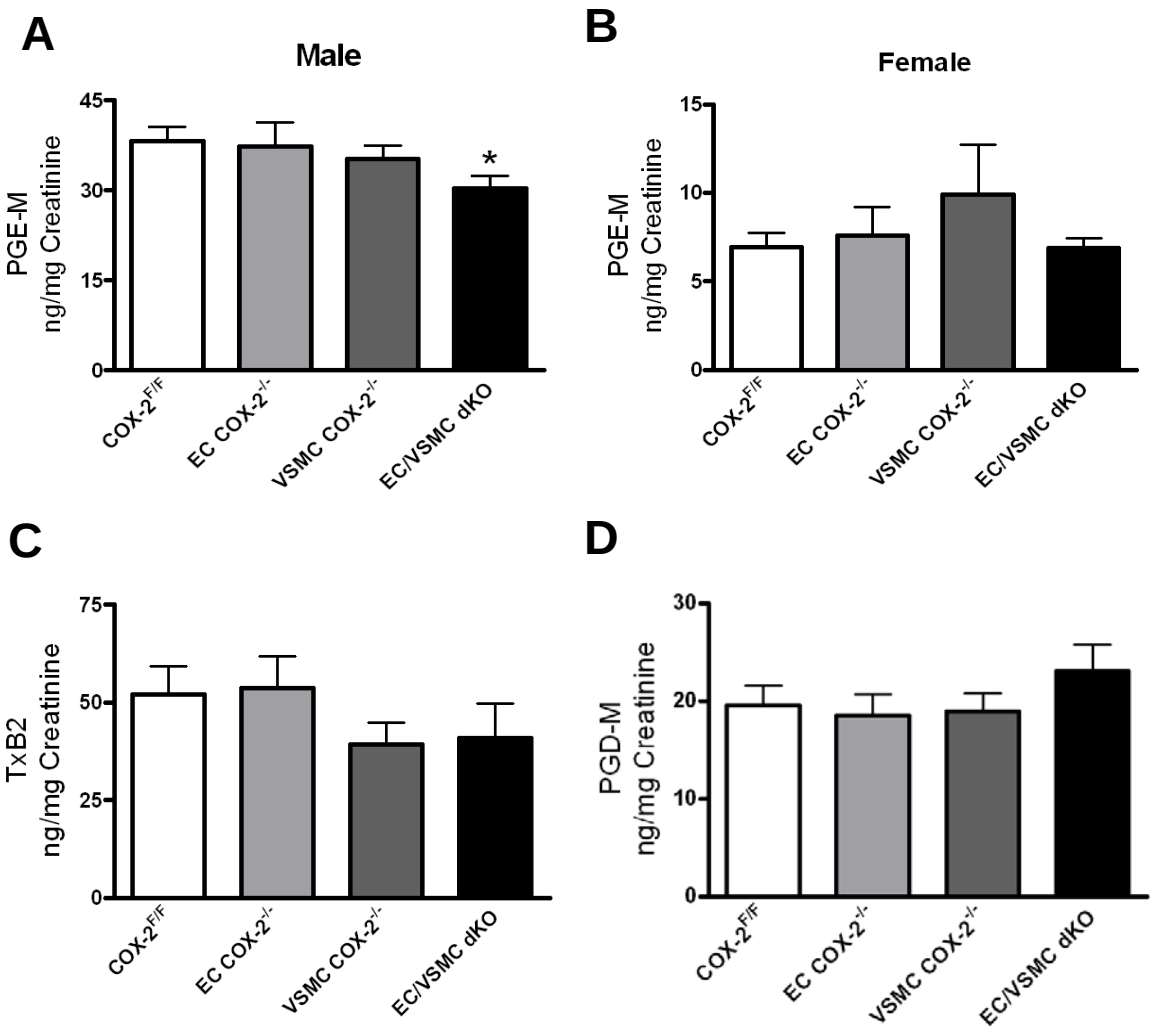

A
Vascular Smooth Muscle Cells
COX-2 KO
VSMC COX-2-/-
EC COX-2-/-
COX-2F/F
PGE2 (ng/mg protein)
150
100
50
0
- LPS
+LPS
6-keto-PGF1α (ng/mg protein)
10.0
7.5
5.0
2.5
0.0
+ LPS
B
Vascular Endothelial Cells
VSMC /COX-2-/-
EC /COX-2-/-
-LPS
20
15
10
5

Fig. S2


A
Male
PGE-M
ng/mg Creatinine
45
30
15
0
*
COX-2F/F
EC COX-2-/-
VSMC COX-2-/-
EC/VSMC dKO
B
Female
PGE-M
ng/mg Creatinine
15
10
5
0
COX-2F/F
EC COX-2-/-
VSMC COX-2-/-
EC/VSMC dKO
C
TxB2
ng/mg Creatinine
75
50
25
0
COX-2F/F
EC COX-2-/-
VSMC COX-2-/-
EC/VSMC dKO
D
PGD-M
ng/mg Creatinine
30
20
10
0
COX-2F/F
EC COX-2-/-
VSMC COX-2-/-
EC/VSMC dKO