**Methods**

2.1. Animal Protocol

Three-month old female Wistar rats (Charles River, L’Arbresles, France) were ovariectomized (OVX) as previously described. After 1 week, rats were submitted to surgery in order to increase blood flow in 1 mesenteric artery as previously described. Three consecutive first-order arteries were used. Ligatures were applied to second-order branches (Figure 1). The artery located between the two ligated arteries was designed as high flow (HF) artery. Arteries located at distance of the ligated arteries were used as control (normal flow, NF). Location of the ligations is shown below:

![Diagram of Superior mesenteric artery and Blood flow](image)

Ovariectomized rats treated were treated with 17β-estradiol (E2, 20µg/kg per day, osmotic minipump, n=12) or with the solvent (control group, n=12). Rats were sacrificed after 2, 4 or 15 days before collection of the mesenteric arteries (n=12 rats per group). In a preliminary series of experiments, rats were submitted to a sham ovariectomy or to sham ligature. Before harvesting the mesenteric arteries, arterial blood pressure was measured as previously described. Blood was also collected for E2 level measurement using a commercially available kit (Estradiol EIA Kit#58225 Cayman Chemical).

In other series of experiments several series of mice were submitted to surgery in order to modify blood flow as described above (n=8 to 12 mice per group). Mice were: ERα−/−, ERβ−/−, eNOS−/− and caveolin-1−/− mice. Finally we used mice with a deficiency for the ERα selectively targeted to the endothelium (Tie2Cre(+)ERαf/f). The latter mice were compared with their littermate wild-type (WT) controls (Tie2Cre(-)ERα) with a C57BL/6J background. In each protocol, rats or mice rats were anesthetized with isoflurane (2.5%). They were treated with buprenorphine (Temgesic®; 0.1 mg/kg, s.c.) before and after surgery. Blood pressure was also measure in mice as described above.

The investigation conforms to the European Community standards on the care and use of laboratory animals (authorization nb 00577) and to the Guide for the Care and Use of
Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The protocol was approved by the ethical committee (Protocol CEEA PdL #2008.10).

Arterial blood pressure was measured in rats before collecting mesenteric arteries for analysis. Blood was collected for E2 level measurement using a commercially available kit (Estradiol EIA Kit#58225 Cayman Chemical). Rats were then sacrificed in a CO2 chamber. The mesentery was quickly removed and placed in an ice-cold physiological salt solution (PSS) of the following composition (in mmol/L): 135.0, NaCl, 15.0, NaHCO3, 4.6 KCl, 1.5, CaCl2, 1.2, MgSO4, 11.0, glucose, 10.0, N-2-hydroxyethylpiperazine-N-2-ethylsulfonic acid. The PSS was maintained at pH 7.4, PO2 160 mmHg, PCO2 37 mmHg3. Mesenteric arteries (HF and NF) were gently dissected and divided into two segments, proximal for the functional study and distal for histological and biochemical studies.

**Pressure-diameter relationship in mesenteric arteries in vitro**

Arterial segments were cannulated at both ends and mounted in a video monitored perfusion system (Living System, LSI, Burlington, VT) as previously described7, 8. Two glass cannulated arterial segments were bathed in a 5 ml organ bath containing a Ca2+-free PSS containing ethylene-bis-(oxyethylenenitrolo) tetra-acetic acid (EGTA, 2 mmol/L) and sodium nitroprusside (SNP, 10 µmol/L). Pressure steps (10 to 150 mmHg) were then performed in order to determine passive arterial diameter. Pressure and diameter measurements were collected using a Biopac data acquisition system (Biopac MP100 and Acqknowledge® software; La Jolla, CA, USA)2.

**Functional assessment of basal NO production in NF and HF arteries**

Two other arterial segments (2-mm long each) were dissected and mounted in a wire myograph (DMT)9. L-NAME-dependent contraction was obtained after precontraction of the arteries with phenylephrine to 5-10% of their maximal contractile response10.

**Endothelium-dependent relaxation**

Segments of NF and HF arteries (2-mm long each) were dissected and mounted in a wire myography (DMT)11. Cumulative concentration-response curves to acetylcholine (0.01 to 10 µmol/L) were performed before and after incubation (20 minutes) with the NO-synthase inhibitor L-NAME (10µmol/L). Acetylcholine-dependent relaxation was performed after precontracted of the arteries with phenylephrine and serotonin to 70% of their maximal contractile response.

**Quantitative Real-time PCR:**

The mRNA levels for the following proteins were determined using Q-RT-PCR: MCP1, CD68, CD11B, COX2, gp91, p22, p47, MMP2, TIMP1, eNOS, CAV1. Mesenteric arteries were dissected in ice-cold physiological salt solution (PSS) and kept in 100 µL RNAlater (Sigma) at -20°C until RNA extraction using the RNeasy® micro kit (Qiagen). 200 ng of total RNA extracted from each artery were used to synthesize cDNA for RT-PCR using the QuantiTect® Reverse Transcription kit (Qiagen) according to the manufacturer instruction. The Quantitative real-time PCR reactions were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using Power SYBR® Green PCR Master Mix (Applied Biosystems) and gene-specific primers designed using Primer3 online software. All data were normalized to the Hprt mRNA. Differences in transcript level were determined using the cycle threshold method as described by the manufacturer.
Western blot analysis of eNOS and phospho-eNOS (ph-eNOS) in HF and NF arteries:

As previously described\textsuperscript{12}, arteries were homogenized and proteins (25 µg total protein from each sample) were separated by SDS-PAGE using a 4% stacking gel followed by a 10% running gel. Proteins were detected with specific antibodies (Transduction Laboratories, eNOS 1:1000, ph-eNOS 1:500, in TBST). Protein expression was visualized using the ECL-Plus Chemiluminescence kit (Amersham) and normalized by actin expression.

Immunostaining of CD11B and F4/80 in whole mesenteric arteries:

As described above, segments of HF and NF arteries, isolated from WT and ER\textalpha--/-- mice were carefully harvested and fixed 15 min in 4% paraformaldehyde and washed 3 times in PBS. Arterial segments were then incubated at 4°C with anti-mouse F4/80 (eBiosciences; 1:200) and anti-mouse Cd11b (eBiosciences; 1:200) for the staining of macrophages. For nuclear staining DAPI was added (1:5000). Vessels were embedded in Mowiol and placed on a glass slide. Fluorescent images were obtained using a confocal microscopy (Nikkon). Excitation wavelengths were 405 nm, 491 nm and 561 nm. Images were acquired and analyzed (Metamorph®).

Statistical Analysis

Results were expressed as means±SEM. Significance of the differences between groups was determined by analysis of variance (ANOVA for consecutive measurements for pressure-diameter curves) or 1-way ANOVA followed by Bonferroni. Probability values less than 0.05 were considered significant.

References


Supplemental figure I: Mean arterial blood pressure was measured in rats and mice.
Panel A: control (CONT) female rats (A) and in ovariectomized (OVX) female rats treated with 17-beat-estradiol (OVX+E2) or raloxifen (OVX+R). Male rats (MR) and male rats treated with letrozole (MR+L).
Panel B: Wild-type (WT) mice or from mice lacking the gene (KO) for ERalpha or Erbeta. Mean ± sem is represented (n=12 rats per group in A and 8 mice per group in B). No significant difference between groups.

E2 blood level (E) and uterus weight (F-G) were measured in intact and OVX rats treated with E2 or left untreated. Mean ± SEM is represented (n=12 rats per group).
#P<0.05, OVX rats versus control rats.
Supplemental figure II: Histomorphometric analysis of HF and NF arteries isolated from wild-type (WT) mice or from mice lacking the gene for ERalpha (KO mice). Arteries were fixed in formaldehyde under an intraluminal pressure of 75mmHg, and sections were used for the measurement of the following parameters: internal diameter (A), external diameter (B), media thickness (C) and media cross-section area (D).

Mean ± SEM is represented (n=8 mice per group).

*P<0.05, HF versus NF arteries.
Supplemental figure III: mRNA levels of MCP1 (A), CD68 (B) and CD11b (C) determined in mesenteric arteries submitted chronically to high flow (HF) or normal flow (NF) and isolated for analysis after 2 or 4 days (day 2 or day 4). Arteries were isolated from ovariectomized rats (OVX) treated with E2 (OVX+E2) or left untreated.

Mean ± SEM is represented (n=12 rats per group).

*P<0.05, HF versus NF arteries.
Supplemental figure IV: mRNA levels of COX2 (A) and COX1 (B) determined in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF) and isolated for analysis after 2 or 4 days (day 2 or day 4). Arteries were isolated from ovariectomized rats (OVX) treated or not with 17-β-estradiol (E2). Mean ± sem is represented (n=12 rats per group).

*P<0.05, HF versus NF arteries
Supplemental figure V: Arterial diameter was measured in response to stepwise increases in pressure in NF and HF mesenteric arteries isolated from rats treated or not with the selective COX2 inhibitor celecoxib for 2 weeks. The time between ligature and collection of arteries for diameter measurement was also 2 weeks. Mean ± sem is represented (n=12 rats per group). *P<0.05, HF versus NF arteries.
**Supplemental figure VI:** Immunofluorescence detection of the nuclei with DAPI (blue), CD11b (green) and F4/80 (red) in a whole segment of mouse mesenteric artery submitted chronically to high flow (HF) in vivo. Arteries were isolated from wild-type (WT) mice. Confocal image acquisition was performed at a 20x magnification (left panel) and then through the arterial wall at a magnification of 63x. This allowed image acquisition in the adventitia, in the tunica media and in the endothelium. The overlay is shown on the top panel. Images shown are representative of 4 different experiments.
**Supplemental figure VII:** Immunofluorescence detection of the nuclei with DAPI (blue), CD11b (green) and F4/80 (red) in a whole segment of mouse mesenteric artery submitted chronically to high flow (HF) in vivo. Arteries were isolated from mice lacking the gene encoding for ERalpha. Confocal image acquisition was performed at a 20x magnification (left panel) and then through the arterial wall at a magnification of 63x. This allowed image acquisition in the adventitia, in the tunica media and in the endothelium. The overlay is shown on the top panel. Images shown are representative of 4 different experiments.
Supplemental figure VIII: Immunofluorescence detection of the nuclei with DAPI (blue), CD11b (green) and F4/80 (red) in a whole segment of mouse mesenteric artery submitted chronically to high flow (HF) in vivo. Arteries were isolated from mice lacking the gene encoding for Eralpha (middle panel) or from wild-type (WT) mice (left panel). Negative control (absence of anti-CD11b or F4/80 antibodies) is shown on the right. Confocal image acquisition was performed at a 10x magnification. The overlay is shown on the top panel. Images shown are representative of 4 different experiments.
Supplemental figure IX: mRNA levels of p47 phox (A), p22phox (B) and gp91 (C) determined in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF) and isolated for analysis after 2 or 4 days (day 2 or day 4). Arteries were isolated from ovariectomized rats (OVX) treated or not with 17-b-estradiol (E2). Mean ± sem is represented (n=12 rats per group). *P<0.05, HF versus NF arteries.
Supplemental figure X: mRNA levels of MMP2 (A) and TIMP1 (B) determined in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF) and isolated for analysis after 2 or 4 days (day 2 or day 4). Arteries were isolated from ovariectomized rats (OVX) treated or not with 17-b-estradiol (E2). Mean ± sem is represented (n=12 rats per group).
*P<0.05, HF versus NF arteries
Supplemental figure XI: Concentration-response curve to L-acetylcholine (A) after precontraction with phenylephrine, before and after incubation with L-NAME. In similar arteries (B), 17b-estradiol (E2) (0.1 µmol/L)-mediated relaxation was investigated. Mesenteric arteries were submitted chronically to high flow (HF) or normal flow (NF) and isolated for analysis after 4 days (day 4). Arteries were isolated from ovariectomized rats (OVX) treated with 17b-estradiol (E2) or left untreated. Mean ± SEM is represented (n=12 rats per group).

*P<0.05, HF versus NF arteries.
Supplemental figure XII: Arterial diameter was measured in HF and NF mesenteric arteries isolated from mice lacking the gene for eNOS (A) or for caveolin-1 (B). Mean ± SEM is represented (n=10 mice per group).
*P<0.05, HF versus NF arteries.
Supplemental figure XIII: mRNA levels of heme-oxigenase-1 (HO-1, A) and TGFbeta1 (B) determined in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF) and isolated for analysis after 2 or 4 days (day 2 or day 4). Arteries were isolated from ovariectomized rats (OVX) treated or not with 17-b-estradiol (E2).

Mean ± sem is represented (n=12 rats per group).

*P<0.05, HF versus NF arteries
Supplemental figure XIV: mRNA levels of HIF1α (A) and VEGF A (B) determined in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF) and isolated for analysis after 2 or 4 days (day 2 or day 4). Arteries were isolated from ovariectomized rats (OVX) treated or not with 17-b-estradiol (E2).
Mean ± sem is represented (n=12 rats per group).
*P<0.05, HF versus NF arteries