Methods

Generation and genotyping of LRP6 mutant mice

Mouse embryonic stem cells (CGR8.8, derived from the 129/Ola mouse strain) were electroporated with the secretory trap vector pGT1.8TM as described[4]. We identified a cell line (Ex187) carrying an insertion in LRP6 by direct sequencing of complementary DNAs obtained by 5′ rapid amplification of cDNA ends (RACE)[22]. Germline chimaeras generated by blastocyst injection were test bred and backcrossed to C57/BL6 mice for four generations before setting up intercrosses of heterozygous mutant animals. To genotype animals at weaning, dot blots of DNA prepared from tail biopsies were probed with vector (*βgeo*) sequences[23]. Mutant embryos before 8.5 d.p.c. were genotyped by the X-gal staining[4] intensity of yolk sacs. Mutant embryos at later stages were identified by morphology or X-gal staining. Murine embryonic fibroblasts were isolated from eviscerated 12.5- and 14.5-d.p.c. embryos using standard procedures[24] and were genotyped by X-gal staining intensity. Northern blot analysis of midgestation embryos and fibroblasts was used to provide independent confirmation of genotypes scored by X-gal staining. Probes: LRP6, a 1.9-kbp *Bgl*II cDNA fragment (3′ of the insertion); LRP5, a 400-bp *Eco*RI/*Not*I cDNA fragment containing the 3′ untranslated region (mouse expressed sequence tag, Genebank no. AI119858); β-actin, 610-bp *Pst*I cDNA fragment.

Skeletal preparation and general histology

We carried out skeletal preparations by standard methods[12]. For histology of tissue sections, embryos were fixed in Bouin's solution for 5–12 h (depending on size of embryo), dehydrated and embedded in paraffin, and 8-μm sections were stained with haematoxylin and eosin according to standard protocols.

vt;LRP6 matings

vt/*vt* mice (Jackson Laboratories, stock no. 000553) were crossed with LRP6 heterozygotes to produce *vt*/+;LRP6^{-}/+ and *vt*/+;+/+ offspring. Compound heterozygotes were subsequently mated to *vt* homozygotes. Inheritance of the LRP6 mutant allele was determined by β-gal staining of yolk sacs or dot blots of tail DNA as described above.

In situ hybridization

Whole-mount *in situ* hybridization of mouse embryos was carried out essentially as described[25]. Whole-mount embryos were sectioned on a cryostat. Probes were provided by A. McMahon (Pax3, Wnt-3a, Wnt-1 and Shh), G. Martin (Fgf8) and R. Harland (Myf-5).

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Correspondence and requests for materials should be addressed to W.C.S. (e-mail: skarnes@socrates.berkeley.edu).

Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase

Tommaso Simoncini*, Ali Hafezi-Moghadam†, Derek P. Brazil‡, Klaus Ley†, William W. Chin§ & James K. Liao*

** Cardiovascular and § Genetics Divisions, Department of Medicine, Brigham & Women's Hospital and Harvard Medical School, 221 Longwood Avenue, Boston, Massachusetts 02115, USA*
† Department of Biomedical Engineering, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908, USA
‡ Howard Hughes Medical Institute, Joslin Diabetes Center, Boston, Massachusetts 02215, USA

Oestrogen produces diverse biological effects through binding to the oestrogen receptor (ER)[1]. The ER is a steroid hormone nuclear receptor, which, when bound to oestrogen, modulates the transcriptional activity of target genes[2]. Controversy exists, however, concerning whether ER has a role outside the nucleus[3], particularly in mediating the cardiovascular protective effects of oestrogen[4]. Here we show that the ER isoform, ERα, binds in a ligand-dependent manner to the p85α regulatory subunit of phosphatidylinositol-3-OH kinase (PI(3)K). Stimulation with oestrogen increases ERα-associated PI(3)K activity, leading to the activation of protein kinase B/Akt and endothelial nitric oxide synthase (eNOS). Recruitment and activation of PI(3)K by ligand-bound ERα are independent of gene transcription, do not involve phosphotyrosine adapter molecules or *src*-homology domains of p85α, and extend to other steroid hormone receptors. Mice treated with oestrogen show increased eNOS activity and decreased vascular leukocyte accumulation after ischaemia and reperfusion injury. This vascular protective effect of oestrogen was abolished in the presence of PI(3)K or eNOS inhibitors. Our findings define a physiologically important non-nuclear oestrogen-signalling pathway involving the direct interaction of ERα with PI(3)K.

PI(3)K mediates the cellular effects of platelet-derived growth factor (PDGF)[5], insulin[6] and vascular endothelial growth factor (VEGF)[7]. The predominant form of PI(3)K comprises p85α, an adapter/regulatory subunit of relative molecular mass 85,000 (M_r 85K), and p110, a catalytic subunit[8] of M_r 110K. PI(3)K catalyses the

formation of lipid mediators[9,10] which recruit signalling molecules containing phosphatidylinositol (PtdIns)-3,4,5-P_3-binding or pleckstrin homology domains such as phosphatidylinositol-dependent kinases and protein kinase Akt[11,12]. The activation of Akt through phosphorylation of Thr 308/Ser 473 (ref. 13) mediates many of the downstream cellular effects of PI(3)K, including stimulation of glucose transporter-4 membrane translocation[14], inactivation of glycogen synthase kinase-3 (ref. 15), and activation of eNOS[16,17] and cell survival pathways[18]. Although oestrogen stimulates eNOS activity[19] and promotes cell survival, it is not known whether PI(3)K mediates these effects of oestrogen.

In human vascular endothelial cells, physiological concentrations of 17β-oestradiol (E_2) increased eNOS activity in a biphasic manner (effector concentration for half-maximal response (EC_{50}) ≈ 0.1 nM) (Fig. 1a, b). The initial increase was mediated by mitogen-activated protein (MAP) kinases[19]; the second increase was completely blocked by the PI(3)K inhibitor, wortmannin. The increase in eNOS activity was also blocked by the ER antagonist ICI 182,780; and the inactive E_2 stereoisomer 17α-oestradiol (αE_2) had no effect. In murine fibroblasts transfected with ERα and eNOS complementary DNAs, E_2 produced an eightfold increase in eNOS activity in wild-type but not in p85α-deficient ($p85\alpha^{-/-}$) fibroblasts[20] (Fig. 1c). Furthermore, in $p85\alpha^{-/-}$ fibroblasts co-transfection of p85α cDNA led to a fourfold increase in E_2-stimulated eNOS activity, whereas in wild-type fibroblasts co-transfection of a dominant-negative p85α mutant cDNA decreased E_2-stimulated eNOS activity by more than 50%.

In non-transfected human endothelial cells, E_2 increased endogenous PtdIns-3,4,5-P_3 levels in a time-delayed manner similar to the wortmannin-sensitive phase of eNOS activation (Fig. 2a). In contrast, insulin rapidly increased endogenous PtdIns-3,4,5-P_3 levels[6] and eNOS activity[21]. Increases in PtdIns-3,4,5-P_3 levels correlated temporally with the ligand-dependent increases in ERα-associated PI(3)K activity (Fig. 2b); events that were blocked by ICI 182,780 and wortmannin (Fig. 2c). Consistent with a rapid, non-nuclear effect of ER on eNOS activation, E_2-stimulated PI(3)K activity was blocked by another ER antagonist, tamoxifen, but not by the MAP kinase inhibitor PD 98059, or by the transcriptional inhibitor actinomycin D (Fig. 2d). Insulin, which uses the phosphotyrosine (p-Tyr) adapter molecule, insulin receptor substrate (IRS)-1, to interact with PI(3)K, increased PI(3)K activity in the p-Tyr and IRS-1 immunoprecipitate (Fig. 2e), but did not increase or augment E_2-associated PI(3)K activity. In contrast, E_2 did not increase p-Tyr- or IRS-1-associated PI(3)K activity (Fig. 2e). These findings suggest that ERα does not recruit PI(3)K that has been already activated by insulin, and that PI(3)K activation by ER and IRS-1 occurs through different mechanisms. Notably, the activation of PI(3)K extended to other steroid hormone nuclear receptors such as the thyroid hormone and glucocorticoid receptors (Fig. 2f). These interactions may explain some of the previously unrecognized functions of these nuclear hormone receptors.

ERα interacted with p85α in a ligand-dependent manner in both non-transfected endothelial cells (Fig. 3a) and $p85\alpha^{-/-}$ fibroblasts

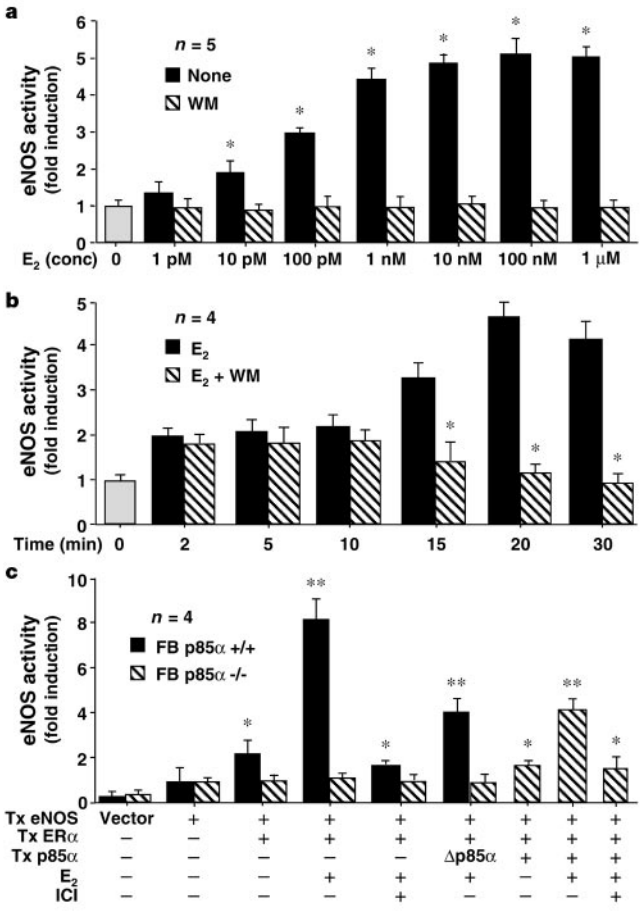

Figure 1 Activation of eNOS by oestrogen is mediated by PI(3)K. **a**, **b**, Concentration-dependent (**a**) and time-dependent (**b**) effects of E_2 and wortmannin (WM, 30 nM) on eNOS activity (fold induction versus baseline) in human vascular endothelial cells. Asterisk indicates $P < 0.05$ compared with unstimulated or E_2 stimulation. **c**, E_2-stimulated NOS activity in murine $p85\alpha^{+/+}$ and $p85\alpha^{-/-}$ fibroblasts (FB) transfected (Tx) with vector (pcDNA3), eNOS, ERα, p85α or dominant-negative p85α (Δp85α) cDNAs. Asterisk indicates $P < 0.05$ compared with transfection with eNOS cDNA alone; two askerisks indicate $P < 0.05$ compared with transfection with ERα and eNOS cDNAs.

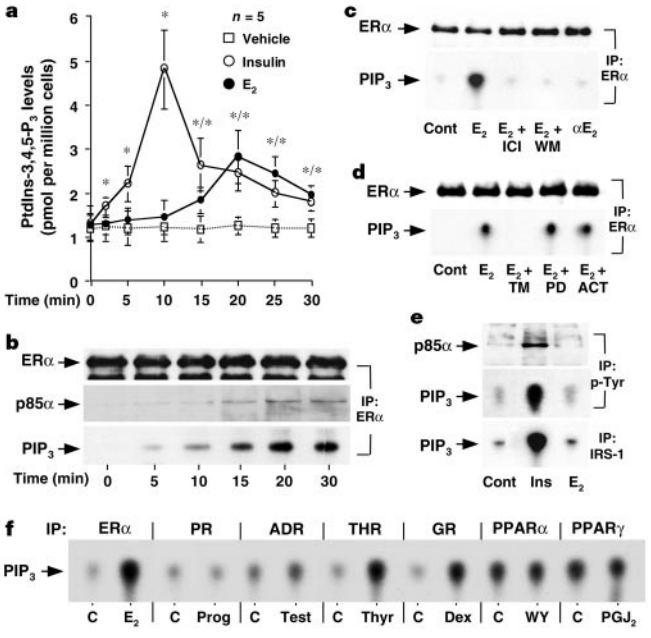

Figure 2 Oestrogen stimulates ERα-associated PI(3)K activity. **a**, Effect of vehicle (ethanol 0.01% v/v), E_2 (10 nM) or insulin (100 nM) on endogenous PtdIns-3,4,5-P_3 levels. Asterisk indicates $P < 0.05$ compared with vehicle. **b**, Time-dependent effect of E_2 on ERα, p85α and PI(3)K activity (PIP_3) in ERα immunoprecipitate (IP). **c**, Effect of ICI (10 μM) or WM on E_2 or 17α-oestradiol (αE_2)-stimulated ERα-associated PI(3)K activity. Cells were pre-treated with ICI or WM for 30 min. **d**, Effect of tamoxifen (TM, 1 μM), PD 98059 (PD, 5 μM) and actinomycin D (ACT, 5 μM) on ERα-associated PI(3)K activity. Inhibitors were added 2 h before E_2 stimulation. **e**, Effect of E_2 or insulin (Ins) on p-Tyr- and IRS-1-associated PI(3)K activity. **f**, Effect of E_2, progesterone (Prog, 10 nM), testosterone (Test, 10 nM), thyroid hormone (Thyr, 10 nM), dexamethasone (Dex, 1 μM), WY14643 (WY, 100 μM) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2, 100 μM) on PI(3)K activity in the corresponding steroid hormone nuclear receptor immunoprecipitates.

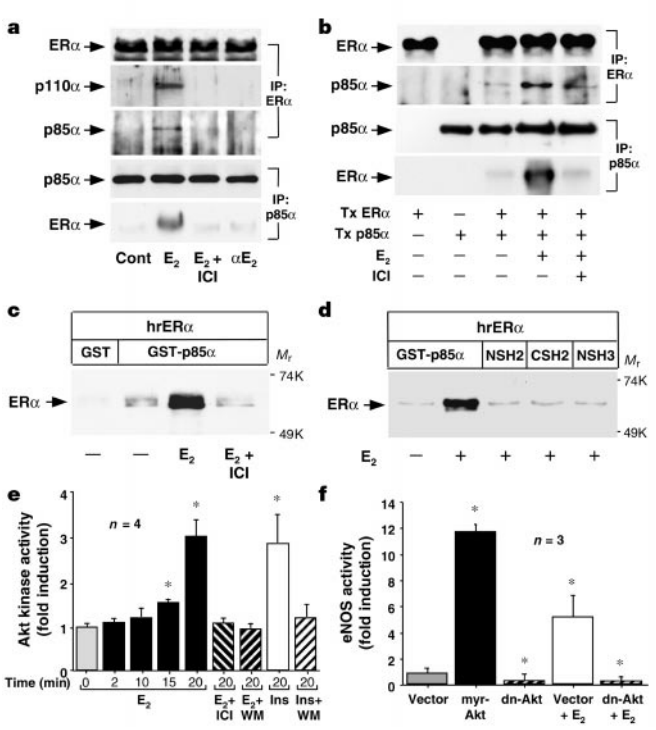

Figure 3 Ligand-dependent interaction of ERα with p85α. **a**, **b**, Effect of E_2 on ERα–p85α co-immunoprecipitation in non-transfected human endothelial cells (**a**) and murine p85α$^{-/-}$ fibroblasts (**b**) transfected (Tx) with ERα and p85α, alone or in combination. **c**, **d**, Affinity purification using agarose-conjugated GST or GST–p85α (**c**), or GST–p85α amino-terminal SH2 domain (NSH2, amino acids 321–470), carboxy-terminal SH2 domain (CSH2, 576–724), or SH3 domain (NSH3, 1–80) fusion protein and human recombinant (hr) ERα (**d**). **e**, E_2- or insulin (Ins)-stimulated Akt kinase activity. Asterisk indicates $P < 0.05$ compared with no stimulation. **f**, Effect of E_2 on eNOS activity (fold induction over baseline) in endothelial cells transfected with adenovirus containing no Akt (vector), constitutively active (myr), or a dominant-negative (dn) Akt. Asterisk indicates $P < 0.05$ compared with vector alone.

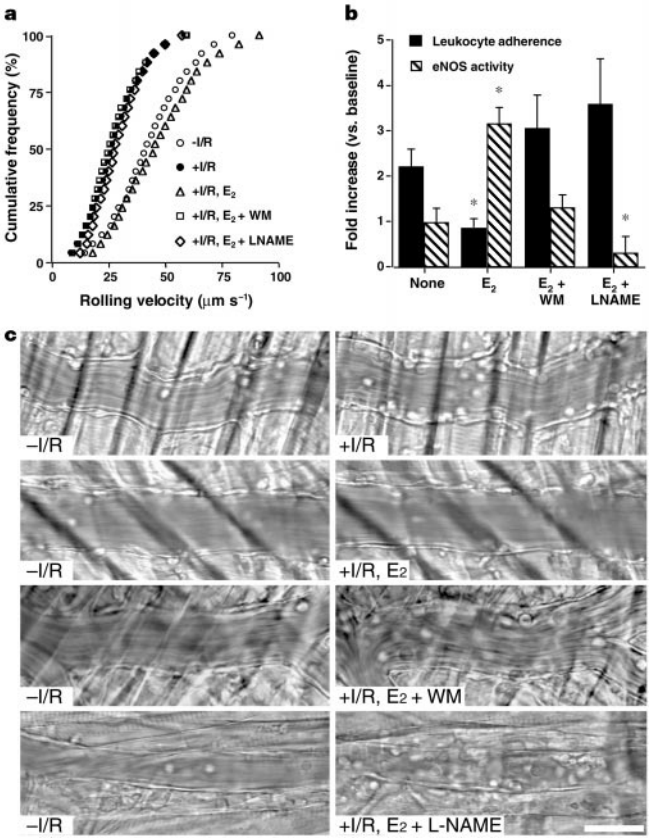

Figure 4 PI(3)K and NO mediate the vascular protective effects of oestrogen. Cumulative histograms of leukocyte rolling velocities before (–) and after (+) ischaemia and reperfusion (I/R) are shown. **a**, **b**, Effect of superfused WM (100 nM) or L-nitroarginine methylester (L-NAME, 0.1 mM) on leukocyte rolling velocity (**a**) and leukocyte adhesion and eNOS activity in the murine cremaster muscle (**b**). Data are expressed as fold increase over baseline before I/R in the same paired venules. Asterisk indicates $P < 0.001$ compared with untreated after I/R (None). **c**, Representative video images showing the same venules before (–) and after (+) I/R with the indicated treatments. Scale bar, 40 μm.

transfected with ERα and p85α cDNAs (Fig. 3b). This ligand-dependent interaction was blocked by ICI 182,780 and was absent in p85α$^{-/-}$ fibroblasts transfected with ERα cDNA alone. However, the ER isoform ERβ, which is thought to mediate some of the cardiovascular effects of oestrogen[4], did not interact with p85α or recruit PI(3)K activity after E_2 stimulation (see Supplementary Information). The interaction of ERα and p85α also occurred in the absence of adapter molecules or accessory proteins, as human recombinant ERα could still interact with glutathione *S*-transferase (GST)–p85α fusion protein in a ligand-dependent manner in a cell-free system (Fig. 3c). This interaction, however, does not involve the *src*-homology SH2/SH3 domains of p85α (Fig. 3d) which interact with p-Tyr residues of growth hormone receptors and adapter molecules[22,23]. Heat shock protein 90, which binds and facilitates the function of ER[24] and eNOS[25], inhibited the interaction of ERα and p85α.

The generation of PtdIns-3,4,5-P_3 leads to the recruitment and activation of Akt[11,26]. E_2 stimulated Akt kinase activity in a time-delayed manner (Fig. 3e), similar to the increases observed in PtdIns-3,4,5-P_3 levels and eNOS activity. To determine whether E_2-stimulated eNOS activation is mediated by Akt, we transiently transfected bovine aortic endothelial cells with adenoviruses containing constitutively active (myr) and dominant-negative (dn) Akt mutants[27]. Transfection of these cells with myr-Akt produced a substantial increase in eNOS activity, whereas overexpression of dn-Akt decreased basal eNOS activity below baseline and completely abolished E_2-stimulated eNOS activity (Fig. 3f).

To determine the physiological significance of this pathway, we used an established model of ischaemia and reperfusion (I/R) injury in the mouse cremaster muscle[28]. I/R leads to leukocyte recruitment to the vascular wall, an event attenuated by NO and exacerbated by eNOS inhibitors such as L-nitroarginine methylester (L-NAME)[29]. I/R reduced median leukocyte rolling velocity by 13.8 μm s^{-1} ($P < 0.003$) and induced a 2.2-fold increase in the number of adherent leukocytes ($P < 0.001$) (Fig. 4a, b). Treatment with E_2 increased eNOS activity 3.2-fold and prevented the subsequent changes in leukocyte accumulation and rolling velocity after I/R. When wortmannin or L-NAME was applied to the cremaster muscle, measurements of leukocyte rolling velocity and accumulation were not different between untreated and E_2-treated mice after I/R, although L-NAME decreased eNOS activity below that of untreated mice (Fig. 4a–c). These findings indicate that the NO-induced vascular protective effect of oestrogen is predominantly mediated by PI(3)K.

Although the nuclear function of ER is clearly established, previous studies regarding the membrane and cytoplasmic effects of oestrogen remain inconclusive[3]. Linking the ER to PI(3)K suggests that the ER may be involved in a critical function outside the nucleus. In addition, the potential biological effects of oestrogen are considerably broadened because PI(3)K is known to mediate various cellular functions[18]. Although most of the ER is localized to the nucleus, we found that there is an increased level of membrane

and cytoplasmic ER after E_2 stimulation (data not shown). Indeed, a study has suggested that membrane-associated ER is involved in mediating NO release from endothelial cells[30]. Thus, it is likely that PI(3)K is being recruited and activated by a small subset of ligand-bound, membrane-associated ERs. It remains to be determined, however, whether oestrogen can also activate PI(3)K indirectly, and whether PI(3)K can account for other rapid, non-nuclear effects of oestrogen. Further studies characterizing the interaction domains of ERα and p85α should help clarify these issues. □

Methods

Cell cultures

Human and bovine aortic endothelial cells were obtained enzymatically with Type IA collagenase (1 mg ml^{-1}). They were cultured and stimulated under serum-starved conditions consisting of phenol-red-free Medium 199 (Gibco BRL, Life Technologies) with 0.4% charcoal-stripped fetal calf serum.

Immunoprecipitations

Cells were washed with ice-cold PBS and lysed with the following buffer: Tris-HCl (20 mM, pH 7.4), EDTA (10 mM), NaCl (100 mM), IGEPAL (1%), Na_3VO_4 (1 mM), NaF (50 mM), PMSF (0.1 mg ml^{-1}) and aprotinin (0.3 mg ml^{-1}). We added the immunoprecipitating antibody (1 μg) to equal amounts of cell lysates (0.5–1 mg) in 500 μl of lysis buffer for 1 h at 4 °C with gentle rocking. Afterwards, 40 μl of 1:1 Protein-A-agarose was added and the entire mixture was rocked gently for another 1 h at 4 °C. The mixture was then centrifuged at 12,000*g* for 5 min at 4 °C. The supernatant was removed and the immunoprecipitate was washed three times with 500 μl of washing buffer, which differs from the lysis buffer in having 150 mM NaCl instead of 100 mM NaCl. We then separated proteins in the washed immunoprecipitate by SDS-PAGE and immunoblotted them with anti-ERα (Ab-10: Clone TE111.5D11, NeoMarkers, Fremont, CA) or anti-p85α (Upstate Biotech., Lake Placid, NY) antibody.

GST fusion protein-affinity purification

Human recombinant GST–p85α fusion protein or GST (Sigma) bound to glutathione-agarose beads (1 μg protein per 20 μl beads) was suspended in 400 μl of *Escherichia coli* protein extract solution (10 mg ml^{-1}) and incubated with 1 μg human recombinant ERα (Panvera, Madison, WI) for 1 h at 4 °C. We pelleted the samples, and washed the beads five times with a buffer containing 50 mM potassium phosphate, pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 10 % (v/v) glycerol and 1% (v/v) Triton X-100 plus protease inhibitors. The beads were re-suspended in 50 μl of 2× Laemmli's buffer and boiled for 5 min. Proteins were separated on SDS-PAGE.

Model of vascular injury

Ten-week-old, 24 g, male C57BL/6 mice (Hilltop, Scottsdale, PA) were subcutaneously implanted with 1.5 mg of slow-release E_2 tablets (Innovative Research of America, Sarasota, FL) 3–5 days before experiments to ensure steady-state serum E_2 levels and to avoid any effects of surgery on baseline haemodynamic parameters. Mice implanted with E_2 tablets had a serum E_2 level of 760 ± 30 pg ml^{-1} compared with that of vehicle-treated mice (24 ± 6 pg ml^{-1}). Mice were anaesthetized and the cremaster muscle was studied under intravital microscopy[28]. Ischaemia was induced by applying pressure to supplying arteries just sufficient to stop blood flow for 30 min. In some experiments, wortmannin (100 nM) or L-NAME (0.1 mM) was applied to the cremaster muscle during the ischaemic period. The pressure was released for reperfusion, and the same vessels were recorded in each animal before and after I/R. The rolling velocities of 25 leukocytes were measured in each venule, sorted and averaged for each rank to construct cumulative histograms. The velocities of 3,750 leukocytes were measured in 150 venules before and after I/R. The number of firmly adherent leukocytes was measured before and after I/R in the same 200-μm long segments of venules. The following number of venules were studied for leukocyte adhesion: untreated, 15 venules; E_2-treated, 20 venules; E_2-treated with wortmannin, 25 venules; E_2-treated with L-NAME, 15 venules. Cremaster eNOS activity was measured in three untreated, four E_2-treated, five E_2-treated with wortmannin and four E_2-treated with L-NAME mice.

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Correspondence and requests for materials should be addressed to J.K.L. (e-mail: jliao@rics.bwh.harvard.edu).