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Endoglin Regulates Cyclooxygenase-2 Expression and Activity

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Abstract—The endoglin heterozygous (Eng+/−) mouse, which serves as a model of hereditary hemorrhagic telangiectasia (HHT), was shown to express reduced levels of endothelial NO synthase (eNOS) with impaired activity. Because of intricate changes in vasomotor function in the Eng−/− mice and the potential interactions between the NO- and prostaglandin-producing pathways, we assessed the expression and function of cyclooxygenase (COX) isoforms. A specific upregulation of COX-2 in the vascular endothelium and increased urinary excretion of prostaglandin E2 were observed in the Eng−/− mice. Specific COX-2 inhibition with parecoxib transiently increased arterial pressure in Eng+/− but not in Eng+/+ mice. Transfection of endoglin in L6E9 myoblasts, shown previously to stimulate eNOS expression, led to downregulation of COX-2 with no change in COX-1. In addition, COX-2 promoter activity and protein levels were inversely correlated with endoglin levels, in doxycyclin-inducible endothelial cells. Chronic NO synthesis inhibition with Nω-nitro-L-arginine methyl ester induced a marked increase in COX-2 only in the normal Eng+/+ mice. Nω-nitro-L-arginine methyl ester also increased COX-2 expression and promoter activity in doxycyclin-inducible endoglin expressing endothelial cells, but not in control cells. The level of COX-2 expression following transforming growth factor-β1 treatment was less in endoglin than in mock transfected L6E9 myoblasts and was higher in human endothelial cells silenced for endoglin expression. Our results indicate that endoglin is involved in the regulation of COX-2 activity. Furthermore, reduced endoglin levels and associated impaired NO production may be responsible, at least in part, for augmented COX-2 expression and activity in the Eng+/− mice. (Circ Res. 2006;99:248-256.)

Key Words: endoglin ■ COX-2 ■ NO ■ TGF-β1

Endoglin (CD105) is a homodimeric membrane glycoprotein that, in association with transforming growth factor (TGF)-β family receptors, binds TGF-β1, TGF-β3, activin, bone morphogenetic protein (BMP)-2, and BMP-7.1 Endoglin is constitutively expressed on endothelial cells of capillaries, veins, and arteries2,3 and can also be observed, albeit at lower levels, in contractile cells such as vascular smooth muscle cells,4 and mesangial cells.5 Mutations in the endoglin (ENG) gene cause hereditary hemorrhagic telangiectasia type 1 (HHT1), also known as Rendu–Osler–Weber syndrome.6 HHT is an autosomal dominant vascular dysplasia that affects 1:10 000 individuals. This disorder is associated with epistasis and telangiectases in the majority of patients and with pulmonary and cerebral arteriovenous malformations that are more frequent, particularly in HHT1 patients. ENG mutations are distributed throughout the gene and lead to haploinsufficiency,7 indicating that endoglin levels are critical in maintaining vascular homeostasis. Endoglin null (Eng−/−) mice die at midgestation of vascular and cardiac defects, demonstrating the critical role of endoglin in cardiovascular development.8,9,10

The vascular endothelium secretes vasodilators including NO, which is produced mostly by endothelial NO synthase (eNOS), and the prostacyclin (PGI2) and prostaglandin E2 (PGE2), which are produced by cyclooxygenases (COXs).11 Endoglin is a regulatory component of the TGF-β receptor system in endothelial cells capable of modulating specific responses to this multipotent growth factor.10,12,13 Its reduced expression may result in disruption of the delicate balance in the secretion of endothelium-derived vasodilators and vasoconstrictors, thus inducing changes in vascular tone regulation. We have previously demonstrated that endoglin haploinsufficiency leads to a complex modification in the regulation of vascular resistance associated with a decrease in eNOS expression and impaired activity in Eng−/− mice.14,15

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Molecular cross-talk between NOS and COX pathways has been documented in several cell types including endothelial cells. However, most studies have focused on inflammatory cells and pharmacological manipulation of both pathways. Therefore, the mechanisms regulating the production, release, and fine balance of NO and prostaglandins (PGs) in endothelial cells are still poorly understood. There are reports of stimulation of PGs by NO in human microvascular and umbilical vein endothelial cells that suggest an additional pathway used by nitrovasodilators to elicit vasodilation. However, chronic inhibition of NO synthesis by N\textsuperscript{-}nitro-L-arginine methyl ester (L-NAME) treatment of rats leads to a decreased flow-induced dilatation in resistance mesenteric arteries, compensated by an increase in COX-2 expression and vasodilatory PGs.

NO stimulates PGE\textsubscript{2} release in arteries, compensated by an increase in COX-2 expression. Arginine methyl ester (L-NAME) treatment of rats leads to a decreased flow-induced dilatation in resistance mesenteric arteries, compensated by an increase in COX-2 expression and vasodilatory PGs. NO stimulates PGE\textsubscript{2} release in arteries, compensated by an increase in COX-2 expression.

However, chronic inhibition of NO synthesis by N\textsuperscript{-}nitro-L-arginine methyl ester (L-NAME) treatment of rats leads to a decreased flow-induced dilatation in resistance mesenteric arteries, compensated by an increase in COX-2 expression and vasodilatory PGs. NO stimulates PGE\textsubscript{2} release in arteries, compensated by an increase in COX-2 expression. Arginine methyl ester (L-NAME) treatment of rats leads to a decreased flow-induced dilatation in resistance mesenteric arteries, compensated by an increase in COX-2 expression and vasodilatory PGs. NO stimulates PGE\textsubscript{2} release in arteries, compensated by an increase in COX-2 expression.

Changes in mean arterial pressure (MAP) in response to vasoactive substances were measured in Eng\textsuperscript{−/−} and Eng\textsuperscript{+/−} mice as described. Mice were kept in ventilated rooms in a germ-free facility. All studies were performed in parallel. The inhibition of COX-2 activity is mediated by COX-1 protein expression was observed between the cell type and state of activation. Elevation of COX-2 expression and activity by chronic in vivo administration of rat NO synthesis inhibitor parecoxib (Dynastat, Pharmacia EEIG, Buckinghamshire, UK; 40 mg/kg b.m. IV) for 30 minutes, or the NO synthesis inhibitor L-NAME (Sigma, I-7378; 5 mg/kg b.m. IV) for 1 hour. Subsequent combined treatments were given as indicated.

Materials and Methods

An expanded materials and methods section is available in the online data supplement at http://circres.ahajournals.org.

Mice

Generation and genotyping of Eng\textsuperscript{+/−} mice on a C57BL/6 background was previously described. Mice were kept in ventilated rooms in a germ-free facility. All studies were performed in parallel. In Eng\textsuperscript{−/−} and Eng\textsuperscript{+/−} littermate female mice aged 4 to 6 months (20 to 25 g). All animal procedures were approved by the University of Salamanca Animal Care and Use Committee. To inhibit NO synthesis in vivo, L-NAME (Sigma) was administered at a dose of 10 mg/kg per day in the drinking water for 28 days.

Blood Pressure Measurements

Changes in mean arterial pressure (MAP) in response to vasoactive substances were measured in Eng\textsuperscript{−/−} and Eng\textsuperscript{+/−} mice as described. Mice were treated with the nonselective COX inhibitor indomethacin (Sigma, 1-7378; 5 mg/kg b.m. SC) for 1 hour, the selective COX-2 inhibitor parecoxib (Dynastat, Pharmacia EEIG, Buckinghamshire, UK; 40 mg/kg b.m. IV) for 30 minutes, or the NO synthesis inhibitor L-NAME (50 mg/kg body mass IV) for 1 hour. Subsequent combined treatments were given as indicated.

Biological Samples, Tissues, and Aortic Endothelial Cell Preparation

Urine PGE\textsubscript{2} was measured using a high-sensitivity immunoassay (R&D Systems), and creatinine was quantified using the Jaffe method. Plasma and urine concentrations of nitrates were determined by a modification of the Griess method. Mice were deeply anesthetized using isoflurane (Forane, Abbott). The kidneys, lungs, and femoral arteries were removed, frozen in liquid nitrogen, individually ground into a fine powder, and stored at −80°C until used for protein and total RNA extraction. Mouse aortic endothelial cells (MAECs) from Eng\textsuperscript{−/−} and Eng\textsuperscript{+/−} mice were isolated and cultured as described.

Cell Culture and Transfections

The rat myoblast cell line L6E9 and the doxycycline-inducible bovine endothelial GM7372-EL cell line were cultured in DMEM, and the human microvascular endothelial (HMEC)-1 cells, grown in eosin/methylene blue medium (Gibco). The generation of doxycycline-inducible endothelin-expressing GM7372-EL cells and stable rat myoblast transfectants expressing human L-endoglin was previously described. The myoblast transfectants were usually cultured in the presence of 400 μg/mL of the G418 antibiotic. COX-2 transcriptional activity was measured using luciferase reporter gene assays and was expressed as relative luciferase units (RLU). To analyze the effect of TGF-β1 on COX-2 expression, L6E9 myoblasts were incubated with different concentrations of recombinant human TGF-β1 (0 to 500 pM) for 24 hours before Western blot analysis. For RNA interference studies, the human endothelial cell line HMEC-1 was transiently transfected with the pX2–COX-2 luc reporter vector and pSUPER-Endo/Ex4 plasmid, encoding an endoglin-specific sequence of small interference RNA (siRNA), or pSUPER-C plasmid, used as negative control. After incubation for 36 hours, 400 pM TGF-β1 was added, and the cells were incubated for 24 hours before measurement of the transcriptional activity.

Western and Northern Blot Analyses

Preparation of tissue and cell extracts for Western blot analysis was described. For Northern blot analysis, total RNA was isolated from tissues using the guanidium thiocyanate–phenol–chloroform method and processed as described. A 4.5-kb fragment of mouse COX-2 EDNA (kindly given by Dr Santiago Lamas, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain) was used as probe. The 28S ribosomal subunit probe (0.7 kb) served as an internal control.

Results

COX Expression in Endoglin Heterozygous Mice

We first examined the expression of COX-1 and COX-2 in tissues of mice by Western blot analysis. No difference in COX-1 protein expression was observed between theEng\textsuperscript{−/−} and Eng\textsuperscript{+/−} kidneys (Figure 1A), isolated femoral arteries (supplemental Figure I), or lung tissues (data not shown). However, expression of COX-2 protein was higher in kidneys (≈2-fold; Figure 1A), femoral arteries (≈2.6-fold; Figure I), and lungs (≈1.8-fold; data not shown) of Eng\textsuperscript{+/−} mice compared with control Eng\textsuperscript{+/−} mice. The rise in COX-2 expression was associated with increased biological activity as confirmed by elevated urinary excretion of PGE\textsubscript{2} in Eng\textsuperscript{+/−} mice (Figure 1B). Expression of COX-2 mRNA was higher in renal and pulmonary tissues of Eng\textsuperscript{+/−} mice when compared with control Eng\textsuperscript{−/−} mice (≈2.2- and 2.1-fold, respectively; Figure 1C).

Immunohistochemical analysis of femoral artery sections revealed that COX-2 is expressed mainly in endothelial cells.
but also in scattered smooth muscle cells in both Eng+/− and Eng−/− mice (Figure 2A and 2B). The cellular staining was specific as demonstrated by the lack of reactivity of the nonimmune serum (Figure 2C). The expression of COX-2 was higher in femoral arteries of Eng−/− mice, in agreement with the elevated levels of COX-2 observed in kidneys and lungs.

**COX-2 Expression Is Increased in Endothelial Cells Isolated From Endoglin Heterozygous Mice**

To confirm the selective increase in COX-2 expression in endothelial cells of Eng+/− mice, we prepared primary cultures of MAECs. As expected for engineered heterozygous mice, Eng+/− MAECs had lower endoglin levels than Eng+/+ cells (Figure 3A). COX-1 expression was unchanged, whereas COX-2 expression was increased by 3-fold in aortic
endothelial cells from Eng+/− mice (Figure 3A). These results indicate that endothelial cells are at least in part responsible for the higher COX-2 expression and activity observed in Eng+/− mice.

**COX-2 Expression Is Decreased in Endoglin Transfected L6E9 Myoblasts**

To determine whether the increase in COX-2 observed in the Eng+/− mice was related to reduced endoglin levels, we assessed COX-2 expression in mock-transfected and human endoglin-transfected L6E9 myoblast cell lines. Figure 3B illustrates the efficiency of transfection in terms of endoglin expression and reveals no changes in COX-1 levels. However, the endoglin-transfected myoblasts showed much reduced COX-2 expression relative to the mock-transfected cells. Thus, the inverse correlation between COX-2 and endoglin levels, seen in endothelial cells from the HHT murine model, was reproduced in endoglin transfected myoblasts.

**COX-2 Expression Is Inversely Correlated With Endoglin Expression in GM7372-EL Cells**

To substantiate the potential modulation by endoglin of COX-2 expression in endothelial cells, a tetracycline-inducible bovine endothelial cell line, GM7372-EL, was used.25 These cells have been engineered to express human endoglin when treated with doxycycline (Dox). Figure 4A shows that the level of endoglin measured by Western blot was proportional to the dose of Dox used. Interestingly, the increase in endoglin was accompanied by a parallel decrease in COX-2 and increase in eNOS expression levels (Figure 4A). We next analyzed the COX-2 promoter-driven transcription in transiently transfected GM7372-EL endothelial cells. Induction of endoglin expression with increasing concentrations of Dox resulted in a marked inhibition of COX-2 transactivity (Figure 4B).

**Chronic NOS Inhibition Leads to Increased COX-2 Levels in Eng+/− But Not in Eng−/− Mice**

To assess whether lower eNOS expression in Eng−/− mice14,15 is responsible for the decreased COX-2 activity, we tested the effect of chronic NOS inhibition by treating mice with L-NAME for 28 days. The effectiveness of chronic NO synthesis inhibition was demonstrated by a decrease in plasma nitrite concentration in Eng+/+ mice (from 2.82±0.48 μmol/L in untreated mice to 0.77±0.20 μmol/L after treatment with L-NAME, P<0.01) and in Eng−/− mice (from 1.67±0.24 μmol/L in untreated mice to 0.90±0.20 μmol/L after L-NAME, P<0.05).

Western blot analysis showed that COX-2 expression in kidneys (Figure 5A) and lungs (Figure 5B) of Eng+/+ mice was higher in the L-NAME–treated group, suggesting that NOS inhibition is associated with an increase in COX-2. In Eng−/− mice, which show higher basal levels of COX-2 than the Eng+/+ mice, no further increase was obtained by chronic treatment with L-NAME. Hence, the COX-2 level in Eng−/− mice chronically treated with L-NAME was equivalent to the basal level observed in Eng+/+ mice. Urinary PGE2 excretion was also measured after chronic L-NAME treatment, as an indicator of COX-2 activity. PGE2 excretion in Eng+/+ mice was higher in the L-NAME–treated (7.2±0.6 ng/mg of creatinine) than untreated (4.2±0.2 ng/mg of creatinine; P<0.005) group. Basal PGE2 excretion in untreated Eng+/− mice (6.4±0.6 ng/ mg of creatinine) was higher than in Eng+/+ mice, as shown in Figure 1B, and these levels were not increased by chronic treatment with L-NAME (7.4±0.6 mg/ mg of creatinine; P>0.1).

**Effect of NO on Endoglin-Dependent Regulation of COX-2 in Endothelial Cells**

We next analyzed the effects of eNOS inhibition on COX-2 promoter activity in GM7372-EL cells, whose endoglin levels were overinduced at the highest Dox concentration. L-NAME selectively increased COX-2 promoter activity in endoglin-induced cells (Figure 6A), suggesting that reduced NOS activity was associated with increased COX-2 expression. By contrast, treatment with the NO donor sodium nitroprusside (SNP) decreased COX-2 promoter activity in both groups of cells, indicating that NO inhibited COX-2 activity.
Western blot analysis also revealed that COX-2 expression was selectively increased by L-NAME in endoglin overexpressing GM cells as compared with controls (Figure 6B). The NO donor decreased COX-2 protein levels in untreated GM cells but had no effect in endoglin overexpressing cells (Figure 6B). L-NAME also increased COX-2 protein levels in MAECs from Eng/H11001/H11001, but not in those from Eng/H11001/H11002 mice (Figure 6C).

Induction of COX-2 Expression By TGF-β1 Is Not Affected By Endoglin

To test the possibility that TGF-β1 is involved in endoglin-dependent COX-2 expression, we assessed COX-2 levels in myoblasts. TGF-β1 induced a dose-dependent increase in COX-2 expression in both mock and endoglin transfectants. The basal level of COX-2 was higher in the mock- versus endoglin-transfected cells, and both basal levels steadily increased after stimulation with increasing concentrations of TGF-β1 (Figure 7A). The relative rise in COX-2 induced by TGF-β1 was similar (1.4- and 1.6-fold at 5 pM TGF-β1; 2.1- and 1.8-fold at 50 pM TGF-β1), indicating that endoglin affected basal levels, but not the TGF-β1-mediated increase in COX-2.

The potential involvement of endoglin in the TGF-β1-induced COX-2 promoter transactivation was further tested
by siRNA experiments in human endothelial cells. Transfection with pSUPER-endoEx4 vector encoding specific sequences of siRNA that suppress endoglin expression increased the basal levels of COX-2 but did not alter the stimulation by TGF-β1 (2.5-fold versus 3.3-fold in the control cells) (Figure 7B). Nevertheless, taking into account the overall absolute values observed in Figure 7, it appears that the COX-2 expression levels in response to TGF-β1 are maximal in the absence of endoglin and decrease in its presence.

**Hemodynamic Studies**

Because we observed an increase in COX-2 expression in tissues of Eng−/− mice and a higher urinary excretion of PGE2, we next assessed if these alterations modify vascular function. Changes in MAP were measured in Eng−/− and Eng−/+ mice after selective COX-2 blockade with parecoxib. A rapid (5 minutes), transient, and statistically significant increase in blood pressure was observed only in Eng−/− mice (Figure 8A). As NO and COX products are both implicated in maintenance of vascular resistance, we determined changes in blood pressure in Eng−/− and Eng−/+ mice after COX and NOS blockade. Before inhibition of NO synthesis with L-NAME further substantiated the effect of parecoxib on Eng−/− mice. A small but significant and sustained increase in MAP was observed in Eng−/+ mice, but not in their littermate controls (Figure 8B), and after 30 minutes no significant differences in MAP were seen between the 2 groups of mice. Administration of the NOS inhibitor L-NAME alone induced
in COX-2 inhibition did not modify the effects of L-NAME to animals pretreated with parecoxib or indomethacin absence of COX-2–derived prostanoids, we administered (45 mm Hg; Figure 8C), whereas almost no response was observed in Eng^-/-^ animals when compared with cells from littermates. This increased COX-2 expression was mainly observed in Eng^-/-^ mice (Figure 8D), as reported previously. To assess the effect of blocking NO synthesis in the absence of COX-2–derived prostanoids, we administered L-NAME to animals pretreated with parecoxib or indomethacin. COX-2 inhibition did not modify the effects of L-NAME in Eng^-/-^ mice (Figure 8C). In contrast, both COX inhibitors (parecoxib and indomethacin) led to a significant increase in the pressor effect of L-NAME in Eng^-/-^ mice (Figure 8D).

**Discussion**

The present study demonstrates that COX-2 was overexpressed in several tissues of Eng^-/-^ mice relative to Eng^+/+^ littermates. This increased COX-2 expression was mainly observed in the vascular endothelium and was not accompanied by changes in COX-1 expression. This finding was supported by the observation that cultured aortic endothelial cells from Eng^-/-^ mice expressed higher COX-2 but similar COX-1 levels when compared with cells from Eng^+/+^ mice. The inverse correlation between endoglin and COX-2 levels was also seen in the L6E9 rat myoblast cell line, which showed reduced COX-2 expression when transfected with endoglin. Also, in the bovine endothelial GM7372-EL line, Dox-inducible endoglin expression was inversely correlated with COX-2 promoter activity and protein levels.

An additional proof of increased COX-2 activity in the Eng^-/-^ mice is their higher urinary excretion of PGE_2_, a major product of COX-initiated arachidonic acid metabolism in the kidney. Because we did not observe differences in renal COX-1 expression between Eng^-/-^ and Eng^+/+^ mice but increased renal COX-2 expression in Eng^-/-^ mice, we suggest that the increased urinary excretion of PGE_2_ is mainly caused by increased COX-2 metabolism.

The increase in COX-2 expression in tissues and the higher urinary excretion of PGE_2_ in Eng^-/-^ mice led to measurements of arterial pressure in Eng^-/-^ and Eng^+/+^ mice after COX-2 blockade. The selective inhibitor parecoxib induced a transient blood pressure increase only in Eng^-/-^ mice, which have higher levels of COX-2. Because this enzyme generates predominantly vasodilators, the rise in pressure on its inhibition suggests a more significant role for COX-2 in the vasoregulation of Eng^-/-^ than in Eng^+/+^ mice. As we previously showed that eNOS expression and activity was lower in these mice and that their hypertensive response to L-NAME was reduced, we measured the effects of combined inhibition of both NOS and COX activities. Pretreatment of Eng^-/-^ mice with parecoxib did not enhance the increase in MAP induced by L-NAME. In contrast, the same treatment applied to Eng^-/-^ mice restored the hypertensive response to L-NAME, suggesting that COX-2–derived vasodilators contribute substantially to MAP regulation in Eng^-/-^ mice. Furthermore, depletion of NO by pretreatment with L-NAME followed by selective COX-2 inhibition induced an increase in MAP.
in MAP only in Eng−/− mice. These findings suggest a critical role for COX-2 derivatives in the vasoregulation of Eng−/− mice, which may compensate for the decreased NO availability.

To investigate the specific mechanism responsible for increased COX-2 expression in Eng−/− mice, we postulated that decreased NO production in Eng−/− mice, 14,15 could stimulate COX-2 expression. Our results corroborate this hypothesis, showing that chronic inhibition of NOS activity in Eng−/− mice leads to a significant increase in COX-2 expression and PGE2 urinary excretion. However, L-NAME had no effect on the already elevated COX-2 found in Eng−/− mice. These findings suggest that chronically decreased NO synthesis in Eng−/− mice might have led to a maximal increase in COX-2 and represents a mechanism of physiological adaptation to endoglin haploinsufficiency.

Because endoglin is a coreceptor for TGF-β, it was of interest to measure the effects of this cytokine on COX-2 expression in cells expressing either no or high endoglin. COX-2 can be stimulated by TGF-β1 in several tissues and cell types, 33–35 and endoglin can neutralize some cellular effects of TGF-β1. 27,36,37 Our experiments with cultured cells demonstrated that although TGF-β1 increased COX-2 expression, the level of induction was not different in mock- and endoglin-transfected myoblasts, despite higher basal levels in endoglin negative cells. Additionally, suppression of endoglin expression in human endothelial cells resulted in increased basal levels in COX-2 promoter activity but did not lead to further induction by TGF-β1. These results suggest that endoglin regulates total levels of COX-2 in basal and TGF-β1–treated cells, but does not alter the TGF-β1–dependent induction of COX-2 expression.

In conclusion, our results demonstrate upregulation of endothelial COX-2 and a consequent increase in vasodilator prostaglandin production in a mouse model of HHT1. The inverse correlation between endoglin and COX-2 expression was confirmed in endothelial and nonendothelial cells and suggests that endoglin modulates COX-2 expression. Our results also indicate that decreased eNOS activity, likely via reduction of NO production, might mediate the rise in COX-2.

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Disclosures
None.

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Supplementary material

Endoglin regulates COX-2 expression and activity
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Materials and Methods

Mice

Generation and genotyping of $Eng^{+/−}$ and littermate control mice (on a C57BL/6 background) was previously described.1 Mice were kept in ventilated rooms, in a germ-free facility. All studies were performed in parallel in $Eng^{+/−}$ and littermate $Eng^{+/+}$ female mice of 4-6 months of age (20-25 g). Mice were treated in accordance with international and national guidelines for the care and use of laboratory animals, as issued by Conseil de l’Europe (Official Daily N. L358/1-358/6, 18th December 1986), Spanish Government (Boletín Oficial del Estado N. 67, pp. 85098512, 18th March 1988, and Boletín Oficial del Estado N. 256, pp. 3134931362, 28th October 1990), and the U.S. National Institutes of Health (Guide for the Care and Use of Laboratory Animals, NIH publication no. 8523). All animal procedures performed were approved by the University of Salamanca Animal Care and Use Committee.

Blood pressure measurements

Arterial blood pressure has been measured in anesthetized mice as previously described.2,3 Mice were anaesthetized with sodium pentobarbital (40 mg/kg b. w., i.p.), body temperature was maintained at 37ºC using a heating pad, and tracheotomy was performed. The right carotid artery was cannulated with PE-10 and PE-50 tubings and connected to a pressure transducer for continuous measurement of arterial blood pressure. Arterial pressure was recorded in a digital data recorder (MacLab/4e, AD Instruments, Australia) and analyzed using Chart v 3.4 (an application program). The left jugular vein was catheterized for the administration of vasoactive substances. Supplemental doses of anesthesia (10 mg sodium pentobarbital/kg b. wt.) were administrated as required. If anesthetic addition caused any detectable change in arterial pressure or heart rate, the animals were discarded.
Changes in mean arterial pressure (MAP) in response to vasoactive substances were measured in *Eng*+/– and *Eng*+/+ mice as described.14 Mice were pretreated with the non-selective cyclooxygenase inhibitor indomethacin (Sigma, I-7378; 5 mg/kg b.m., s.c.) for 1 hour, the selective COX 2 inhibitor parecoxib (Dynastat, Pharmacia EEIG, Buckinghamshire, U.K; 40 mg/kg b.m., i.v.) for 30 min., or the NO synthesis inhibitor L-NAME (50 mg/kg b.m., i.v.) for 1 hour. The reason for the pre-treatment duration of each inhibitor is to allow the time necessary to obtain a stable MAP. Subsequent combined treatments were given as indicated. Animals showing any sign of hemorrhage and animals with a systolic blood pressure lower than 60 mmHg were excluded.

**Tissue and aortic endothelial cells preparation.**

Mice were deeply anesthetized using isoflurane (Forane, Abbott Lab). The kidneys, lungs and femoral arteries were removed, frozen in liquid nitrogen, individually ground into a fine powder and stored at –80ºC until used for protein and total RNA extraction. Mouse aortic endothelial cells (MAEC) from *Eng*+/+ and *Eng*+/− mice were isolated and cultured as previously described.2 In all experiments, cells did not exceed passage 3, and were characterized using antibodies to von Willebrand factor and CD31/PECAM-1. In each experiment, a pool of cells from three different aortas was used to decrease inter-individual variation.

**Chronic nitric oxide synthase inhibition**

To inhibit NO synthesis *in vivo*, L-NAME was administered at a dose of 10 mg/kg/day in the drinking water during 28 days, based on an average water intake of 4-5ml per mouse per day. For the last two days of L-NAME administration, mice were transferred to metabolic cages for urine collection. The efficiency of NO synthesis inhibition was assessed by measuring plasma and urine nitrite levels as described.32
**PGE2, creatinine and nitrite detection**

Urine was transferred to eppendorf tubes containing indomethacin (10 µg/mL), centrifuged and stored at -40°C. PGE2 was measured using a high sensitivity immunoassay (R&D Systems) and creatinine was quantified colorimetrically using a modified Jaffe’s method. Plasma and urine concentrations of nitrites were determined by a modification of the Griess method as described.3

**Immunohistochemistry**

Immunostaining was performed on buffered formalin fixed, paraffin-embedded tissues, following antigen retrieval with a citrate solution (BioGenex) in a microwave oven for 10 minutes. Endogenous peroxidase was blocked by incubation in 3% H2O2 and the sections were incubated with an affinity purified polyclonal antibody to COX-2 (Santa Cruz Biotechnology; catalogue # sc-1747) and processed using the goat ABC Staining system (Santa Cruz Biotechnology; catalogue # sc-2023) combined with the chromogen diaminobenzidine (DAB, BioGenex). Negative controls were prepared using a non-immune serum. Sections were counterstained with hematoxylin-eosin.

**Cell transfectants expressing endoglin and endothelial cell lines**

The rat myoblast cell line L6E9, and the tetracycline-inducible bovine endothelial GM7372-EL cell line4 were cultured in DMEM, while the human microvascular endothelial HMEC-1 cells5 were grown in EMB (Gibco). Generation of doxycyclin-inducible endoglin-expressing GM7372-EL cells and of stable rat myoblast transfectants expressing human L-endoglin was previously described.4,6 The myoblast transfectants were cultured in the presence of 400 µg/ml of the G418 antibiotic. GM7372-EL cells, plated at 5 x 10^5/dish in 60-mm culture plates, were incubated with or without Dox for 24 hours and treated or not with L-NAME (5mM) or SNP (100µM) for 24 hours prior to Western blot analysis. To analyze the effect of TGF-β1 on
COX-2 expression, L6E9 myoblast were incubated with different concentrations of recombinant human TGF-β1 (0-500 pM) for 24 hours prior to immunodetection analysis.

**Western blot analysis**

Preparation of tissue and cell extracts for Western blot analysis was previously described. COX-1 and COX-2 expression was assessed using a mouse monoclonal anti-COX-1 and a goat polyclonal anti COX-2 (Santa Cruz Biotechnology). Human endothelial (Transduction Laboratories) and RAW 264.7 phorbol ester activated (Santa Cruz Biotechnology) cell lysates were used as positive controls for COX-1 (70-72kDa) and COX-2 (74kDa), respectively. Murine and human endoglin were detected using the rat monoclonal antibody MJ7/18 or the mouse monoclonal antibody P4A4, respectively. eNOS was detected with a rabbit polyclonal anti-endothelial type constitutive NOS (Santa Cruz Biotechnology ; catalogue # sc-653). Specific protein levels were normalized to either α-tubulin or β-tubulin levels as indicated.

**Northern blot Analysis**

Total RNA was isolated from lungs and kidneys using the guanidinium thiocyanate-phenol-chloroform method, as previously described. A 4.5-kb fragment of mouse COX-2 cDNA in pcDNA I/neo (kindly given by Dr. Santiago Lamas, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain) was used as probe. The 28S ribosomal subunit probe (0.7-kb) served as an internal control.

**Reporter assays**

COX-2 transcriptional activity was measured using a pXP2LUC reporter plasmid containing the –1795 to +104 region of the human COX-2 promoter. When necessary, cells incubated for 24 hours with L-NAME (5mM) or SNP (sodium nitroprussiate; 100µM). Transactivation was measured using the luciferase reporter assay. All transient transfections were carried out...
using Superfect (Qiagen). Relative luciferase units (RLU) were determined in a TD20/20 luminometer (Promega). Samples were co-transfected with the SV40-ß-galactosidase expression plasmid to correct for transfection efficiency. ß-galactosidase activity was measured using the Galacto-Light kit (Tropix). Experiments were performed in triplicate, at least three times; representative ones are illustrated.

RNA interference studies

The pSUPER-Endo-Ex4 vector was generated by inserting a double-stranded oligonucleotide corresponding to human endoglin exon 4 into pSUPER plasmid.11 Upon transfection, the pSUPER-Endo-Ex4 generates intracellular expression of small endoglin RNA molecules that silence the human endoglin gene. The pSUPER-C vector containing an endoglin-unrelated sequence was used as a negative control.11 To suppress the expression of endoglin, HMEC-1 cells were transfected with pSUPER plasmids 36 hours prior to functional analysis. After incubation for 36 hours, 400 pM TGF-ß1 was added and the cells were incubated for 24 hours. COX-2 transcriptional activity was measured using the luciferase reporter assay.

Statistics

Data are expressed as mean ± SEM. Significance was tested using Student’s t test when two groups are compared, and 2-way repeated-measures ANOVA followed by Scheffe’s test for multiple intra- and inter-group comparisons. \( P <0.05 \) was considered significant.

References


**Figure Legend**

**Online Figure 1.** Expression of COX-1 and COX-2 in femoral arteries of *Eng* \(^{+/+}\) and *Eng* \(^{+/-}\) mice. **A,** Representative Western blot analysis of renal tissues with antibodies specific for COX-1, COX-2, or β-tubulin (loading control). **B,** Densitometry results are expressed as COX/tubulin ratios. Data shown are mean ± SEM of 8 mice per group. \(*P < 0.05\) vs. *Eng* \(^{+/+}\) group (Student’s t test).
Online Figure 1