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 E_2 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 N^{ω}-nitro-L-arginine methyl ester induced a marked increase in COX-2 only in the normal $Eng^{+/+}$ mice. N^{ω}-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.

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 arteries[2,3] 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 epistaxis 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 (PGI_2) and prostaglandin E_2 (PGE_2), 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.[16] 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.[16] However, chronic inhibition of NO synthesis by N^{ω}-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.[17,18] NO stimulates PGE_2 release in COX-2–deficient cells, but inhibits such release in COX-1 null cells. The inhibition of COX-2 activity is mediated by nitration of this enzyme in tyrosine residues.[19] It was also reported that the diverse effects of NO on COX-2 expression can be: (1) mediated directly or via other stimuli[20,21]; (2) related to the cell type and state of activation[22]; and (3) dependent on NO concentration, being stimulatory at low levels and inhibitory at high levels.[23]

In the current study, we measured COX isoforms expression and activity in tissues and endothelial cells isolated from $Eng^{+/-}$ mice and identified an inverse relationship between endoglin and COX-2 levels. We assessed the effect of NOS and COX-2 inhibition on arterial pressure in $Eng^{+/-}$ and $Eng^{+/+}$ mice. We also tested the effects of L-NAME on COX-2 expression and activity by chronic in vivo administration in the murine model of HHT or by in vitro treatment of cells expressing various levels of endoglin. Our data indicate that $Eng^{+/-}$ mice, shown previously to have impaired eNOS activity, had elevated COX-2, suggesting that endoglin plays a role in the maintenance of vascular homeostasis and the fine balance between eNOS and COX-2 in endothelial cells.

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 $Eng^{+/-}$ mice on a C57BL/6 background was previously described.[8,24] Mice were kept in ventilated rooms in a germ-free facility. All studies were performed in parallel in $Eng^{+/-}$ and $Eng^{+/+}$ 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 $Eng^{+/-}$ and $Eng^{+/+}$ mice as described.[14] Mice were treated with the nonselective COX inhibitor indomethacin (Sigma, I-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_2 was measured using a high-sensitivity immunoassay (R&D Systems), and creatinine was quantified using the Jaffe method. Plasma and urine concentrations of nitrites were determined by a modification of the Griess method.[14] 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 $Eng^{+/+}$ and $Eng^{+/-}$ mice were isolated and cultured as described.[14]

Cell Culture and Transfections

The rat myoblast cell line L6E9 and the doxycycline-inducible bovine endothelial GM7372-EL cell line[25] were cultured in DMEM, and the human microvascular endothelial (HMEC)-1 cells,[26] were grown in eosin/methylene blue medium (Gibco). The generation of doxycyclin-inducible endoglin-expressing GM7372-EL cells and stable rat myoblast transfectants expressing human L-endoglin was previously described.[25,27] 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[28] 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 pXP2–COX-2 luc reporter vector[28] and pSUPER-Endo/Ex4 plasmid,[29] encoding an endoglin-specific sequence of small interference RNA (siRNA), or pSUPER-C plasmid,[29] 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.[5,30] For Northern blot analysis, total RNA was isolated from tissues using the guanidinium thiocyanate–phenol–chloroform method and processed as described.[14] A 4.5-kb fragment of mouse COX-2 cDNA (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 the $Eng^{+/+}$ and $Eng^{+/-}$ 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 $Eng^{+/-}$ mice compared with control $Eng^{+/+}$ mice. The rise in COX-2 expression was associated with increased biological activity as confirmed by elevated urinary excretion of PGE_2 in $Eng^{+/-}$ mice (Figure 1B). Expression of COX-2 mRNA was higher in renal and pulmonary tissues of $Eng^{+/-}$ mice when compared with control $Eng^{+/+}$ 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

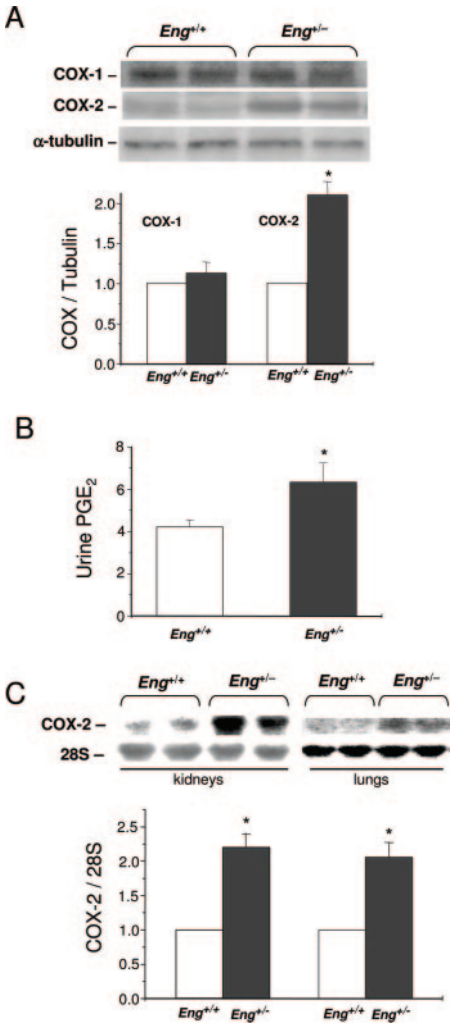


Figure 1. Expression and activity of COX-1 and COX-2 in $Eng^{+/+}$ and $Eng^{+/-}$ mice. A, Western blot analysis of renal tissues with antibodies specific for COX-1, COX-2, or α-tubulin (loading control). Densitometry results are expressed as COX/tubulin ratios. B, Urinary PGE_2 excretion was measured by ELISA and expressed in ng/mg creatinine. C, Northern blot analysis of kidney and lung total RNA; COX-2 mRNA levels are expressed relative to the 28S RNA band. Data shown in each panel are mean±SEM of 8 mice per group. *$P<0.05$ vs $Eng^{+/+}$ group (Student t test).

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

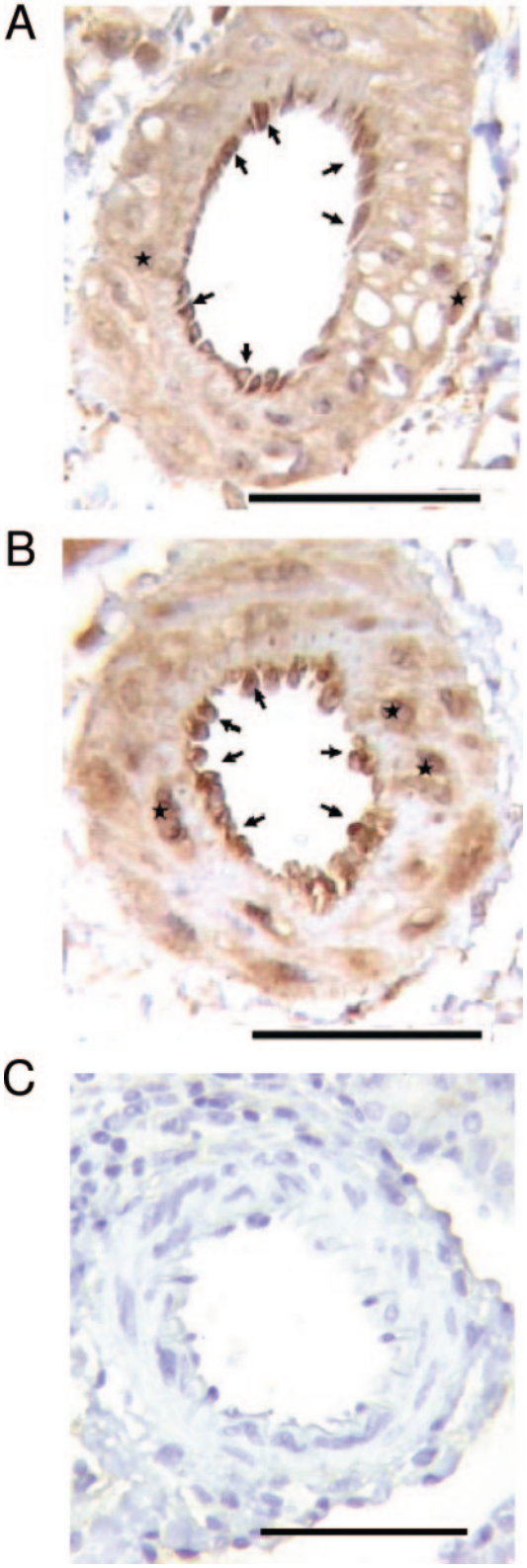


Figure 2. COX-2 immunostaining in arteries of $Eng^{+/+}$ and $Eng^{+/-}$ mice. Femoral arteries from $Eng^{+/+}$ (A) and $Eng^{+/-}$ (B) mice were stained for COX-2. Positive staining was observed in endothelial cells (arrows) and smooth muscle cells (asterisks). No staining was seen with a non-immune rabbit serum control (C). Bar: 100 μm.

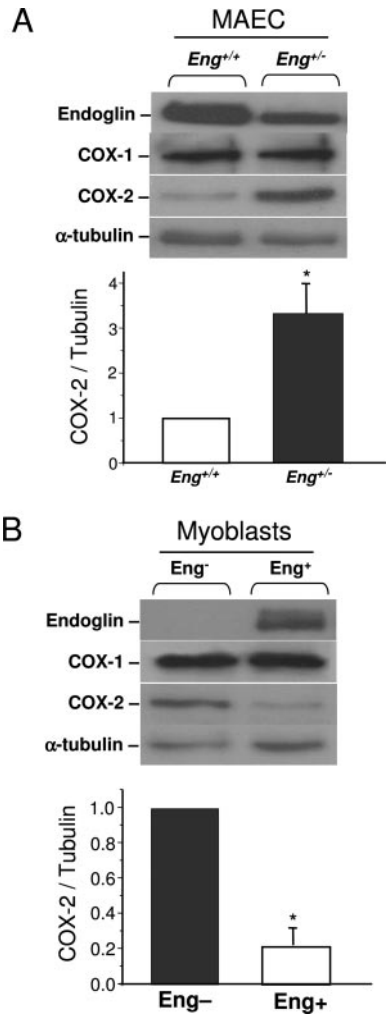


Figure 3. Western blot analysis of endoglin, COX-1, COX-2, and tubulin in murine aortic endothelial cells and L6E9 myoblasts. A, Extracts of $Eng^{+/+}$ and $Eng^{+/-}$ murine aortic endothelial cells. B, Extracts of L6E9 myoblasts transfected with vector alone (Eng^{-}) or with human L-endoglin (Eng^{+}). Densitometry results are expressed as the relative ratio of COX-2 to α-tubulin and represent the means±SEM of at least 3 paired experiments. *Significant difference with control group ($P<0.05$, Student t test).

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 $Eng^{+/-}$ mice[14,15] 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 PGE_2 excretion was also measured after chronic L-NAME treatment, as an indicator of COX-2 activity. PGE_2 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 PGE_2 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 ng/ 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.

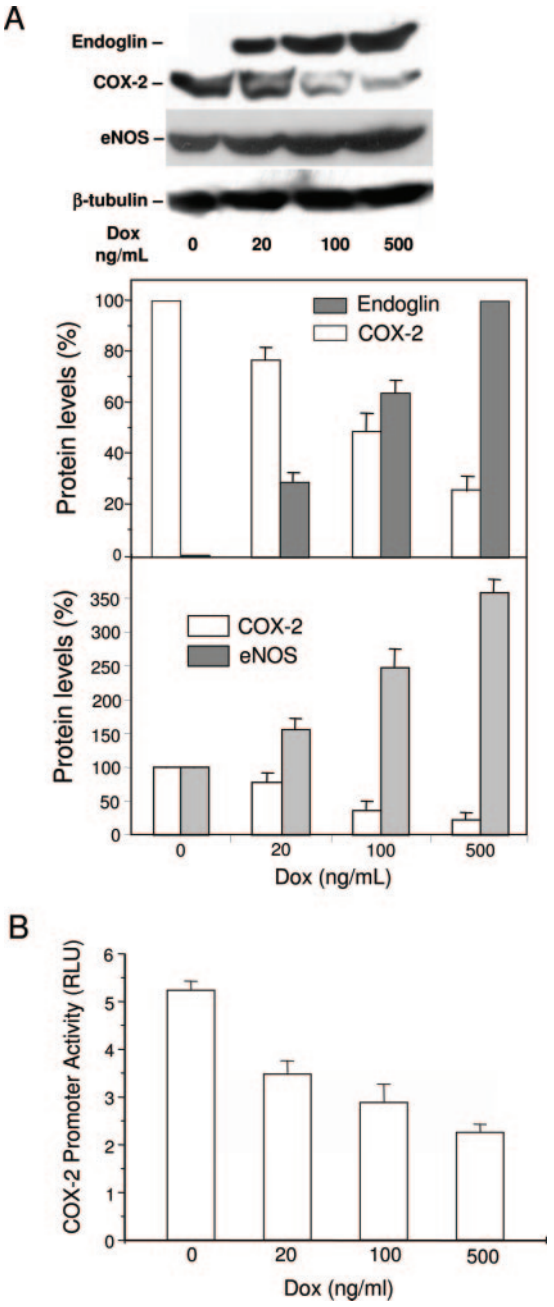


Figure 4. Relationship between endoglin and COX-2 in GM7372-EL endothelial cells. A, Bovine endothelial GM7372-EL cells were incubated with increasing concentrations of doxycyclin (Dox) for 48 hours to induce endoglin expression. Endoglin, COX-2, eNOS, and β-tubulin were detected in total cell extracts by Western blot analysis. Figure is representative of 3 separate experiments. Densitometry results are expressed as % of maximal COX-2/β-tubulin and endoglin/β-tubulin ratios (upper histogram) or % of basal COX-2/β-tubulin and eNOS/β-tubulin ratios (lower histogram). B, Bovine endothelial GM7372-EL cells were transfected with the pXP2-COX-2 luc reporter vector and incubated with increasing concentrations of Dox for 48 hours. COX-2 promoter transactivity was measured using the luciferase reporter assay. The data are representative of 3 separate experiments.

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

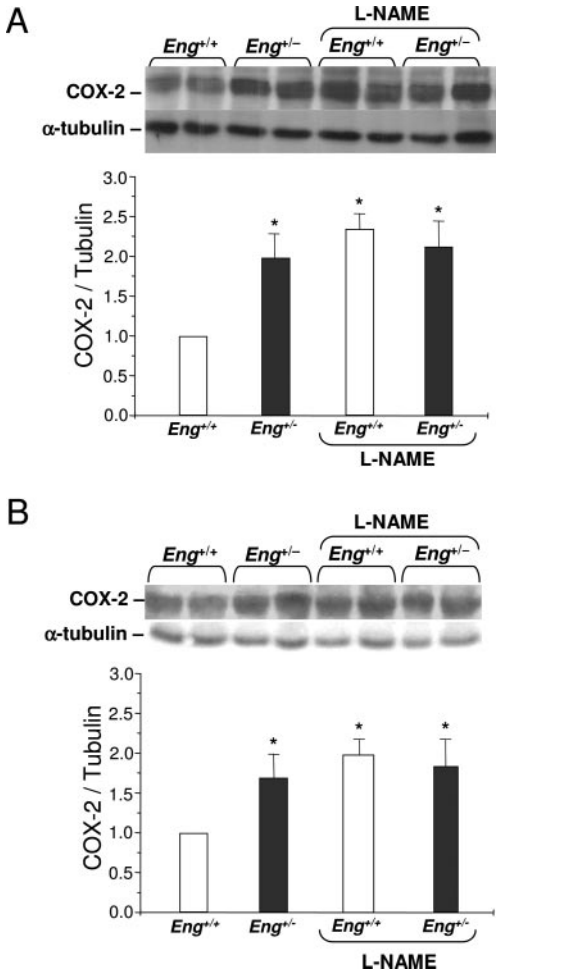


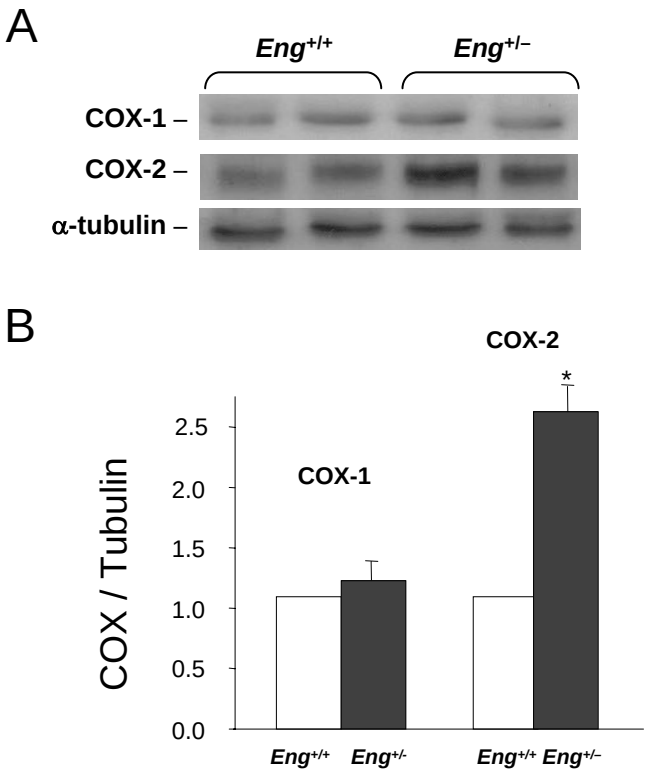
Figure 5. Differential COX-2 response of *Eng*$^{+/+}$ and *Eng* $^{+/-}$ mice to chronic treatment with L-NAME. Mice were treated with or without L-NAME in their drinking water for 28 days. COX-2 expression in renal tissue (A) or lungs (B) is shown. The data are expressed as mean±SEM (n=8) of the ratio COX-2/α-tubulin relative to the untreated *Eng*$^{+/+}$ mice. *$P<0.05$ vs *Eng*$^{+/+}$ without L-NAME; 2-way ANOVA and Scheffé test.

GM cells but had no effect in endoglin overexpressing cells (Figure 6B). L-NAME also increased COX-2 protein levels in MAECs from *Eng*$^{+/+}$, but not in those from *Eng*$^{+/-}$ 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-β–induced COX-2 promoter transactivation was further tested



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