Regulation of Bone Morphogenetic Protein-2 Expression in Endothelial Cells

Role of Nuclear Factor-κB Activation by Tumor Necrosis Factor-α, H_2O_2, and High Intravascular Pressure

Anna Csiszar, MD, PhD; Kira E. Smith; Akos Koller, MD, PhD; Gabor Kaley, PhD; John G. Edwards, PhD; Zoltan Ungvari, MD, PhD

Background—Recent studies suggest that bone morphogenetic protein-2 (BMP-2), a transforming growth factor-β superfamily member cytokine, plays an important role both in vascular development and pathophysiological processes, including endothelial activation that is likely to contribute to the development of coronary atherosclerosis, yet the factors that regulate arterial expression of BMP-2 are completely unknown. We tested the hypothesis that BMP-2 expression in endothelial cells is governed by an H_2O_2 and nuclear factor (NF)-κB–dependent pathway that can be activated by both proinflammatory and mechanical stimuli.

Methods and Results—The proinflammatory cytokine tumor necrosis factor (TNF)-α induced NF-κB activation and elicited significant increases in BMP-2 mRNA and protein in primary coronary arterial endothelial cells and human umbilical vein endothelial cells that were prevented by NF-κB inhibitors (pyrrolidine dithiocarbamate and SN-50), silencing of p65 (siRNA), or catalase. Administration of H_2O_2 also elicited NF-κB activation and BMP-2 induction. In organ culture, exposure of rat arteries to high pressure (160 mm Hg) elicited H_2O_2 production, nuclear translocation of NF-κB, and upregulation of BMP-2 expression. Although high pressure upregulated TNF-α, it appears that it directly regulates BMP-2 expression, because upregulation of BMP-2 was also observed in vessels of TNF-α knockout mice.

Conclusions—Vascular BMP-2 expression can be regulated by H_2O_2-mediated activation of NF-κB both by inflammatory stimuli and by high intravascular pressure. (***Circulation*. 2005;111:2364-2372.)**

Key Words: free radicals ■ cytokines ■ growth factors

The transforming growth factor superfamily member cytokine, bone morphogenetic protein-2 (BMP-2), was originally detected in cartilage and bone[1]; however, recent studies from this and other laboratories demonstrated that vascular endothelial and smooth muscle cells are also a significant source of BMPs.[2–6] First genetic analysis of patients with primary pulmonary hypertension indicated that a vascular BMP-2/BMP receptor system plays an important role in vascular physiology.[7,8] Indeed, BMP-2 has been shown to regulate a host of cellular functions,[2,4,5,9] including cardiovascular development,[9] neovascularization in tumors,[10] and smooth muscle cell chemotaxis in response to vascular injury.[2] Both BMP-2 (unpublished observation, 2004) and BMP-4, which are closely related by their amino acid sequence and act on the same receptor,[4,6] were shown to exert proinflammatory effects by inducing expression of adhesion molecules and enhancing monocyte adhesion. Endothelium-derived BMPs are also osteoinductive,[5] and hypotheses have been put forward that they may also contribute to vascular calcification during the development of atherosclerotic plaques.[3,5,11] Importantly, recent studies confirmed a striking upregulation of BMP-2/4 in atherosclerotic lesions.[2–4]

Despite the growing evidence for the physiological/pathophysiological importance of BMP-2 in blood vessels, the mechanisms regulating endothelial BMP-2 expression have not yet been elucidated. Previously, we[12] have demonstrated that in coronary arteries in hyperhomocysteinemia, vascular inflammation and upregulation of TNF-α are associated with an increased vascular BMP-2 expression. On the basis of these findings, we hypothesized that expression of BMP-2 in endothelial cells is regulated by inflammatory stimuli, such as TNF-α.[13] There are also data that show a mechanosensitive, stretch-induced BMP expression in chondrocytes.[14] Thus, we aimed to elucidate whether mechanical loading of cells by increased wall tension/high intraluminal pressure regulates endothelial BMP-2 expression. Previous studies by us and others suggested that both proinflammatory cytokines and high intraluminal pressure elicit vascular oxidative stress.[12,15] Importantly, an increased level of reactive oxygen species (in particular an increased H_2O_2 production) has been linked to

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From the Department of Physiology, New York Medical College, Valhalla, NY.

Correspondence to Zoltan Ungvari, MD, PhD, Department of Physiology, New York Medical College, Valhalla, NY 10595. E-mail zoltan_ungvari@nymc.edu

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nuclear factor (NF)-κB activation and proinflammatory phenotypic alterations in various cell types. Thus, we hypothesized that in endothelial cells, tumor necrosis factor (TNF)-α and high pressure increase H_2O_2 production, which elicits NF-κB activation and consequential upregulation of BMP-2 expression.

Methods

Studies on Endothelial Cell Cultures: TNF-α Stimulation, Pharmacological Inhibition, and RNA Interference (RNAi) Silencing of NF-κB

Primary rat coronary arterial endothelial cells (CAECs; Celprogen) and human umbilical vein endothelial cells (HUVECs; Cell Applications Inc) were maintained in culture as described previously.[16] After passage 4, the cells were treated with recombinant TNF-α (0.1 to 30 ng/mL for 24 hours)[16] in the presence or absence of an NF-κB inhibitor (pyrrolidine dithiocarbamate[17] [PDTC]; 10^{-5} mol/L), SN-50 (a cell-permeable peptide that inhibits nuclear translocation of NF-κB; 50 μg/mL, Calbiochem), SN-50M (an inactive form of SN-50), the H_2O_2 scavenger catalase (500 U/mL), the O_2^- scavengers PEG-SOD (200 U/mL) and Tiron (10 mmol/L), or the NAD(P)H oxidase inhibitors diphenyleneiodonium (DPI; 10^{-5} mol/L) and apocynin (3×10^{-4} mol/L).

In separate experiments, downregulation of the p65 subunit of NF-κB in HUVECs and CAECs was achieved by RNA interference with the proprietary sequences of the TranSilent NFkB siRNA Vector (Panomics) and the siLentGene U6 Cassette RNA Interference System (Promega), as we have reported previously.[16] Cell density at transfection was 30%. Specific gene silencing was verified with quantitative real-time reverse-transcription polymerase chain reaction (QRT-PCR) and Western blotting as described previously.[16] Cells transfected with anti-p65 small interfering RNA (siRNA) or scrambled siRNA were treated with TNF-α on day 4 after the transfection, when gene silencing was optimal.

Transient Transfection and Luciferase Assays

The effect of TNF-α on NF-κB activity in CAECs was tested by a reporter gene assay. We used an NF-κB reporter that comprised an NF-κB response element upstream of firefly luciferase (NF-κB -Luc, Stratagene) and a renilla luciferase plasmid under the control of the cytomegalovirus promoter (as an internal control). All transfections were performed with Novafector (Venn Nova LLC) according to the manufacturer's protocols. Firefly and renilla luciferase activities were assessed after 42 hours with the Dual Luciferase Reporter Assay Kit (Promega) and a luminometer.

Vessel Culture Studies

Male Wistar rats (n=24, Taconic Biotechnology, Germantown, NY) were euthanized by injection of sodium pentobarbital (50 mg/kg IP). Branches of the left and right femoral arteries of rats were isolated and maintained in vessel culture as reported previously.[12,15,16,18] In brief, arteries were cannulated on both sides in a stainless steel vessel culture chamber (Danish Myo Technology) under sterile conditions and superfused continuously with F12 medium (GIBCO BRL) that contained antibiotics (100 IU/L penicillin, 100 mg/L streptomycin, and 10 μg/L Fungizone), supplemented with 5% fetal calf serum (Boehringer-Mannheim), as described previously.[12,15,16,18] Arteries were exposed to 80 or 160 mm Hg for 24 hours. Minimal intraluminal flow was maintained only to renew the culture medium within the intraluminal space, with maintenance of minimal shear stress (<0.5 dyne/cm^2). Some segments were treated with PDTC (10^{-5} mol/L), SN-50, or DPI.

In another set of experiments, carotid arteries from 8-week-old male TNF-α–deficient mice and wild-type control mice (n=8, Jackson Laboratory, Bar Harbor, Me) were cultured at 80 or 160 mm Hg. At the end of the culture period, the arterial segments were removed from the organ culture bath and processed as described below.

Oligonucleotides for Real-Time RT-PCR

mRNA Targets	Sense	Antisense
Rat BMP-2	TCAAGCCAAACACAAACAG	CGCTAAGCTCAGTGGG
Rat TNF-α	TCGTAGCAAACCACCAAG	CTGACGGTGTGGGTGA
Rat p65	ATGGACGATCTGTTTCCC	GTCTTAGTGGTATCTGTGCT
Rat β-actin	GAAGTGTGACGTTGACAT	ACATCTGCTGGAAGGTG
Mouse BMP-2	ACACAAACAGCGGAAG	AGAGTCTGCACTATGGC
Mouse GAPDH	CCCAATGTGTCCGTCGTGG	AGCCCCGGCATCGAAG
Human BMP-2	GGTGGAATGACTGGATTG	GCATCGAGATAGCACTG
Human p65	GCAGTTTGATGATGAAGACC	CTGTCACTAGGCGAGT
Human GAPDH	AACGAATTTGGCTACAGC	AGGGTACTTTATTGATGGTACAT
Human β-actin	GATAGCATTGCTTTCGTGT	TTCAACTGGTCTCAAGTCAG

Animal Models of Hypertension

To study the effects of high pressure in vivo, hypertension was induced in male Wistar rats by abdominal aortic banding (n=8) as described previously (sham-operated control animals were used as controls).[18] The abdominal aortic banding model of hypertension provides the advantage that blood vessels proximal to the coarctation are exposed to high pressure, although in distal vascular beds, pressure does not exceed normotensive levels. Because both vascular beds are exposed to the same circulating factors, the in vivo effects of the chronic presence of high blood pressure on vascular BMP-2 expression could be assessed independently. Hypertension was also induced in separate groups of rats by infusion of angiotensin II (for 1 week) according to the methods of Laursen et al.[19]

Quantitative Real-Time PCR

Total RNA from the arteries was isolated with a Mini RNA Isolation Kit (Zymo Research) and was reverse transcribed with Superscript II RT (Invitrogen) as described previously.[20,21] The QRT-PCR technique was used to analyze mRNA expression with the Strategene MX3000, as reported previously.[12,16,20,21] The housekeeping gene β-actin or GAPDH was used for internal normalization. Oligonucleotides used for real-time QRT-PCR are listed in the Table. Fidelity of the PCR reaction was determined by melting temperature analysis and visualization of product on a 2% agarose gel.

Western Blotting

Western blotting was performed as described previously,[20,21] with a primary antibody that recognizes both the more abundant glycosylated (18 kDa) and the nonglycosylated (13 to 15 kDa) forms of BMP-2 (R&D Systems). The detection limit for recombinant, glycosylated BMP-2 (R&D Systems) was ≈10 ng/well (Figure 1B). Anti-β-actin (Novus Biologicals) was used for normalization purposes. IκB content was analyzed in cytoplasmic extracts that contained 10 μg of protein with an anti-IκBα antibody (Novus Biochemicals).

Nuclear Extraction and NF-κB Activity Assay

Nuclei were isolated from arteries pressurized to 80 or 160 mm Hg or treated with 10 ng/mL TNF-α (1 hour at 37°C) with the Nuclear Extraction kit from Active Motif. In brief, vessel segments were homogenized with a Dounce tissue homogenizer in 500 mL of ice-cold hypotonic lysis buffer, followed by 2 centrifugation steps (500*g* for 30 seconds at 4°C) to exclude tissue debris. Then, nuclear proteins (≈10 μg/vessel segment) were extracted according to the manufacturer's protocol. Cytosolic fractions were retained to assay

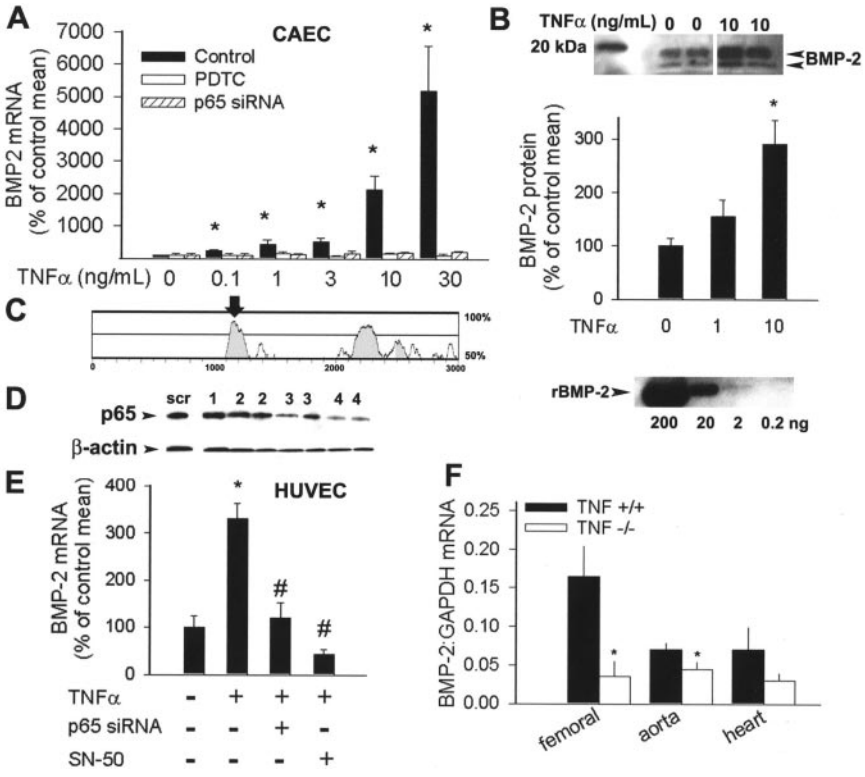

Figure 1. A, Effect of TNF-α (from 0.1 to 30 ng/mL, for 24 hours) on expression of BMP-2 mRNA in cultured primary rat CAECs under control conditions or in presence of PDTC (10^{-5} mol/L) or after anti-p65 siRNA treatment. Analysis of mRNA expression was performed by real-time QRT-PCR. Data are mean±SEM (n=4 to 5 for each group). *$P<0.05$. B, Representative Western blot showing increased BMP-2 protein expression in TNF-α–treated CAECs (first lane: molecular weight marker). Bar graphs are summary data of 4 independent experiments. Data are mean±SEM *$P<0.05$. Bottom, Demonstration of sensitivity of Western blot method with serial dilutions of glycosylated recombinant BMP-2 (rBMP-2, 18 kDa). *$P<0.05$. C, rVISTA plot showing percent of conservation between mouse and human at 5′ flanking region (3000 bp) of BMP-2 gene. Arrow points to location of highly conserved NF-κB binding site.[26] D, Representative Western blot showing downregulation of p65 subunit of NF-κB in HUVECs by day 4 after p65 siRNA treatment (scr indicates scrambled siRNA control). E, TNF-α–induced upregulation of BMP-2 mRNA in HUVECs is prevented by p65 siRNA treatment or by SN-50, an NF-κB inhibitor peptide. Data are mean±SEM (n=4 to 5 for each group). *$P<0.05$ vs untreated control; #$P<0.05$ vs TNF-α. F, Expression of BMP-2 mRNA in femoral artery, aorta, and heart of wild-type and TNF-α −/− mice, as determined by real-time QRT-PCR. GAPDH was used for normalization. Data are mean±SEM (n=3 to 5 for each group). *$P<0.05$.

IκBα content. Protein concentrations in samples were equalized with a Bradford protein assay (Bio-Rad). The nuclear extract obtained was used to assay NF-κB binding activity with the TransAM NF-κB ELISA kit (Active Motif) according to the manufacturer's guidelines.

In Situ Detection of Activated Form of NF-κB

Nuclear translocation of the activated form of NF-κB was detected in situ according to the methods of Au-Yeung et al.[22] In brief, femoral arterial branches were pressurized to 80 or 160 mm Hg or treated with 10 ng/mL TNF-α (for 1 hour at 37°C), then embedded in OTC medium and cryosectioned. The sections were fixed in acetone, permeabilized in PBS containing Triton X and Tween 20. Immunolabeling was performed with a primary antibody against the activated p65 subunit of NF-κB (Chemicon International). This antibody is specific for the activated form of NF-κB, thus allowing identification of active NF-κB.[22] A fluorescein (FITC)-conjugated goat anti-rabbit IgG (Zymed Laboratories) was used as secondary antibody (1:50, at 37°C for 45 minutes). Because the activated NF-κB is translocated in the nuclei, the sections were also stained with DAPI to identify cell nuclei.

H_2O_2 Measurements

H_2O_2 production was measured by the methods of Werner[23] in cultured endothelial cells exposed to the following treatments: TNF-α, TNF-α plus DPI, TNF-α plus apocynin [10^{-4} mol/L, to inhibit NAD(P)H oxidase[15,18]], or untreated control. Cells were incubated with an assay mix that consisted of homovanillic acid (100 μmol/L) and horseradish peroxidase (5 U/mL) in HEPES-buffered salt solution (pH 7.5) with or without catalase at 37°C for 1 hour. The reaction was stopped with 80 μL of glycine solution (0.1 mol/L, pH 10, 0°C). H_2O_2-induced fluorescent product was assessed with a fluorimeter (excitation 321 nm, emission 421 nm), and the background-corrected fluorescent signal was normalized to the cell count. Calibration curve was constructed with 0.01- to 100-μmol/L H_2O_2 standards in assay mix (1 hour at 37°C) with or without catalase (200 U/mL). This method also was used to measure H_2O_2 production in intact blood vessels. In 1 mL of assay mix, cannulated vessels were pressurized in the presence and absence of DPI, apocynin, or catalase. At a constant pressure (80 or 160 mm Hg), the vessels were superfused and intraluminally perfused with the assay mix at a minimal flow rate (shear stress <0.1 dyne/cm^2).

Data Analysis

Data were normalized to the respective control mean values. Data are expressed as mean±SEM. Statistical analyses of data were performed by Student's t test or by 2-way ANOVA followed by the Tukey post hoc test, as appropriate. $P<0.05$ was considered statistically significant.

Results

TNF-α Promotes BMP-2 Expression in Endothelial Cells: Role of NF-κB

In CAECs, TNF-α elicited substantial increases in the mRNA and protein expression of BMP-2 in a concentration-dependent manner (Figures 1A and 1B). Identical results were obtained in HUVECs as well (Figure 1E). Administration of PDTC, which completely abolished TNF-α–induced NF-κB activation (Figure 2E), prevented TNF-α–induced BMP-2 expression in CAECs (Figure 1A). TNF-α–induced BMP-2 expression in HUVECs was also prevented by the NF-κB inhibitor peptide SN-50 (Figure 1E), but not by its inactive form, SN-50M (not shown).

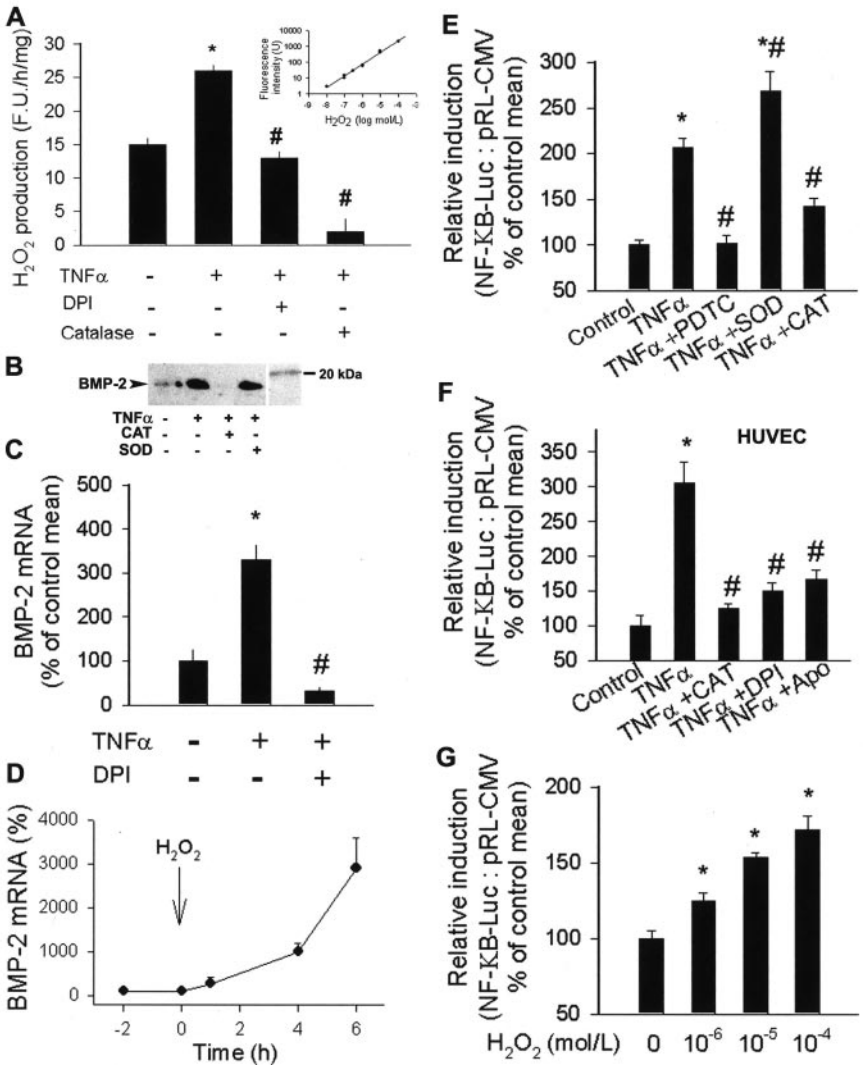

Figure 2. A, Demonstration of TNF-α–induced, DPI-sensitive H_2O_2 generation in CAECs by homovanillic acid/horseradish peroxidase method. Results are expressed as arbitrary fluorescent units (F.U.). Data are mean±SEM, n=4 to 5 for each group. *$P<0.05$ vs untreated control; #$P<0.05$ vs TNF-α treatment. Inset, Exogenous H_2O_2 was used to construct calibration curve, which was linear in 10^{-7} to 10^{-4} mol/L range. B, Original Western blot showing that TNF-α–induced BMP-2 expression was prevented by catalase (CAT) but not by SOD (representative of 3 independent experiments; last lane is molecular weight marker). C, DPI inhibits TNF-α–induced BMP-2 expression in CAECs. Real-time QRT-PCR. Data are mean±SEM; n=4 to 5 for each group. *$P<0.05$ vs untreated control; #$P<0.05$ vs TNF-α treatment. D, Time course of H_2O_2 (10^{-4} mol/L)-induced BMP-2 mRNA expression in CAECs (real-time QRT-PCR). Data are mean±SEM; n=4 for each time point. E, Reporter gene assay showing that TNF-α–induced NF-κB reporter activity in CAECs is suppressed by PDTC and catalase (CAT), whereas it is increased by SOD. CMB indicates cytomegalovirus. *$P<0.05$ vs control; #$P<0.05$ vs TNF-α treatment. F, TNF-α–induced NF-κB reporter activity in HUVECs is suppressed by catalase (CAT), DPI, and apocynin (Apo). *$P<0.05$ vs control; #$P<0.05$ vs TNF-α treatment. G, H_2O_2 induces NF-κB reporter activity in CAECs in concentration-dependent manner. Endothelial cells were transiently cotransfected with NF-κB–driven firefly luciferase and cytomegalovirus (CMV)-driven renilla luciferase constructs followed by TNF-α stimulation. Cells were then lysed and subjected to luciferase activity assay. After normalization, relative luciferase activity was obtained from 4 to 7 independent transfections. Data are mean±SEM *$P<0.05$ vs control.

We used rVISTA (http://www-gsd.lbl.gov/vista), a tool that combines a transcription factor binding sites database search with a comparative sequence analysis,[24] to confirm the presence of a putative NF-κB binding site in an evolutionary, highly conserved region of the human and rat BMP-2 promoter (Figure 1C).

To further explore the regulation of BMP-2 expression, we used RNA interference (RNAi). Both in HUVECs and CAECs, p65-siRNA treatment resulted in a significant downregulation of the mRNA (by ≈90%, QRT-PCR) and protein expression of the p65 subunit of NF-κB (Figure 1D), which substantially decreased TNF-α–induced BMP-2 expression both in CAECs (Figure 1A) and in HUVECs (Figure 1E). The siRNA treatment did not affect β-actin expression, and administration of a scrambled siRNA sequence did not alter the expression of p65 protein (Figure 1D). Basal mRNA expression of BMP-2 was significantly lower in femoral and carotid arteries of TNF-α −/− mice than in vessels of TNF +/+ mice (Figure 1F), which suggests a role for TNF-α in regulation of basal BMP-2 production in vivo.

Role of H_2O_2 in TNF-α–Induced NF-κB Activation and BMP-2 Expression

Previous studies by others and ourselves have shown that TNF-α can induce significant vascular reactive oxygen species production.[12] Indeed, we found that TNF-α elicited a significant increase in H_2O_2 production in CAECs, which was inhibited by catalase and DPI (Figure 2A). Administration of catalase and DPI, but not superoxide dismutase (SOD), prevented TNF-α–induced upregulation of BMP-2 (Figures 2B and 2C). We demonstrated that TNF-α significantly enhanced the transcriptional activity of NF-κB (as indicated by an increase in the luciferase activity), which was inhibited by catalase and PDTC but not SOD in CAECs (Figure 2E). We also found that TNF-α–induced NF-κB activation was similarly inhibited by catalase, DPI, and apocynin, a specific NAD(P)H oxidase inhibitor in HUVECs (Figure 2F). Collectively, these findings suggest that TNF-α promotes NAD(P)H oxidase–derived H_2O_2 production, which plays a key role in NF-κB activation in endothelial cells. This view is further supported by the observations that administration of exoge-

nous H_2O_2 induced NF-κB activation (Figure 2G) and significantly upregulated BMP-2 mRNA expression (Figure 2D).

High Intravascular Pressure Promotes Arterial BMP-2 Expression

Exposure of arteries to high pressure in vitro elicited significant upregulation of vascular BMP-2 mRNA (Figure 3A) and protein (Figure 3B) expression in cultured arteries that could be prevented by inhibition of NF-κB activation with PDTC and SN-50 (Figure 3A). To test the in vivo role of high pressure, hypertension was induced in rats by abdominal aortic banding (mean arterial pressure proximal to the coarctation: 147±3 mm Hg, distal: 94±7 mm Hg). Compared with normotensive femoral arteries, hypertensive brachial arteries exhibited an upregulated BMP-2 expression (Figure 3C). In contrast, BMP-2 expression was similarly increased in forelimb arteries and femoral arteries of rats with angiotensin II–induced hypertension (Figure 3C; mean arterial pressure 148±5 mm Hg) compared with vessels of normotensive control rats (mean arterial pressure 116±2 mm Hg), which suggests a role for high pressure itself in regulation of BMP-2 expression.

Role of TNF-α in High Pressure–Induced BMP-2 Expression

Exposure of cultured arterial segments to high pressure resulted in a slight but significant increase in TNF-α expression (Figure 3D). We also extended earlier observations that compared with normotensive vessels, there is also increased (by ≈70%) TNF-α expression in hypertensive arteries of aortic banded rats, predominantly located in the vascular smooth muscle cells (data not shown). To test the hypothesis that pressure-induced TNF-α promotes BMP-2 expression, arteries of TNF-α −/− mice were pressurized to 80 or 160 mm Hg. These studies showed that high pressure also resulted in significant upregulation of BMP-2 expression in TNF-α–deficient vessels (Figure 3E), which suggests that high pressure predominantly regulates BMP-2 expression directly, via a TNF-α–independent pathway.

Demonstration of High Pressure–Induced Activation of NF-κB in Vascular Endothelial Cells

Using an ELISA-based NF-κB binding assay, we found that after pressurization to 160 mm Hg, the DNA-binding capacity of NF-κB from arterial extracts was increased significantly compared with extracts from vessels at 80 mm Hg (Figure 4A). The short time course of NF-κB activation strongly suggests that it is elicited directly by high pressure and does not involve new protein synthesis (eg, that of TNF-α).

One of the key steps in the activation of the NF-κB pathway involves its translocation to the nucleus, which first requires phosphorylation and subsequent degradation of its inhibitor IκBα.[25] Thus, IκBα degradation is a useful marker of vascular NF-κB pathway activation.[25] We evaluated the protein levels of IκBα isoform in cytoplasmic extracts of vessels exposed to 80 or 160 mm Hg (for 20 minutes). In high pressure–exposed arteries, levels of IκBα were reduced

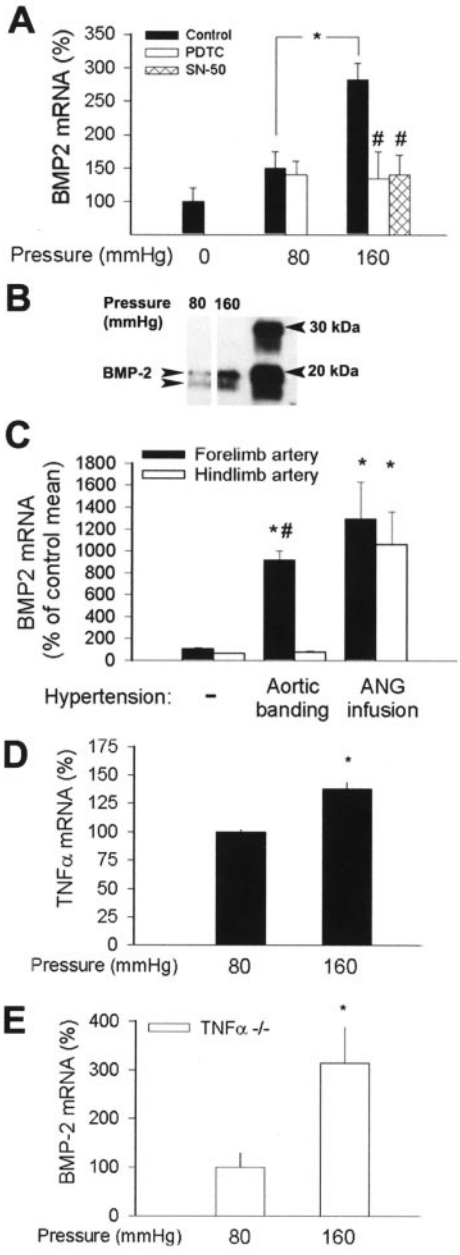

Figure 3. A, High intraluminal pressure (160 mm Hg) increases expression of BMP-2 mRNA in rat femoral arterial branches maintained in organoid culture (for 24 hours). Analysis of mRNA expression was performed by real-time QRT-PCR and normalized to mean of control values obtained in nonpressurized arteries. Data are mean±SEM (n=4 to 5 for each group). *$P<0.05$, 160 vs 80 mm Hg; #$P<0.05$, PDTC treated vs untreated. B, Representative Western blot showing BMP-2 protein content in cultured arteries exposed to 80 or 160 mm Hg pressure (last lane: molecular weight marker). C, Expression of BMP-2 mRNA was selectively upregulated in hypertensive forelimb arteries of rats with aortic banding hypertension, whereas BMP2 expression was normal in normotensive hindlimb arteries (β-actin was used for normalization). In angiotensin II (ANG)-infused rats, both vascular beds were hypertensive and exhibited increased BMP-2 expression. Data are normalized to mean value of normotensive controls (n=4 to 6 for each group). D, High pressure (160 mm Hg) elicited slight increases in expression of TNF-α in cultured arteries. Data are mean±SEM (n=4 to 5 for each group). *$P<0.05$ E, High pressure also induced BMP-2 expression in carotid arteries of TNF-α knockout mice. Data are mean±SEM (n=4 to 5 for each group). *$P<0.05$.

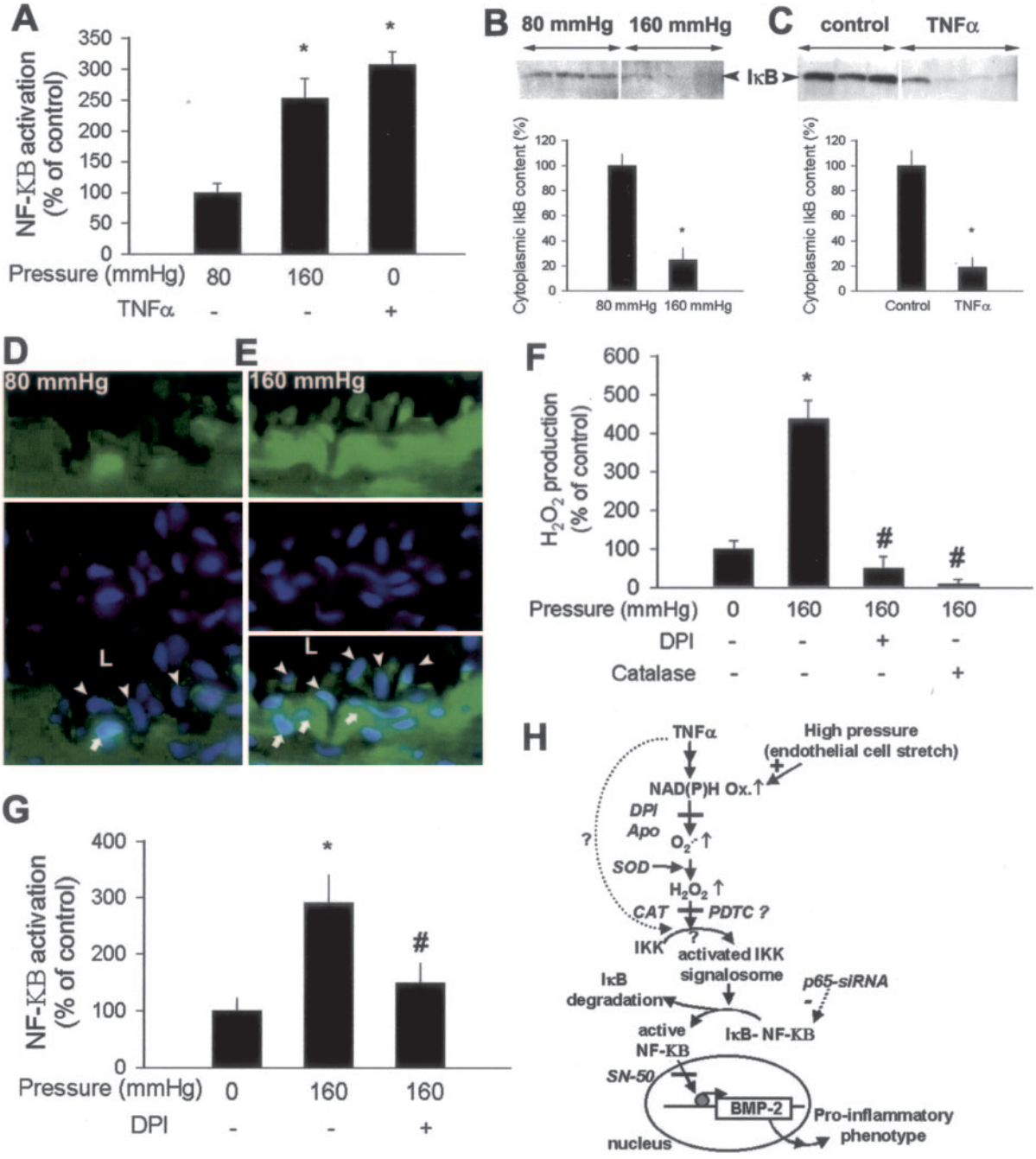

Figure 4. A, ELISA-based demonstration of high pressure– and TNF-α (10 ng/mL)–induced NF-κB activation in nuclear extracts from isolated arteries. Data are mean±SEM (n=4 to 5 for each group). *$P<0.05$. vs 80 mm Hg. B and C, Demonstration of high pressure–induced (B) or TNF-α–induced (C) decreases in cytoplasmic IκBα content by Western blotting. Bar graphs are summary data. Data are mean±SEM (n=4 to 5 for each group). *$P<0.05$ vs 80 mm Hg. D and E, Demonstration of high pressure–induced nuclear translocation of activated NF-κB. Superimposed images of immunofluorescent staining of activated form of NF-κB (green) with nuclear counterstaining (blue, DAPI) shows that high pressure treatment causes marked increase in nuclear staining intensity for activated NF-κB in endothelial cells, which indicates translocation of NF-κB into nucleus (arrowheads in E). In contrast, little active NF-κB can be detected in nuclei of endothelial and smooth muscle cells in arteries exposed to 80 mm Hg (arrowheads in D). Increased nuclear translocation of NF-κB was also observed more frequently in smooth muscle cells of high pressure–exposed arteries (arrows in E) than in vessels exposed to physiological pressure (arrow in D). Representative images were obtained from 6 separate experiments (original magnification ×40). L indicates lumen. F, Increased H_2O_2 generation (measured by homovanillic acid/horseradish peroxidase method) in high pressure–exposed arteries was inhibited by NAD(P)H oxidase inhibitor DPI. Data are mean±SEM (n=4 to 5 for each group). *$P<0.05$ vs control; #$P<0.05$ vs high pressure treated. G, Inhibition of high pressure–induced NF-κB activation by DPI in isolated arteries (ELISA). *$P<0.05$ vs no pressure; #$P<0.05$ vs untreated. H, Proposed scheme for common redox-sensitive pathway leading to activation and nuclear translocation of NF-κB in endothelial cells induced by increased H_2O_2 production due to activation of NAD(P)H oxidase by inflammatory cytokines and high pressure. Binding of NF-κB to its target sequences promotes expression of proinflammatory mediators, such as BMP-2, which induce proatherogenic phenotypic changes in endothelial cells. Ox indicates oxidase; Apo, apocynin; and CAT, catalase.

substantially compared with that in vessels pressurized to 80 mm Hg (Figure 4B). As a positive control experiment, we confirmed that TNF-α treatment also elicited considerable decreases in vascular IκBα content (Figure 4C).

Using a monoclonal antibody that specifically recognized the activated p65 subunit of NF-κB, we found that high pressure treatment caused a marked increase in the nuclear staining intensity for activated NF-κB in endothelial cells, which indicates the translocation of NF-κB into the nucleus (Figure 4E, arrowheads). In contrast, little active NF-κB was detected in the nuclei of endothelial and smooth muscle cells in arteries exposed to 80 mm Hg (Figure 4D, arrowheads). For quantitative comparison, the captured green fluorescent images were converted to grayscale images, and the ratio of total pixel intensities in the nucleus (DAPI-positive area) and total pixel intensities of the cytoplasm (DAPI-negative area) was calculated with Scion image software (arbitrary units; 80 mm Hg 0.7 ± 0.1, 160 mm Hg 2.8 ± 0.5, $P<0.05$). Increased nuclear translocation of NF-κB also was frequently observed in smooth muscle cells of high pressure–exposed arteries (Figure 4E, arrows). Pretreatment with PDTC prevented high pressure–induced nuclear translocation of NF-κB in both endothelial and smooth muscle cells (not shown).

High Pressure–Induced Vascular H_2O_2 Generation

Exposure to high pressure elicited substantial increases in vascular H_2O_2 generation (Figure 4F) that were inhibited by both catalase and DPI. Hypertensive arteries of aortic banded rats also exhibited an increased DPI-sensitive H_2O_2 production ($234\pm37\%$) compared with vessels of normotensive rats ($100\pm41\%$), which suggests that NAD(P)H oxidase is a dominant source of pressure-induced H_2O_2 generation both in vivo and in vitro.[15,18] The present data show that high pressure–induced vascular NF-κB activation is inhibited by DPI (Figure 4G), which suggests a direct link between high pressure–induced activation of NAD(P)H oxidase-derived H_2O_2 production, NF-κB activation, and BMP-expression.

Discussion

There are 3 important findings in this study. First, TNF-α provoked a robust increase in BMP-2 mRNA and protein expression in endothelial cells (Figures 1A and 1B). The findings that inhibition of NF-κB signaling by pharmacological and molecular methods prevented TNF-α–induced upregulation of BMP-2 (Figures 1A and 1D) provide strong evidence that NF-κB plays a central role in regulation of endothelial BMP-2 expression. Indeed, NF-κB binding sites appear to be present in the promoter region of the rat BMP-2 gene, and a recent study reported that NF-κB binds an NF-κB response element in the 5′ flanking region of the BMP-2 gene in mouse chondrocytes.[26] Interestingly, we found that this NF-κB binding site is located in an evolutionary, highly conserved region of the BMP-2 promoter (Figure 1C), which provides an explanation for the similar upregulation of BMP-2 expression in human and rat endothelial cells by TNF-α (Figures 1A and 1D). The in vivo significance of the regulation of vascular BMP-2 expression by TNF-α and NF-κB is supported by the lower level of BMP-2 expression in vessels of TNF-α–deficient mice (Figure 1G). Importantly, endothelial NF-κB activation[27] and an enhanced coexpression of BMP-2 and TNF-α[12] have been demonstrated recently in pathophysiological conditions, such as hyperhomocysteinemia, that are known to promote vascular inflammation and atherosclerosis.

The second significant finding in the present study was that TNF-α elicited substantial NAD(P)H oxidase–derived H_2O_2 production in endothelial cells (Figure 2A), which is an essential signaling event underlying BMP-2 induction (Figures 2B and 2C). We have provided direct evidence that inhibition of NAD(P)H oxidase and/or elimination of H_2O_2 inhibits NF-κB activation in TNF-α–stimulated endothelial cells (Figures 2E and 2F) and that exogenous H_2O_2 induces robust NF-κB activation (Figure 2G) and BMP-2 expression (Figure 2D). These findings are also in line with the observation that SOD, which increases H_2O_2 levels by enhancing breakdown of O_2^-, had the opposite effect on NF-κB activity and BMP-2 expression (Figures 2B and 2E). A role for NAD(P)H oxidase–derived H_2O_2 is further supported by the observation that TNF-α stimulation did not increase BMP-2 levels in arteries of mice with genetic deficiency of the NAD(P)H oxidase subunit gp91^{phox} (A. Csiszar, unpublished data, 2004).

The third and perhaps most interesting finding was that high intraluminal pressure per se plays an important role in endothelial H_2O_2 production, NF-κB activation, and regulation of BMP-2 expression. The finding that high pressure itself is sufficient to induce BMP-2 expression in vitro, in the absence of circulating factors (Figures 3A and 3B), has been confirmed by the ex vivo observations that in forelimb arteries of aortic banded rats, which are exposed to high pressure and exhibit an increased ROS production,[18] BMP-2 is upregulated (Figure 3C), whereas normotensive arteries (located downstream from the coarctation but which are exposed to the same circulating factors) of the same animals exhibit normal phenotype. Also, in angiotensin II–induced hypertension, in which there are no regional differences in blood pressure, BMP-2 expression was increased similarly in the brachial and femoral arteries (Figure 3C). Interestingly, pressure- or stretch-induced increased expression of the BMP-related cytokine transforming growth factor-β has been also documented in blood vessels,[28] and mechanosensitive expression of BMPs has been shown in bone[1,29] and chondrocytes.[14]

Because the chronic presence of high pressure may increase plasma TNF-α levels[30] and/or vascular TNF-α expression[31] (Figure 3D), one could assume that high pressure activates BMP-2 expression indirectly, via upregulation of TNF-α. However, this hypothesis could be refuted, because genetic absence of TNF-α did not prevent high pressure–induced upregulation of BMP-2 in mouse carotid arteries (Figure 3E). Instead, the present data support the view that pressure directly activates NF-κB. Because inhibition of NF-κB prevented pressure-induced BMP-2 expression (Figure 3A), it is likely that inflammatory and mechanical stimuli regulate BMP-2 expression by activating a common, NF-κB–dependent pathway. One of the key steps in activation of the NF-κB pathway involves its translocation to the nucleus. Indeed, we demonstrated that both administration of

TNF-α and imposition of a high intraluminal pressure in arterial segments were associated with rapid nuclear translocation of NF-κB (Figure 4A), which extends recent findings by Lemarie et al.[25] Immunofluorescent labeling confirmed that high pressure–induced nuclear accumulation of activated NF-κB was particularly prevalent in the nuclei of endothelial cells (Figure 4D). Interestingly, NF-κB binding to its target sequence was also shown to increase in hypertension,[32] and recent studies suggest that in vitro stretching may activate NF-κB in cultured endothelial and smooth muscle cells.[33] In resting cells, NF-κB is bound in the cytoplasm by its redox-sensitive inhibitor, IκB, which masks the nuclear localization sequence on the transcription factor. We and others[25] showed that high pressure elicits rapid degradation of IκBα[25] (Figure 4B), which is likely responsible for the pressure-induced nuclear translocation of NF-κB. The data presented in Figure 2 indicated that H_2O_2 production is an important mediator of NF-κB activation in endothelial cells. Importantly, we found that high pressure can elicit significant increases in vascular H_2O_2 generation,[34] likely by activation of NAD(P)H oxidase (Figure 4F).[15] The view that high pressure–induced NAD(P)H oxidase may initiate NF-κB activation in endothelial cells even in the absence of proinflammatory cytokines is supported by the finding that high pressure–induced activation of NF-κB was decreased significantly by DPI (Figure 4G).

In conclusion, we propose that in endothelial cells, both inflammatory and mechanical stimuli (TNF-α and high pressure–related cell stretch) promote NAD(P)H oxidase–derived H_2O_2 generation, which leads to IκB degradation and nuclear translocation of NF-κB (Figure 4H). Oxidative stress–induced NF-κB activation upregulates the expression of BMP-2, which is likely to promote proatherogenic endothelial activation and monocyte adhesion.[4,6] Future studies should elucidate the role of BMP receptor subtypes and downstream signaling mechanisms (eg, SMAD-dependent and -independent pathways, such as mitogen-activated protein kinases) induced by BMPs, which mediate their proinflammatory effects. More information on the phenotypic consequences of high pressure–induced NAD(P)H oxidase–derived H_2O_2 production and NF-κB activation should improve our understanding of vascular remodeling and development of early atherosclerosis in hypertension.[2–4,12]

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