Bone Morphogenetic Proteins Protect Pulmonary Microvascular Endothelial Cells From Apoptosis by Upregulating α-B-Crystallin

Mariana Ciumas,* Mélanie Eyries,* Odette Poirier,* Svetlana Maugenre, France Dierick, Natalia Gambaryan, Kevin Montagne, Sophie Nadaud, Florent Soubrier

Objective—To investigate the role of bone morphogenetic proteins (BMPs) on α-B-crystallin (CRYAB) expression and its physiological consequences on endothelial cells (ECs).

Approach and Results—We report that the gene encoding for the small heat shock protein, CRYAB, is a transcriptional target of the BMP signaling pathway. We demonstrate that CRYAB expression is upregulated strongly by BMPs in an EC line and in human lung microvascular ECs and human umbilical vein ECs. We show that BMP signals through the BMPR2-ALK1 pathway to upregulate CRYAB expression through a transcriptional indirect mechanism involving Id1. We observed that the known antiapoptotic effect of the BMPs is, in part, because of the upregulation of CRYAB expression in EC. We also show that cryab is downregulated in vivo, in a mouse model of pulmonary arterial hypertension induced by chronic hypoxia where the BMP pathway is downregulated.

Conclusions—We demonstrate a cross-talk between BMPs and CRY AB and a major effect of this regulatory interaction on resistance to apoptosis. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: ACVRL1 protein, human ■ anoxia ■ BMP2 protein, human ■ Id1 protein, human ■ RNA, small interfering ■ Smad proteins

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β superfamily of extracellular signaling proteins and play crucial roles in embryonic development, adult tissue homeostasis, and in the pathogenesis of a variety of diseases.1 The highly conserved BMP signaling pathway comprises the BMP ligands, 2 types of receptors (type I: ALK1, 2, 3, and 6 and type II: BMPR2, ACTR2A, and ACTR2B), and signal transducers, the Smad proteins. Once activated, the receptor complex phosphorylates the carboxy terminus of the receptor-regulated Smad proteins, Smad1, 5, and 8. Activated receptor-regulated Smad proteins interact with the common partner Smad, Smad4, and translocate to the nucleus, where the Smad complex directly binds defined DNA sequences and regulates target gene expression.2

BMPs were reported to promote survival of human pulmonary arterial endothelial cells (ECs) and circulating endothelial progenitor cells isolated from normal subjects. This antiapoptotic effect induced by the BMP signaling pathway on vascular EC is, in part, because of the decrease in caspase-3 activity.3 CRYAB is a soluble cytoplasmic protein and a member of the small heat shock protein (sHSP) family. CRYAB was identified initially as an abundant structural eye lens protein but seemed later to be expressed in the heart, skeletal muscle, central nervous system, kidney, and lung of mice.4 It is induced by heat shock, proinflammatory cytokines, and oxidative stress and plays a role in cellular growth, migration, differentiation, and development.5–7 CRYAB has chaperone-like properties, binds to both desmin and cytoplasmic actin, and helps to maintain cytoskeletal integrity.8 As in the case of other chaperones and sHSPs, CRYAB can bind to unfolded proteins and can prevent their denaturation and aggregation.9 CRYAB has been shown to protect cells against apoptosis induced by different factors, such as DNA-damaging agents, tumor necrosis factor-α, TRAIL, hydrogen peroxide, etc.10–12 CRYAB is known to protect vascular EC from apoptosis, in part, by binding to caspase-3 and blocking its autoproteolytic maturation.10,13,14 CRYAB is also considered as an oncogenic protein, by protecting cancer cells against apoptosis induced by chemotherapeutic agents, by interacting with proapoptotic...
proteins, or inhibiting caspase-3 activation. In cardiomyocytes, it was shown that CRYAB silencing promoted cell death after exposure to H2O2, and that CRYAB has a protective role against apoptosis, by a mechanism involving translocation of CRYAB into mitochondria and by interacting with different proteins, including voltage-dependent anion channels and caspase-3.

Here, we show that BMPs induce CRYAB expression by a transcriptional, indirect molecular mechanism, which requires the BMPR2 and ALK1 receptors and the transcription factor Id1. BMPs protect EC from serum starvation and hypoxia-induced apoptosis and the protecting effect is, in part, because of a strong upregulation of the antiapoptotic protein CRYAB. Cryab is downregulated in the lung of a mouse model of pulmonary hypertension induced by hypoxia, a condition in which the BMP signaling pathway has decreased activity.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**BMPs Upregulate CRYAB in Human Microvascular ECs**

Microarray RNA profiling experiments were performed to identify BMP target genes in human microvascular EC-1 (HMEC-1) stimulated for 7 or 24 hours by 10 ng/mL of BMP4. Among the modulated genes, including Id1, Id2, and Id3, which were markedly upregulated (data not shown), we observed a strong upregulation of CRYAB expression by BMP4 at both 7 and 24 hours of stimulation.

We confirmed, by real-time polymerase chain reaction (PCR), the upregulation of CRYAB expression in HMEC-1 treated by 10 ng/mL of BMP4 for 24, 48, and 72 hours (4.3±4.1, 8.6±0.7, and 7.5±1, respectively; Figure 1A). CRYAB mRNA was also increased by other members of the BMP growth factors family, BMP7 (fold increase at 4.8±0.9 at 24 hours, 4.8±1.8 at 48 hours, and 3.4±0.06 at 72 hours) and BMP9 (fold increase at 86.5±6.3 at 24 hours, 25.1±0.8 at 48 hours, and 15.1±1.5 at 72 hours), similar to BMP4 (Figure 1A). In human lung microvascular ECs (HLMEC), BMP7 and BMP9 increased CRYAB expression, whereas BMP4 had no effect on CRYAB expression (Figure 1 in the online-only Data Supplement). BMP9 also induced CRYAB expression in human umbilical vein ECs (HUVEC; Figure I in the online-only Data Supplement). By Western blot, we observed that CRYAB levels were increased strongly in HMEC-1 treated with BMP4, BMP7, and BMP9 compared with control cells (Figure 1B).

**CRYAB Induction by BMPs Is Transcriptional Indirect**

To characterize the molecular mechanism involved in the BMP-induced CRYAB upregulation, we inhibited DNA transcription or mRNA translation by pretreating cells with actinomycin D or cycloheximide, respectively, before BMP stimulation. When protein translation was stopped by cycloheximide, CRYAB expression was not upregulated in response to BMP (Figure 2A), showing that this regulation depends on the induction of the expression of a protein and is therefore indirect. Blocking mRNA transcription by actinomycin D treatment also abolished the induction of CRYAB expression by BMP (Figure 2A), showing that this regulation depends on mRNA transcription. However, this actinomycin D experiment does not differentiate between CRYAB mRNA transcription and the transcription of an intermediate protein mRNA. To directly study the transcription of CRYAB mRNA, we analyzed the transcriptional level of the CRYAB gene by measuring its pre-mRNA expression by real-time PCR using primers that hybridize to intronic sequences of the gene and, therefore, specifically recognize pre-mRNA. After 5 hours of

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**Nonstandard Abbreviations and Acronyms**

<table>
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<tr>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>HMEC</td>
<td>human lung microvascular EC</td>
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<td>HLMEC</td>
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<td>HUVEC</td>
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<td>sHSP</td>
<td>small heat shock protein</td>
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**Figure 1.** Bone morphogenetic proteins (BMPs) upregulate CRYAB in human microvascular endothelial cell-1 (HMEC-1). Serum-restricted HMEC-1 was stimulated with 10 ng/mL of BMP4, BMP7, or BMP9 for 24, 48, or 72 hours. A, Expression of CRYAB was quantified by real-time reverse transcription polymerase chain reaction analysis. The relative CRYAB mRNA level was normalized to RPL32 mRNA level and expressed as a fold change relative to untreated cells. Results are means±SD values of 3 independent experiments. **P<0.05, ***P<0.01, and ****P<0.001 relative to untreated cells. B, Immunoblotting for CRYAB. Blot was reprobed for β-actin to control for differences in protein loading.
Cryab induction by bone morphogenetic proteins (BMPs) is transcriptional indirect. A, Serum-restricted human microvascular endothelial cell-1 (HMEC-1) was pretreated or not with cycloheximide (CHX) or actinomycin D (ActD) for 15 minutes before being stimulated with BMP4, BMP7, or BMP9 (10 ng/mL) for 5 hours. Cryab expression was quantified by real-time reverse transcription polymerase chain reaction (PCR) analysis. The relative Cryab mRNA level was normalized to RPL32 levels and expressed as a fold change relative to untreated cells. Results are mean ± SD values of 4 independent experiments. *P < 0.05 relative to untreated cells. B, Serum-restricted HMEC-1 was treated with 10 ng/mL of BMP4 or BMP7 for 5 hours. Nuclear RNAs were isolated and analyzed by real-time PCR using primers that hybridize to intron 1 and exon 2 allowing only the detection of the Cryab pre-mRNA. Cryab pre-mRNA expression was normalized to GAPDH mRNA expression and expressed as a fold change relative to untreated cells. Results are mean ± SD values of 3 independent experiments. *P < 0.05 relative to untreated cells.

Cryab Upregulation by BMPs Is Mediated by the BmpR2–Alk1–Id1 Pathway

We knocked down the expression of BmpR2 using a BmpR2-targeting siRNA (Figure II in the online-only Data Supplement). Cryab upregulation at the mRNA level by BMPs was nearly abolished in HMEC-1 transfected with BmpR2-directed siRNA compared with those transfected with nonspecific siRNA (19.4 ± 8.9% of control siRNA value) compared with those transfected with nonspecific siRNA (Figure 3A). In contrast, inhibiting Alk1 by siRNA did not significantly decrease Cryab mRNA upregulation (Figure 3A). Altogether, these results demonstrate that the upregulation of Cryab expression by BMPs is mediated by BmpR2 and Alk1, but not by Alk3. Alk6 was not tested because it is not expressed in EC (data not shown).

The main intracellular signaling pathway activated by BMPs is the Smad pathway. We observed an induction of Smad 1/5/8 phosphorylation after BMP treatment of HMEC-1 in a time-dependent manner concomitantly with BMP-induced Cryab expression (Figure III in the online-only Data Supplement). We tested the implication of the transcription factors Id1, Id2, and Id3, which are strongly induced by BMPs. After silencing these mRNA targets using specific siRNAs (Figure II in the online-only Data Supplement), we observed that Id1 expression knockdown reduces Cryab mRNA induction in HMEC-1 by 55 ± 14%, whereas Id2 and Id3 knockdown did not modify significantly this induction (Figure 3B). These data corroborate the actinomycin D and cycloheximide experiments, which suggested the transcription and translation of an inducer factor.

Role of Cryab in the Antiapoptotic Effect of BMPs on Serum-Starved HLMEC

To determine whether the upregulation of Cryab mRNA by BMPs plays a role in the antiapoptotic effect induced by BMPs on serum-starved HLMEC, we inhibited Cryab expression using a specific siRNA targeting Cryab mRNA (Figure II in the online-only Data Supplement). In cells transfected with a nonspecific siRNA, we observed an antiapoptotic effect of BMP7 in serum-free conditions. This effect was abolished in cells whose Cryab expression is knocked down by a specific siRNA (Figure 4A). This observation was confirmed by flow cytometry because the percentage of annexin V–positive cells is higher when Cryab-targeting siRNA is transfected in cells treated with BMP7 (6.9 ± 2.2% in siCT-transfected cells versus 13.5 ± 4.52% in siCryab-transfected cells) and BMP9 (7.2 ± 2.4% in siCT-transfected cells versus 13.7 ± 4.7% in siCryab-transfected cells) compared with cells transfected with a control siRNA (Figure 4B and 4C).

These results suggest that BMPs protect EC from apoptosis, in part, by upregulating the antiapoptotic factor Cryab.

Role of BMP and Cryab in Hypoxia-Induced Apoptosis in HUVEC

To corroborate results obtained on HLMEC by serum-starvation–induced apoptosis, we used hypoxia as another inducer of apoptosis in EC. HUVEC were cultured in hypoxic conditions (1% O2). Apoptosis was measured by Hoechst 33342 staining to quantify condensed pyknotic nuclei in apoptotic cells. In hypoxic conditions, BMP9 induced a significant decrease of apoptotic cells whose Cryab mRNA induction is knocked down by a specific siRNA (Figure 3A). This observation was confirmed by flow cytometry because the percentage of annexin V–positive cells is higher when Cryab-targeting siRNA is transfected in cells treated with BMP7 (6.9 ± 2.2% in siCT-transfected cells versus 13.5 ± 4.52% in siCryab-transfected cells) and BMP9 (7.2 ± 2.4% in siCT-transfected cells versus 13.7 ± 4.7% in siCryab-transfected cells) compared with cells transfected with a control siRNA (Figure 4B and 4C).

These results suggest that BMPs protect EC from apoptosis, in part, by upregulating the antiapoptotic factor Cryab.
Decreased BMP Activity Correlates With the Downregulation of CRYAB Expression In Vivo

BMP signaling is known to be altered in pulmonary hypertension. Thus, we studied the BMP signaling pathway and Cryab expression in the mouse hypoxia model of pulmonary arterial hypertension (PAH). Mice were exposed to chronic hypoxia for 35 days leading to the development of pulmonary hypertension as assessed by hemodynamic parameter measurements (Figure IV in the online-only Data Supplement). In this model, a significant reduction of Bmpr2 expression in mouse lung was observed (Figure 6A). In these conditions, we also observed a decrease of Smad 1/5/8 phosphorylation compared with control conditions (Figure 6C and 6D). These results confirmed that the BMP signaling pathway is downregulated in the mouse hypoxia model of PAH in our experimental conditions.

We observed a significant downregulation of the Cryab mRNA and protein in the lung of animals exposed to chronic hypoxia compared with those exposed to normal oxygen conditions (Figure 6B, 6C, and 6D). These results show that Cryab expression is downregulated in vivo during chronic hypoxia, and this downregulation correlates with the decreased BMP signaling pathway activity.

We analyzed the expression of Cryab in the mouse lung by immunofluorescence staining. The expression of Cryab in EC of small vessels was shown by double staining with antibodies against the EC-specific Von Willebrandt factor and CRYAB (Figure 6E). We observed that Cryab is expressed in the mouse lung in various types of cells but at different levels. In the lung parenchyma, the highest levels of expression are observed in EC, in smooth muscle cells (from vessels and airways) and in bronchiolar epithelial cells (Figure 6E; Figure VI in the online-only Data Supplement). We confirmed the specificity of the CRYAB antibody by performing a parallel hybridization in similar conditions on mouse kidney. The results show specific expression of Cryab in kidney vessels in EC and smooth muscle cells (Figure VII in the online-only Data Supplement).

Discussion

We demonstrate in this study a cross-talk between the BMP pathway and the sHSP family regulation, namely the strong induction of the CRYAB gene transcription and expression by BMPs. Experiments in vitro favor an indirect mechanism of transcription induction by BMPs because a protein biosynthesis is required, as shown by cycloheximide experiments. Therefore, a transcription factor upregulated by BMPs is likely to be involved, and our results confirm this conclusion because Id1 knockdown strongly, but not totally, inhibits the induction. The absence of total inhibition could be because of either incomplete mRNA knockdown by siRNAs or the involvement of other intermediate transcription factors. Knockdown experiments of intracellular intermediates of BMP signaling show that BMPR2 and ALK1 are required for the induction.

We initially identified the CRYAB transcriptional upregulation by BMPs in an EC line (HMEC-1), and this result was...
extended to HLMEC, and HUVEC. 2 types of human primary culture of EC from different origins. However, we found some contrasted effects between BMPs in these cell types because BMP4, which induces CRYAB in HMEC-1, is not efficient in HLMEC, and BMP9 is clearly the most active inducer in all cell types.

We evaluated the physiological effect of BMPs in EC taking into account the known physiological effect of CRYAB. Indeed, CRYAB is a sHSP known to inhibit apoptosis through inhibition of caspase-3 activation. Because BMPs are known to inhibit apoptosis in EC, an effect which contrasts with the documented proapoptotic effects of BMPs during embryogenesis and also in adult vascular smooth muscle cells, we searched for a specific effect of BMPs on EC apoptosis through CRYAB. We observed a strong apoptotic effect induced by serum deprivation of HLMEC, and this effect was antagonized by BMP7 and BMP9 as attested by the lower percentage of annexin V–positive cells measured by flow cytometry. This effect is dependent on the induction of CRYAB expression as the knockdown of CRYAB expression resulted in a nearly complete loss of the BMP anti-apoptotic effect. We confirmed these results using hypoxia as a stress for inducing apoptosis in HUVEC. In this model, BMP9 also significantly decreased apoptosis, an effect suppressed by inhibiting CRYAB expression. To eliminate an indirect effect mediated by VEGF, we measured VEGF mRNA induction by BMP9 in HMEC-1, and we did not observe any induction (Figure V in the online-only Data Supplement).

Figure 4. Knockdown of CRYAB expression prevents the inhibition by bone morphogenetic protein (BMP) of serum-depletion–induced endothelial cell apoptosis. Human lung microvascular endothelial cells were transfected with a nonspecific siRNA (siCT) or a CRYAB–specific siRNA (siCRYAB). After transfection, cells were cultured in normal conditions (FCS), in serum-free medium (SF), and in serum-free medium with 200 ng/mL BMP7 or BMP9 for 24 hours. A. Apoptosis levels were assessed by colorimetric ELISA measuring histone-associated DNA fragments in the cytoplasmic fraction of cell lysates. Results are expressed as a fold change relative to cells transfected with siCT and cultured in FCS. Results represent mean±SD of 3 independent experiments. *P<0.05 relative to untreated cells cultured in SF medium transfected with siCT (B). C. Cells were labeled with annexin V-FITC and PI, and apoptosis was assessed by flow cytometry. C, Representative scatter plots of PI (y axis) vs annexin V-FITC (x axis): Absence of both markers (lower left quadrants) indicates viable cells; PI-positive alone (upper left quadrants) indicates cellular necrosis, whereas annexin V staining alone or together with PI (upper right and lower right quadrants) is indicative of early and late-stage apoptosis, respectively. B, Graph showing the quantification of annexin V–positive cells. Results are expressed as the percentage of annexin V–positive cells (PI+ and PI−). Results are mean±SD of 6 independent experiments. *P<0.05 vs serum-free siCT-transfected cells and $P<0.05 siCT-transfected cells treated with BMP7 and BMP9 vs siCRYAB-transfected cells treated with BMP7 or BMP9.

Figure 5. Knockdown of CRYAB expression prevents the inhibition by bone morphogenetic protein 9 (BMP9) of hypoxia–induced endothelial cell apoptosis. Human umbilical vein endothelial cells were transfected with 50 nmol/L of siCT or siCRYAB, and treated or not with BMP9 (10 ng/mL). After 24 hours, cells were submitted to 24 hours of hypoxia (1% O2) followed by labeling with Hoechst 33342. The nuclear chromatin morphological changes of apoptotic cells were analyzed, and the proportion of apoptotic nuclei was counted by fluorescent microscopy. Results are mean±SD of 4 independent experiments. **P<0.01 relative to cells transfected with siCT, but not treated with BMP9.
We looked for a correlation between the activation status of the BMP signaling pathway and Cryab expression in pulmonary tissues by investigating the expression and regulation of Cryab in the mouse hypoxia model of PAH. In this model, we observed a downregulation of the BMP pathway, as shown by the pSmad1/5/8 decrease after chronic exposure to hypoxia, confirming previously published results. In this experimental condition, Cryab is also decreased in the mouse lung as measured by RNA expression and by Western blot. The expression of CRYAB was detected in pulmonary EC, but also localized in other cell types. Therefore, these results show that, in vivo, a BMP signaling decrease is associated with a decrease in Cryab expression, which is consistent with the BMP-induced CRYAB regulation found in cultured HMEC-1, HLMEC, and HUVEC.

A few hereditary vascular diseases result from decreased BMP signaling and are linked to genetic mutations abrogating a single allele function of a gene belonging to the BMP pathway. Hereditary hemorrhagic telangiectasia is mainly caused by ALK1 (ACVRL1), Endoglin, and Smad4 genes mutations, and heritable PAH is linked to BMPR2 mutations but can also complicate ACVRL1-linked hereditary hemorrhagic telangiectasia.

Figure 6. CRYAB expression in mouse lung exposed to normoxia or hypoxia. Total RNAs were isolated from lungs of mice exposed to normoxia or hypoxia (10% O2) for 35 days, and mRNA levels were quantified by real-time reverse transcription polymerase chain reaction analysis. Results are mean±SD values of 5 animals per group. A, BMPR2 expression was normalized to RPL13a levels and expressed as a fold change relative to normoxia. *P<0.05 relative to normoxic group. B, CRYAB expression was normalized to RPL13a levels and expressed as a fold change relative to normoxia. *P<0.05 relative to normoxic group. C, Immunoblotting of pSmad1/5/8 protein extracted from the lung of mice exposed to normoxia or hypoxia. All blots were reprobed with β-actin to correct for protein loading. D, Graph showing densitometry of immunoblots of CRYAB and pSMAD1/5/8. Band intensities were normalized to the β-actin blots. Results are represented as fold change relative to normoxia. Results are mean±SD values of 5 animals per group. *P<0.05 relative to normoxia. E, Immunofluorescence analysis of (a) endothelial cells (visualized by Von Willebrandt factor [VWF], green); (b) CRYAB expression (red) in normal mouse lung; (c) 4′,6-diamidino-2-phenylindole nucleus staining; and (d) merged images. Expression of CRYAB in endothelial cells of small lung vessels is indicated by arrows. Scale bar, 20 μm.
telangiectasia.25 These 4 genes are receptors or coreceptors for the BMP ligands or intermediate signaling molecules. The cross-talk identified in our study between BMP and CRYAB might be of importance for understanding the physiopathology of these diseases because under stress conditions, a deficient BMP signaling might result in increased endothelial apoptosis, at least in part because of decreased CRYAB expression. It has been proposed that, after a stress to pulmonary EC in the lung, the selection of EC resistant to apoptosis and subsequent vascular smooth muscle cell proliferation could explain the remodeling observed in PAH.26

The cross-talk between BMP and CRYAB could have implication beyond resistance to apoptosis because CRYAB has wide physiological functions as sHSP. Indeed, CRYAB is known to be involved in resistance to oxidative stress because of high glucose concentration in EC and to prevent apoptosis in mouse neonatal cardiomyocytes exposed to H2O2.14,16 BMP7 was reported recently to protect mesangial cells from oxidative stress, and it can be speculated that it might be through CRYAB induction.27

The BMP-CRYAB cross-talk might also be of importance for the abnormal angiogenesis observed when the BMP signaling pathway is decreased by an ACVR1I or Endoglin inactivating mutation such as in hereditary hemorrhagic telangiectasia because angiogenesis is a balance between vascular cell apoptosis and proliferation.28 BMP signaling plays a major role on the balance between endothelial tip cells, present at the tip of growing capillaries, and stalk cells, which have a proliferative behavior, form tubes and vessel branching.29 CRYAB null mice present with decreased retinal vessel density and subtle defects in vascular pattern morphogenesis.30 After experimental retinopathy, CRYAB null mice show greater EC apoptosis, reduced neovascular tufts in the retina, and vessel leakage.30 CRYAB also stabilizes vascular endothelial growth factor and favors neovascularization in retinopathies.30 Taken together, these results and ours suggest that CRYAB might be one of the BMP targets whose decreased expression can lead to the abnormal angiogenesis observed in hereditary hemorrhagic telangiectasia. We, and others, previously showed a strong inhibition by BMP9 of Apelin gene expression, encoding a peptide highly expressed in endothelial tip cells, which binds to its receptor present on stalk cells, where it promotes cell proliferation and vessel branching through mTOR signaling.29,31 Therefore, the expected increase in apelin expression when BMP signaling is decreased might contribute synergistically with CRYAB decrease to the abnormal angiogenesis observed in mutation carriers of the BMP pathway genes.

In conclusion, we have detected a molecular link between the BMP signaling pathway and a chaperone protein acting in different cell processes, in particular, in resistance to apoptosis, and this relationship might play a major role in the physiopathology of vascular diseases linked to BMP signaling deficiency.

Acknowledgments

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Disclosures

None.

References


Significance

We show the strong induction of CRYAB, a chaperone protein which is a small heat shock protein, by bone morphogenetic proteins. This induction is transcriptional indirect and is dependent on BMPR2, ALK1, and the transcription factor Id1. We show that, in vitro, bone morphogenetic protein inhibits endothelial cell apoptosis induced by serum starvation and by hypoxia, and that this inhibition is dependent on CRYAB induction. We also show in the mouse hypoxia model a decrease in the bone morphogenetic protein pathway activity and a decrease in CRYAB expression. We speculate that the CRYAB decrease observed in this model can favor initial apoptosis and secondary endothelial cell proliferation.
Bone Morphogenetic Proteins Protect Pulmonary Microvascular Endothelial Cells From Apoptosis by Upregulating α-B-Crystallin
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Materials and Methods

BMPs up-regulate α-B-crystallin in endothelial cells, and that mediates in part their anti-apoptotic effect.

**Reagents.** Recombinant human BMP4, BMP7 and BMP9 were purchased from R&D Systems (Minneapolis, MN). Cycloheximide and Actinomycin D were obtained from Boehringer Ingelheim GmbH (Ingelheim, Germany). Primary antibodies used were: αB-crystallin: SPA-222, for Western blot and SPA-223 for immunofluorescence, (both from Enzo LifeScience, Villeurbanne, France), pSmad1/5/8, SMAD5 (Cell Signaling Technology, Danvers, MA), β-actin (Sigma-Aldrich, St Louis, MO), Von Willebrandt Factor (#ab11713 from Abcam, Cambridge, UK).

**Cell culture.** The human dermal microvascular endothelial cell line HMEC-1 was obtained from Thomas J. Lawley (Emory University, School of Medicine, Atlanta, GA) and grown as previously described¹. Human lung microvascular endothelial cells (HLMEC) were obtained from Clonetics (Baltimore, MD) and cultured in endothelial cell Basal Medium 2 (EBM-2) supplemented with EGM-2MV Single Quots (Clonetics, Baltimore, MD). HUVEC (Human Umbilical Vascular Endothelial Cell) were extracted from umbilical cord as previously described² and cultured in endothelial cell basal medium 2 (EBM-2) supplemented with EGM-2MV (Lonza, Basel Switzerland).

**siRNA transfection.** Synthetic small interfering RNA (siRNA) targeting the human BMPR2 mRNA, CRYAB mRNA, ALK1 mRNA, ALK3 mRNA, Id1 mRNA, Id2 mRNA and Id3 mRNA were purchased from Dharmacon Research (Lafayette, CO). Non-targeting siRNA 2 (Dharmacon #D-001210-02) was used as a non-specific control. SiRNA were transfected using Dharmafect-1 transfection reagent according to manufacturer’s recommendation. After transfection, cells were starved for 24h then treated with BMPs for indicated time before lysis or staining.

**Real-time RT-PCR.** Real-time RT-PCR assay was performed as previously described³. Primers used for real-time RT-PCR are indicated below:

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<td>hCRYAB</td>
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**Western Blot.** Cells were lysed with 100μl of Ripa buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail 1 and 2 (Sigma-Aldrich, St Louis, MO), snap frozen and stored at -80°C until use. Frozen tissues were homogenized in the same lysis buffer, centrifuged for 5 minutes at 14000 rpm at 4°C in a chilled micro centrifuge and the supernatant was stored at -80°C until use. Samples were subjected (20μg of proteins per lane for cell extracts and 50μg of proteins per lane for tissue extracts) to SDS-polyacrylamide gel electrophoresis (PAGE) in Tris-Glycine gels. The proteins were transferred to the PVDF membrane using XCell II Blot Module (Invitrogen, Carlsbad, CA). Membranes were then incubated overnight with corresponding antibodies (CRYAB 1:1000, pSMAD1/5/8 and SMAD5 1:1000, β-actin 1: 40000) followed by an anti-rabbit IgG or anti-mouse IgG secondary antibody conjugated to horse-radish peroxidase (1:40000, 1h; Jackson ImmunoResearch Laboratories, West Grove, PA). Bands were visualized using an enhanced chemiluminescence substrate system ECL+ detection system (GE Healthcare, Buckinghamshire, UK) using either High Performance Chemiluminescence Films or Ettan DIGE Imager (GE Healthcare, Buckinghamshire, UK). The intensity of each band was analyzed by densitometry using ImageJ software or using ImageQuant software (GE Healthcare, Buckinghamshire, UK) respectively.

**Transcription assay of the CRYAB gene.** Gene transcription was measured by quantifying pre-mRNA as previously described4. CRYAB pre-mRNA was measured by real-time PCR using the following intron-exon primers: upstream 5'-TTGGTCTCACCTAAGGGGA-3' located in intron 1 and downstream 5'-TGTCATCTGATGGGCTCA-3' located in exon 2. CRYAB pre-mRNA expression was normalized to GAPDH expression measured by real-time PCR using the following primers: upstream 5'-GAAGGTGAAGGTCGGAGT-3' placed on exon 2 and downstream 5'-GAAGATGCTGCTGAGGATT-3' placed on exon 4.

**Apoptosis assay.** HLMEC were plated at a density of 2.5x10^5 cells/well in 6-well plates for 24h. Cells were then cultured for 48h in complete medium or SF medium, in the presence of BMP7 (200 ng/ml). Cell lysates were tested for apoptosis by measurement of cytoplasmic nucleosomes using a Cell Death Detection ELISA kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's recommendation. This assay quantitatively detects histone-associated DNA fragments in the cytoplasmic fraction of cell lysates.

**Annexin assay.** Apoptosis was assessed in HLMEC by flow cytometry analysis using the Annexin V:FITC Apoptosis Detection Kit II (BD Pharmingen, San Diego, CA) under control or apoptosis-inducing conditions (SF), in the presence or absence of BMPs and siRNA targeting CRYAB mRNA or a non-targeting siRNA. At the end of the treatment period (72h), cells were suspended in Enzyme Free Cell Dissociation Buffer (InVitrogen, Carlsbad, CA) and washed twice with cold PBS. Annexin assay was performed according to the manufacturer's
Fluorescence was measured on a Beckman Coulter XL analyzer (Beckman Coulter, Fullerton, CA).

**Nuclear chromatin condensation assay.** HUVEC were seeded in 6-well plates at a density of $2 \times 10^5$ per well, and cultured in normoxia or hypoxia (1%O2) conditions for 24h. Plates were washed twice with PBS, and cells were fixed using 4% paraformaldehyde for 15 min, followed by another two washes of PBS and incubated in 500 μl of Hoechst 33342 solution (5 μg/ml in PBS) for 15 min in the dark. Analysis was performed by fluorescent microscopy. Blue cells were scored as apoptotic or normal based on morphological criteria: small, round, condensed cells were scored as apoptotic, and all other cells were scored as healthy. For each sample, about 1000 cells were scored in a blinded manner.

**Animals.** Eight weeks-old male C57Bl/6 mice (CERJ, Orléans, France) were used for this study and were divided into two groups: a control group exposed to normoxic conditions, and one group exposed to hypoxic conditions (10% O2) in a normobaric hypoxic chamber for 35 days. After 35 days, mice were euthanized immediately after hemodynamic assessment. Lungs were washed in PBS, immediately frozen in liquid nitrogen and stored at -80° before RNA and protein extraction, or inflated with diluted OCT (1:2 in PBS, 2ml) and frozen in isopentane dipped into liquid nitrogen for immunofluorescence analysis. Mice were treated in accordance with our institutional guidelines (Ministère de l’Agriculture, France; authorization 75-1206) and conformed to the Directive 2010/63/EU of the European Parliament.

**Immunofluorescence staining and microscopy.** Frozen mouse lung sections (6 μm) were fixed in 4% PFA for 15 minutes and washed in PBS. Sections were blocked in 3% BSA for 1h at room temperature followed by incubation with primary antibodies (SPA-223 1:100, VWF 1:1000) in blocking solution over night at 4°C. Sections were washed in PBS and incubated with Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) in PBS buffer for 1h, followed by nuclear staining with DAPI. Sections were mounted using Fluorescent Mounting Medium (Dako, Denmark A/S). Slices were analyzed using an Olympus XL microscope and images were then deconvoluted, using MetaMorph softwear (MDS Analytical Technologies, Downingtown, PA).

**Statistical Analysis-** Statistical analysis was performed with XLSTAT (Addinsoft, Paris, France) using a one-way analysis of variance for all data, followed by a Student Newman-Keuls post-hoc analysis for multiple tests. Pairwise comparisons were analysed using the non parametric Mann-Whitney test. The null hypothesis was rejected for $p<0.05$. Data are expressed as mean ± S.D.

**References**


Supplementary Figure I. BMPs up-regulate CRYAB in endothelial cells. (A) Serum-restricted HLMEC were stimulated with 200ng/ml of BMP7 or BMP9 for 24, 48 or 72h. (B) Serum-restricted HUVEC were stimulated with 10ng/ml of BMP9 for 24h. Expression of CRYAB was quantified by real-time RT-PCR analysis. The relative CRYAB mRNA level was normalized to RPL32 mRNA level and expressed as a fold change relative to untreated cells. Results are mean ± S.D. values of 3 independent experiments. * P<0.05 relative to untreated cells.
Supplementary figure II. Knock-down expression of targeted genes by specific siRNA. HMEC-1 were transfected with a non specific siRNA (Si CT) or specific siRNA targeting ALK1, ALK3, BMPR2, CRYAB, ID1, ID2 or ID3. Each specific gene expression was quantified by real time RT-PCR analysis. The targeted gene mRNA level was normalized to GAPDH levels and expressed as a fold change of its expression in untreated cells.
**Supplementary figure III. SMAD pathway is activated by BMPs in HMEC-1.** Serum-restricted HMEC-1 were stimulated with 10ng/ml of BMP4, BMP7 or BMP9 for 24, 48 or 72h. (A) Protein extracts were analyzed by Western blot using specific phospho- SMAD1/5/8 and SMAD5 antibodies. (B) Densitometric analysis of SMAD1/5/8 activation. SMAD activation is defined as the ratio between the phospho-SMAD 1/5/8 signal and SMAD5 signal.
Supplementary figure IV. Hemodynamic parameters in mice exposed to normoxia or hypoxia for 35 days. (A) Hematocrit. (B) Right Ventricule Systolic Pressure (RVSP) (C) Fulton index.
Supplementary Figure V. Absence of VEGF mRNA induction in HMEC-1 treated with BMP9. Serum-restricted HMEC-1 were stimulated with 10 ng/ml BMP9 for 24h. Expression of VEGF mRNA was quantified by real-time RT-PCR analysis. The relative VEGFmRNA level was normalized to GAPDH mRNA level and expressed as a fold change relative to untreated cells. Results are mean ± S.D. values of 5 independent experiments. ns, not significant.
Supplementary Figure VI CRYAB expression in mouse lung exposed to normoxia or hypoxia. (E) Immunofluorescence analysis of endothelial cells (visualized by Von Willebrandt factor (VWF) -green) shows CRYAB expression (in red) in endothelial cells of lung vessels but also in vascular and airway smooth muscle cells and in bronchiolar (br) epithelial cells normal mouse lung; nuclei are stained with DAPI; Expression of CRYAB in endothelial cells of small muscularized (mv) and non muscularized (nmv) lung vessels is indicated by arrows. Scale bar 50µm.
Supplementary Figure VII. CRYAB expression in mouse kidney vessels showing the specificity of the Cryab antibody. Immunofluorescence analysis of endothelial cells (visualized by Von Willebrandt factor (VWF) -green) shows CRYAB expression (in red) in endothelial cells of kidney vessels (v) but not in other kidney cells. Scale bar 50µm.