Induction of activin B by inflammatory stimuli up-regulates expression of the iron-regulatory peptide hepcidin through Smad1/5/8 signaling

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Anemia is very common in patients suffering from infections or chronic inflammation and can add substantially to the morbidity of the underlying disease. It is mediated by excessive production of the iron-regulatory peptide hepcidin, but the signaling pathway responsible for hepcidin up-regulation in the inflammatory context is still not understood completely. In the present study, we show that activin B has an unexpected but crucial role in the induction of hepcidin by inflammation. There is a dramatic induction of Inhbb mRNA, encoding the activin βb-subunit, in the livers of mice challenged with lipopolysaccharide, slightly preceding an increase in Smad1/5/8 phosphorylation and Hamp mRNA. Activin B also induces Smad1/5/8 phosphorylation in human hepatoma–derived cells and, synergistically with IL-6 and STAT-3 signaling, up-regulates hepcidin expression markedly, an observation confirmed in mouse primary hepatocytes. Pretreatment with a bone morphogenetic protein type I receptor inhibitor showed that the effect of activin B on hepcidin expression is entirely attributable to its effect on bone morphogenetic protein signaling, most likely via activin receptor-like kinase 3. Activin B is therefore a novel and specific target for the treatment of anemia of inflammation. (Blood. 2012;120(2):431-439)

Introduction

Anemia of inflammation develops as a complication of an acute or chronic activation of the immune response. It is particularly common in hospitalized patients and in the elderly and has a negative impact on recovery and survival. Most chronic bacterial, fungal, viral, or parasitic infections with systemic manifestations can cause anemia of inflammation, as do rheumatologic disorders, systemic autoimmune disorders, inflammatory bowel disease, chronic kidney diseases, and some malignancies. The limitation of iron supply to erythropoiesis is a major factor in the development of this anemia. Treatment includes administration of erythropoiesis-stimulating agents and intravenous iron. However, iron is rapidly trapped in the macrophage compartment, rendering it unavailable for erythropoiesis once the initial iron bolus is incorporated into RBCs and body stores.

Anemia of inflammation appears to be caused, at least in part, by the induction of the iron-regulatory hormone hepcidin. Hemic acts by binding to the sole known iron-export channel, ferroportin, which is found on duodenal enterocytes, macrophages, and hepatocytes, the cell types that export iron into plasma. Binding of hepcidin to ferroportin induces its internalization and degradation, which progressively inhibit iron efflux from these cells, leading to hypoferremia. The fundamental mechanisms causing increased hepcidin production by the liver during inflammation are still incompletely understood, but a cross-talk with the pathway through which hepcidin is regulated by iron is not excluded.

Iron overload induces the expression of bone morphogenetic protein 6 (BMP6), a member of the TGF-β superfamily. Binding of BMP6 to paired serine/threonine kinase type I and type II receptors results in phosphorylation of receptor-associated SMAD1, SMAD5, and SMAD8 proteins, which, after complexing with the common mediator protein SMAD4, translocate to the nucleus to bind specific regulatory elements in the promoter of the hepcidin gene, increasing hepcidin transcription. Hemojuvelin (HJV) acts as a BMP6 coreceptor and is as critical as BMP6 to hepcidin expression. Interestingly, although mice deficient for Bmp6 or for its coreceptor, HJV, have markedly reduced hepcidin synthesis, they are able to induce hepcidin production when challenged with lipopolysaccharide (LPS). Therefore, neither BMP6 nor HJV are required for hepcidin up-regulation in response to inflammation. In contrast, STAT-3 signaling is important for the induction of hepcidin by inflammatory stimuli. The interaction of IL-6 or other mediators of the IL-6 family with its receptor results in phosphorylation of the intracellular signaling molecule STAT-3. Phospho-STAT-3 then dimerizes and is translocated to the nucleus, where it interacts with a characterized response element in the hepcidin promoter.

Interestingly, however, transcriptional activation of hepcidin by IL-6 is abrogated in mice with liver-specific conditional knock-out of Smad4, and a BMP-responsive element in the hepcidin promoter is required to control hepcidin expression in response to IL-6. Furthermore, pharmacologic inhibition of BMP type I receptors by dorsomorphin or an optimized derivative, LDN-193189, blocks induction of hepcidin expression by IL-6, turpentine, or group A streptococcal peptidoglycan-polysaccharide not just in cultured hepatoma-derived cells, but also in zebrafish.
mouse, or rat. These observations provide evidence supporting a role not only for STAT-3, but also for BMP/Smad signaling, in the induction of hepcidin in response to inflammation. However, the molecule that activates BMP signaling in the inflammatory context is unknown.

Whereas pharmacologic inhibition of all BMP type I receptors has been shown to attenuate the induction of hepcidin expression in different animal models of inflammation, targeting global BMP signaling may have broad effects that preclude the use of LDN-193189 in human therapy. In search of a more specific target, the goal of the present study was to identify the ligand responsible for the activation of the BMP signaling pathway in the inflammatory context.

Methods

Murine models

To examine the effect of LPS (serotype 055:B5; Sigma-Aldrich) in the regulation of hepcidin and to evaluate the importance of Bmp6 in this regulation, 7- to 8-week-old wild-type mice and Bmp6-null mice (Bmp6lox/lox on a CD1 background were challenged with an IP injection of LPS (1 µg/g body weight) or an equivalent volume of saline, and livers were harvested at different time points (30 minutes and 1, 1.5, 2, 4, 6, 15, 24, and 48 hours) after injection. To examine the role of IL-6 in the inflammatory response, 7- to 8-week-old B6.129S2-Il6tm1Krup/J mice and wild-type controls on a C57BL/6J background were challenged with LPS or saline, and livers were harvested 4 hours after injection. All experiments were performed on male mice and, unless otherwise specified, mice received a diet with standard iron content (250 mg iron/kg; SAFE). Experimental protocols were approved by the Midi-Pyrénées animal ethics committee.

HepG2 cells

Human hepatoma cells (HepG2; ATCC) were cultured in high-glucose DMEM supplemented with 10% FCS and antibiotics. For stimulation experiments, cells were transferred to 6-well dishes and starved with DMEM containing 0.1% FCS for 5 hours before exposure to IL-6 (50 ng/mL; R&D Systems) and/or activin A (50 ng/mL; R&D Systems) or activin B (50 ng/mL; R&D Systems) for 1.5, 2.5, 4, 6, and 24 hours. The pharmacologic inhibitor LDN-193189 (100 nM; Axon Medchem) was included in the culture medium, and LPS (50 ng/mL; R&D Systems) and/or activin A (50 ng/mL; R&D Systems) or activin B (50 ng/mL; R&D Systems) were added 30 minutes before activin B exposure.

Quantitation of mRNA levels

Total RNA from mouse liver, human hepatoma cells, or mouse primary hepatocytes, was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized using MMLV-RT (Promega). The sequences of the primers for target genes and the reference gene Hprt are listed in supplemental Table 1 (available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Quantitative PCR reactions were prepared with LightCycler 480 DNA SYBR Green I Master reaction mix (Roche Diagnostics) and run in duplicate on a LightCycler 480 instrument (Roche Diagnostics).

Protein extraction

Livers were homogenized in a FastPrep-24 Instrument (MP Biomedicals) for 15 seconds at 4 m/s. The lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, pH 8, and 0.1% NP-40) included inhibitors of proteases (complete protease inhibitor cocktail; Roche Applied Science) and of phosphatases (phosphatase inhibitor cocktail 2; Sigma-Aldrich). Liver proteins were quantified using a protein assay kit (Bio-Rad). HepG2 cells and mouse primary hepatocytes were lyzed in RIPA buffer (Sigma-Aldrich) supplemented with protease and phosphatase inhibitors, and proteins were quantified using the Bio-Rad DC Protein Assay.

Western blot analysis

Fresh protein extracts were diluted in Laemmli buffer (Sigma-Aldrich), incubated for 5 minutes at 95°C, and subjected to SDS-PAGE. Proteins were then transferred to PVDF membranes (Millipore). Membranes were blocked with 5% of dry milk in TBS-T buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.15% Tween 20), incubated with rabbit Abs to phospho-Smad1/5/8 (Cell Signaling Technology) or phospho–Stat-3 (Cell Signaling Technology) at 4°C overnight, and washed with TBS-T buffer. After incubation with a goat anti–rabbit IgG Ab (Cell Signaling Technology) conjugated to HRP, enzyme activity was visualized by using an ECL-based detection system (Millipore). Blots were then stripped and reprobed with rabbit Abs to Smad5 (Epitomics) or Stat-3 (Cell Signaling Technology) for 2 hours at room temperature before incubation with the goat anti–rabbit HRP-linked Ab. Densitometry of western blots was performed using the ImageJ 1.45s gel-analysis method.

Statistical analyses

Data were first normalized to the invariant control Hprt and, for each sample and each target gene, the ΔCt = [Ct target gene – Ct Hprt] was calculated. Because the numerical value of Ct is inversely related to the amount of amplicon in the reaction, the higher the ΔCt value, the greater the amount of target amplicon. Target gene expression in an individual is proportional to 2−ΔCt. However, individual expression values are usually shown on a log scale and, because log2 (2−ΔCt) = −ΔCt, −ΔCt data rather than 2−ΔCt data are plotted on the y-axes. An increase of 1 on the y-axis thus corresponds to a 2-fold increase in target gene expression. 

ΔCt data are the observed values from experimental procedures and it is recommended that ΔCt data rather 2−ΔCt data be the subject of statistical analysis. Means of ΔCt values in LPS-challenged and unchallenged mice were thus compared by Student t tests. Because, in addition to inflammation, mouse genotype (wild-type versus Bmp6−/−) may affect mean ΔCt values, we also used 2-way ANOVA to test for the effect of both factors and their interaction.

All target and Hprt genes had PCR amplification efficiencies close to 2, so that ΔCt values were compared with 0 by paired 2-sample t tests. The fold changes were obtained by raising 2 to the ΔCt. However, individual expression values are usually shown on a log scale and, because log2 (2−ΔCt) = −ΔCt, −ΔCt data rather than 2−ΔCt data are plotted on the y-axes. An increase of 1 on the y-axis thus corresponds to a 2-fold increase in target gene expression.

Inflammation up-regulates hepcidin independently of BMP6

To investigate the detailed timing of the inflammatory response induced by the Gram-negative bacterial cell wall component, groups of 6 mice on a CD1 background (3 wild-type and 3 Bmp6−/−) were thus compared by Student t tests. Because, in addition to inflammation, mouse genotype (wild-type versus Bmp6−/−) may affect mean ΔCt values, we also used 2-way ANOVA to test for the effect of both factors and their interaction.
were killed at different time points (30 minutes and 1, 1.5, 2, 4, 6, 15, 24, and 48 hours) after challenge with LPS. One group of 6 CD1 mice (3 wild-type and 3 Bmp6−/−) was killed to provide baseline values. As expected, LPS induced a rapid and massive elevation of Tnf and Il-6 mRNA expression in the livers of both wild-type and Bmp6−/− mice (supplemental Figure 1). The magnitude of this induction was similar in the 2 groups of mice and was followed by an increase in the liver acute-phase protein Ctr mRNA (supplemental Figure 2A). As shown in Figure 1A, wild-type mice exhibited increased hepcidin (Hamp) mRNA levels after LPS administration, hepcidin mRNA levels were maximum (ie, 4-6 hours after LPS administration; supplemental Figure 2B). (A) Hepcidin (Hamp) mRNA levels were measured by quantitative RT-PCR. Values shown are means of −ΔΔCt (ie, −(ΔCt Hamp − ΔCt Hprt)) ± SD. The higher the −ΔΔCt, the greater the amount of Hamp amplion. Four hours after LPS administration, hepcidin mRNA levels were increased on average 2.5-fold (−ΔΔCt = 6.31-6.97 = 1.34; 2−ΔΔCt = 2.53) in wild-type mice and approximately 40-fold (−ΔΔCt = 5.62-0.25 = 5.37; 2−ΔΔCt = 41.35) in Bmp6−/− mice. Means of −ΔΔCt values between LPS-challenged and unchallenged mice of each genotype were compared by Student tests. ***P < .001; **P < .01; *P < .05. (B) Protein extracts were prepared from the livers of 6 mice at each time point. Phospho–Stat3, total Stat3, phospho–Smad1/5/8, and total Smad5 were detected by immunoblot techniques in wild-type (left) and Bmp6−/− (right) mice. The blots shown are representative of 3 independent experiments for each time point and each mouse genotype. Densitometry was performed using the ImageJ 1.45s gel-analysis method and results are shown in supplemental Figure 6.

Figure 1. Inflammation up-regulates hepcidin expression via phosphorylation of the Smad effectors 1, 5, and 8 independently of Bmp6. Groups of 6 CD1 mice (3 wild-type and 3 Bmp6−/−) were killed at different time points after administration of LPS (1 μg/g of body weight). (A) Hepcidin (Hamp) mRNA levels were measured by quantitative RT-PCR. Values shown are means of −ΔΔCt (ie, −(ΔCt Hamp − ΔCt Hprt)) ± SD. The higher the −ΔΔCt, the greater the amount of Hamp amplion. Four hours after LPS administration, hepcidin mRNA levels were increased on average 2.5-fold (−ΔΔCt = 6.31-6.97 = 1.34; 2−ΔΔCt = 2.53) in wild-type mice and approximately 40-fold (−ΔΔCt = 5.62-0.25 = 5.37; 2−ΔΔCt = 41.35) in Bmp6−/− mice. Means of −ΔΔCt values between LPS-challenged and unchallenged mice of each genotype were compared by Student tests. ***P < .001; **P < .01; *P < .05. (B) Protein extracts were prepared from the livers of 6 mice at each time point. Phospho–Stat3, total Stat3, phospho–Smad1/5/8, and total Smad5 were detected by immunoblot techniques in wild-type (left) and Bmp6−/− (right) mice. The blots shown are representative of 3 independent experiments for each time point and each mouse genotype. Densitometry was performed using the ImageJ 1.45s gel-analysis method and results are shown in supplemental Figure 6.

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LPS challenge increases phosphorylation of Smad1/5/8 effectors in the livers of both wild-type and Bmp6−/− mice

As expected, LPS stimulated Stat-3 activation rapidly. Phosphorylation of Stat-3 reached a peak 1 hour after LPS administration, 3 hours ahead of hepcidin maximal induction, and then returned gradually to basal levels (Figure 1B). Because pharmacologic inhibition of BMP type I receptors blocks the induction of hepcidin expression by various inflammatory stimuli,25,26,28 we also investigated whether LPS was able to induce BMP/Smad signaling even in the absence of Bmp6. As shown in Figures 1B and supplemental Figure 6, phosphorylation of the Smad1/5/8 effectors was significantly increased 2-4 hours after LPS challenge, slightly ahead of the increase in Hamp mRNA expression, in the livers of both wild-type and Bmp6−/− mice and returned to basal levels at 6 hours. This provides evidence supporting a role for BMP/Smad signaling in the induction of hepcidin by LPS and suggests that a ligand of the TGF-β/BMP superfamily other than BMP6 activates hepcidin production during inflammation.

None of the ligands of the BMP subfamily is up-regulated after LPS administration

Because Bmp6 is not necessary for the induction of hepcidin by inflammation, we suspected that another ligand of the Bmp subfamily was involved in this process and that its expression was induced by inflammation (like the expression of Bmp6 expression was induced by iron). We therefore compared liver mRNA expression of Bmp2, Bmp4, Bmp5, Bmp6 (in wild-type mice), Bmp7, and Bmp9 between LPS-challenged and control mice. All of these Bmp molecules had been shown previously to increase hepcidin mRNA expression robustly in hepatoma-derived cells.35 However, rather than being increased by LPS, expression of these Bmp ligands was either unchanged or even significantly decreased 2-15 hours after LPS administration, as shown for Bmp2, Bmp5, and Bmp9 in supplemental Figure 3. On the assumption that BMP gene expression at the mRNA level is informative for that at the protein level, these ligands of the BMP subfamily do not appear to play a significant role in the induction of hepcidin by inflammation.

In contrast to Bmp mRNAs, activin B expression is highly up-regulated by inflammation in the livers of both wild-type and Bmp6−/− mice

In hepatoma-derived cell lines, the endogenous ligands BMP2, BMP4, and BMP6 have been shown to signal through the BMP
type I receptors ALK3 and/or ALK2 to regulate hepcidin expression, a process facilitated by HIV. Interestingly, the expression of other members of the TGF-β superfamily, activin A and activin B, is increased in inflammatory diseases such as septicemia, inflammatory bowel disease, and rheumatoid arthritis, which raises the possibility that activins play a significant role in the acute inflammatory response. We therefore hypothesized that, although activins conventionally use their own receptors, ALK4 or ALK7, they could under some circumstances bind type I receptors used by BMP ligands and activate hepcidin through phosphorylation of the Smad effectors 1, 5, and 8. Therefore, we investigated whether the mRNA expression of Inhba, Inhbb, Inhbc, and/or Inhbe, encoding the βα, ββ, βc, and βε activin subunits, respectively, was induced by LPS. As shown in Figure 2A, there was a dramatic increase in Inhbb mRNA, starting 1 hour after LPS administration and peaking at 4 hours. The average induction was greater than 35-fold in both wild-type and Bmp6−/− mice. In contrast, Inhba mRNA undergoes a profound decrease (more than 8-fold in both strains) between 4 and 6 hours after the LPS challenge (Figure 2B). Expression of Inhbc mRNA was only modestly affected by LPS (supplemental Figure 4A), but that of Inhbe was strongly reduced up to 4 hours after LPS administration (supplemental Figure 4B). These findings show that, among all activin molecules, activin B is the only candidate ligand for which expression increases after an LPS challenge.

The up-regulation of activin B transcription is independent of IL-6

Because IL-6 is sufficient for the induction of hepcidin during inflammation, we next examined whether IL-6−/− mice were able to increase activin B transcription in response to an LPS challenge. IL-6−/− and IL-6+/+ controls of the same C57BL/6 background (5 mice/group) were killed 4 hours after LPS administration and their activin B and hepcidin levels were compared with those of unchallenged IL-6−/− and IL-6+/+ animals (5 mice/group, respectively. Interestingly, as shown in Figure 3A, IL-6−/− mice were able to up-regulate both Inhbb and Hm7m mRNA in response to LPS to a level similar to that of LPS-challenged wild-type mice. These data show that induction of activin B and hepcidin by LPS occurs independently of IL-6. As for the wild-type and Bmp6−/− mice, Inhba and Hjv mRNA expression was strongly reduced in the livers of IL-6−/− mice 4 hours after LPS administration (data not shown), whereas phosphorylation of both Stat-3 and Smad1/5/8 was significantly increased (Figure 3B). Activation of Stat-3 signaling in IL-6−/− mice was likely due to LPS-induced mediators of the IL-6 family such as leukemia inhibitory factor (LIF), IL-11, or oncostatin M, which was described previously to be an hepcidin inducer. Up-regulation of Inhbb mRNA, together with activation of Smad1/5/8 signaling in these mice, also suggests a role for activin B in signal transduction.

Activin B induces hepcidin expression and SMAD1/5/8 phosphorylation in human hepatoma-derived cells

We next examined the role of activin B, alone or in combination with IL-6, on hepcidin expression in the human hepatoma-derived HepG2 cells. Cells were treated with IL-6, activin B, or both for periods of time ranging from 1.5-24 hours. We found that activin B increased hepcidin mRNA expression on average 13-fold, with peak levels achieved 4 hours after treatment. Its effect on hepcidin up-regulation was larger than that of IL-6 (only approximately 6-fold on average) and, interestingly, the combination of activin B and IL-6 increased hepcidin mRNA levels much more (more than 43-fold on average) than the additive effects of the 2 molecules (Figure 4A). We then evaluated the ability of activin B, alone or in combination with IL-6, to activate different signaling pathways in HepG2 cells after 2.5, 4, or 6 hours of treatment. As expected, IL-6 induced STAT-3 phosphorylation. However, more interestingly, activin B induced the phosphorylation of the SMAD effectors 1, 5, and 8 after an exposure of 2.5 (Figure 4B), 4, or 6 hours (data not shown). The 2 signaling pathways are independent of each other.
and the capacity of activin B to induce SMAD1/5/8 phosphorylation is not shared with activin A (Figure 4B). These data suggest that activin B could bind to BMP type I receptors rather than to the conventional activin type I receptors ALK4 and ALK7, which have so far been reported to signal only through Smad2 and Smad3. To investigate this possibility, we treated HepG2 cells with the BMP type I receptor inhibitor LDN-193189 (100nM) 30 minutes before adding activin B. At this concentration, LDN-193189 was shown to inhibit activity of BMP type I receptors, but not that of activin and TGF-β type I receptors. As shown in Figure 5,

Figure 3. Inflammation up-regulates activin B and hepcidin transcription in the absence of IL-6. Groups of C57BL/6 Il-6−/− and Il-6+/+ mice were injected with LPS (1 µg/g of body weight) or saline (5 mice/group) and were killed 4 hours later. (A) mRNA levels of Inhbb and Hamp were measured by quantitative RT-PCR. Values shown are means of −ΔΔCt (i.e., −ΔCt target gene − ΔCt housekeeping gene) ± SD. Four hours after LPS administration, Inhbb mRNA levels were increased on average 35-fold both in Il-6−/− mice (−ΔΔCt = −3.15 ± 8.27 = 5.12; 2−ΔΔCt = 35.10) and in Il-6+/+ mice (−ΔΔCt = −3.26 ± 8.39 = 5.13; 2−ΔΔCt = 34.75). Hamp mRNA levels were increased on average 3.1-fold in both Il-6−/− mice (−ΔΔCt = 7.90-6.27 = 1.63; 2−ΔΔCt = 3.10) and Il-6+/+ mice (−ΔΔCt = 8.02-6.38 = 1.63; 2−ΔΔCt = 3.10). The effect of LPS on Inhbb and Hamp gene expression was assessed by the Student t test. **P < .01; ***P < .001. (B) Protein extracts were prepared from the mouse livers of the Il-6−/− mice injected with LPS or saline. Phospho–Stat-3, total Stat-3, phospho–Smad1/5/8, and total Smad5 were detected by immunoblot techniques. Densitometry was performed using the ImageJ gel-analysis method and results are shown in supplemental Figure 6.

Figure 4. Activin B induces hepcidin expression and SMAD1/5/8 phosphorylation in human hepatoma-derived cells. HepG2 cells were treated with IL-6 (50 ng/mL) and/or activin B (50 ng/mL) or activin A (50 ng/mL) for 1.5, 2.5, 4, 6, and 24 hours. (A) Hepcidin (Hamp) mRNA levels were measured by quantitative RT-PCR. Values shown are means of −ΔΔCt (i.e., −ΔΔCt treatment − ΔΔCt vehicle) ± SD obtained from 4 independent experiments. Means of −ΔΔCt were compared with 0 by Student t tests. ***P < .001; **P < .01; *P < .05. The fold changes induced by treatment can be obtained by raising 2 to the power −ΔΔCt. (B) Protein extracts were prepared from HepG2 cells treated with the different activators for 2.5 hours. Phospho–Smad1/5/8, total Smad5, phospho–Stat-3, and total Stat-3 were detected by immunoblot techniques. Similar patterns were observed after 4 and 6 hours of treatment (data not shown).
pretreatment with LDN-193189 completely abolished the induction of hepcidin gene expression and SMAD1/5/8 phosphorylation 2.5, 4, or 6 hours after stimulation with activin B. This demonstrates that, in human hepatoma-derived cells, the effect of activin B on hepcidin expression is entirely attributable to its effects on BMP signaling. We then used soluble BMP type I receptors to identify the receptor involved in activin B signaling. Alk1 is predominantly expressed in the endothelium, and we were not able to detect Alk6 mRNA expression in the mouse liver or in HepG2 cells. We therefore focused on the 2 other BMP type I receptors and pretreated HepG2 cells with soluble forms of ALK2 (ALK2-Fc) or ALK3 (ALK3-Fc). As shown in supplemental Figure 5, only ALK3-Fc prevented the induction of hepcidin gene expression by activin B, suggesting that ALK3 is the most likely type I receptor involved in activin B signaling.

**Activin B also induces hepcidin expression and Smad1/5/8 phosphorylation in mouse primary hepatocytes**

To further evaluate the impact of activin B on the regulation of hepcidin gene expression, we measured basal hepcidin expression and the ability of activin B to induce this expression in primary hepatocytes isolated from Bmp6-deficient mice. Because of the lack of functional Bmp6, these mice are insensitive to iron and are a valuable resource in identifying the molecular pathways through which hepcidin expression is up-regulated specifically by inflammation. Activin B rapidly increased hepcidin mRNA expression in mouse primary hepatocytes, with peak levels achieved 2.5 hours after stimulation. Hepcidin expression then remained stable up to at least 4 hours (data not shown). Interestingly, despite a strong activation of Stat-3 signaling (Figure 6A), the effect of IL-6 on hepcidin up-regulation (approximately 1.8-fold increase on average) was relatively modest (Figure 6B) compared with that of activin B (approximately 7-fold increase on average). However, similar to HepG2 cells, there was a clear synergy between Smad1/5/8 and Stat-3 signaling, with a 17-fold increase on average when primary hepatocytes were treated with both activin B and IL-6. Pretreatment of mouse primary hepatocytes with the BMP type I receptor inhibitor LDN-193189 prevented induction of Smad1/5/8 phosphorylation (Figure 6C) and up-regulation of Hamp gene expression by activin B, confirming that the effect of activin B on hepcidin expression is attributable to its effects on BMP signaling not only in hepatoma-derived cells, but also in primary hepatocytes.

**Discussion**

In the present study, we first observed that hepcidin up-regulation in the livers of LPS-challenged mice coincided with an increase in Smad1/5/8 phosphorylation. Activation of the Smad1/5/8 signaling cascade does not appear to be restricted to this model of acute inflammation. Indeed, phosphorylation of Smad1/5/8 was also found to be increased significantly in rats injected with group A streptococcal peptidoglycan-polysaccharide, which resulted in the development of chronic arthritis associated with chronic persisting inflammatory anemia bearing the typical features of anemia of chronic disease. Interestingly, the increase in Smad1/5/8 phosphorylation was independent of Bmp6, unaffected by iron in the 7- to 8-week-old Bmp6−/− mice, and not related to the induction of any of the BMP molecules expressed in the liver, at least at the mRNA level. We therefore examined the expression of other ligands that could share type I receptors with BMP molecules and found that Inhibb mRNA was the only one to be rapidly induced in the liver of mice challenged with LPS; its peak coincided with maximum phosphorylation of Smad1/5/8 and slightly preceded that of Hamp gene expression. So far, activins have only been shown to signal through Smad2 and Smad3. Indeed, whereas canonical BMP signaling is mediated via the ALK1/2/3/6 type I receptors that phosphorylate Smad1, Smad5, and Smad8, canonical activin signaling is mediated via the ALK4/7 type I receptors that phosphorylate Smad2 and Smad3. Therefore, we used human HepG2 hepatoma cells and primary mouse hepatocytes to demonstrate that activin B could induce hepcidin transcription through a noncanonical signaling pathway involving phosphorylation of the Smad effectors 1, 5, and 8. Pretreatment of HepG2 cells and mouse primary hepatocytes with the BMP type I receptor inhibitor LDN-193189 completely abolished activin B-mediated induction of Smad1/5/8 phosphorylation and hepcidin gene expression, confirming that activin B uses the classic BMP type I receptors to regulate signaling and hepcidin expression in the liver. In addition, pretreatment with soluble BMP type I receptors suggested that activin B was most likely signaling via ALK3.

Interestingly, signaling through a noncanonical pathway has already been reported for another member of the activin subfamily. Although TGFβ signals predominantly through a receptor complex comprising ALK5 and TβRII to activate Smad2 and Smad3 phosphorylation, it can sometimes activate Smad1 and Smad5 in endothelial cells, as well as in several normal epithelial cell lines, fibroblasts, and cell lines of tumor origin. TGFβ induction
Figure 6. Activin B uses the classic BMP type I receptors to induce SMAD1/5/8 phosphorylation and hepcidin expression in mouse primary hepatocytes. Mouse primary hepatocytes were treated with IL-6 (50 ng/mL) and/or activin B (50 ng/mL) for 2.5 hours. (A) Protein extracts were prepared from these hepatocytes. Phospho–Smad1/5/8, total Smad5, phospho–Stat-3, and total Stat-3 were detected by immunoblot techniques. The blot shown is representative of 3 independent experiments. (B) Hepcidin (Hamp) mRNA levels were measured by quantitative RT-PCR. Values shown are means of −ΔΔCt (ie, −ΔΔCt [treatment − ΔCt vehicle]) + SD obtained from 4 independent experiments. Means of −ΔΔCt were compared with 0 by Student t tests. **P < .01. The fold change induced by treatment can be obtained by raising 2 to the power −ΔΔCt. (C) Mouse primary hepatocytes were treated with activin B (50 ng/mL) for 2.5 hours in the presence or absence of LDN-193189 (1 μM). Phospho–Smad1/5/8 and total Smad5 were detected by immunoblot techniques. The blot shown is representative of 3 independent experiments.

of Smad1/5 phosphorylation appears to be mediated by a heterotrameric receptor complex comprising, in addition to ALK5, the BMP receptors ALK1 in endothelial cells and ALK2 or ALK3 in other cells.42 This shows that BMP type I receptors can bind different ligands in different contexts and very likely bind activin B to induce hepcidin transcription in the inflammatory setting.

As observed previously,43 Hjv gene expression is strikingly down-regulated by inflammation in the mouse liver. Curiously, phosphorylation of the Smad effectors 1, 5, and 8 is only transient and returned to baseline levels 6 hours after LPS injection in the present study, even though activin B expression was still high at this time point. This coincided with the marked down-regulation of Hjv and, as expected, was followed by a decrease in hepcidin expression. Further work is needed to determine whether Hjv down-regulation could serve as a negative feedback signal to prevent excessive hepcidin production.

Activation of the hepcidin promoter by inflammatory stimuli was shown previously to require not only STAT-3 activation and binding to a motif located at position −72/−64 bp, but also SMAD1/5/8 activation and binding of heteromeric complexes with SMAD4 to a BMP-responsive element at position −84/−79 bp.20 In human hepatoma-derived cells HepG2 and mouse primary hepatocytes, the effect of activin B on hepcidin up-regulation is larger than that of IL-6, and the combination of activin B and IL-6 increases hepcidin mRNA levels much more than the additive effects of the 2 molecules. Further analyses will determine whether this synergistic effect on hepcidin gene expression results from the combined independent effects of Stat-3 and Smad1/5/8 pathways or, in contrast, to the cooperative interaction between them. Interestingly, the formation of a complex between STAT-3 and Smad1, bridged by the adaptor CREB-binding protein p300, has been shown previously to be involved in the cooperative signaling of LIF and BMP2 and the subsequent induction of astrocytes from neural progenitors.44 A similar mechanism could be involved in the induction of hepcidin by inflammatory stimuli.

Although phosphorylation of the Smad effectors 1, 5, and 8 returned to baseline levels 6 hours after LPS injection, hepatocyte STAT-3 activation was sustained slightly longer. This is probably explained by independent regulatory mechanisms of the 2 signaling pathways and/or by a role of STAT-3 on the regulation of inflammatory response in addition to that on hepcidin transcription. Indeed, it has been shown previously45 that activation of hepatocyte STAT-3 protects against hepatocellular damage and subsequently reduces liver necrosis associated with inflammation. Hepatocyte STAT-3 also inhibits IFN-γ–activated Stat-1, a key proinflammatory signal in the liver, thereby playing an important role in attenuating inflammatory responses.

Activins were originally characterized as reproductive factors, and their involvement in inflammatory processes is a relatively newly described phenomenon. So far, activin A has been the most extensively studied family member in the context of inflammation and immunity. Gene expression, synthesis, and release are stimulated by bacterial and viral molecular products through activation of the IL-1/TLR signaling pathway, by various inflammatory cytokines, and by oxidative stress.46 Activin A increases in the serum and various tissues in acute and chronic inflammatory diseases and models, and there is evidence that activin A has both proinflammatory and immunoregulatory functions. In the inflammatory setting, the expression of the βB-subunit has received relatively little attention compared with activin A, but there is some evidence that at least some cell types increase the production of activin B in response to inflammatory stimuli, most notably hepatic stellate cells,47 pituitary cells,48 and microglial cells.49 In rat anterior pituitary cells, IL-1β, but not LPS, stimulates expression and synthesis of both βB-subunit mRNA and protein to form...
activin B dimers. Interestingly, the cytokine that is most potent in up-regulating hepcidin in mouse primary hepatocytes is IL-1. It was suggested that the effect of IL-1 on activin up-regulation was indirect and independent of IL-6. Activin B could thus be the missing link between IL-1 and hepcidin. Whereas activin B shares many of the functions of activin A, possibly acting as a slightly weaker activin agonist in many cases, it may also exert functionally distinct effects from those of activin A. This is exemplified in the present study where, in contrast to Inhibh, Inhiba mRNA was strongly down-regulated in the livers of the LPS-challenged mice. It was shown previously that blocking activin A actions in the inflammatory response with follistatin alters the magnitude and kinetics of proinflammatory cytokine release and reduces mortality to severe inflammatory insults. Therefore, it can be speculated that the observed down-regulation of activin A would contribute to reducing the severity of inflammation. In summary, the results of the present study provide evidence for a unique role of activin B in the up-regulation of hepcidin by inflammation and identifies activin B as a more specific target than the global BMP type I receptor inhibitor LDN-193189 for the treatment of anemia of inflammation.

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Authorship

Contribution: C.B.-F. and C.L. performed the Western blot experiments; C.L., L.K., and J.B. generated the mouse models and conducted the RT-PCR experiments; C.B.-F. and J.B. performed the in vitro experiments on HepG2 cells and mouse primary hepatocytes, respectively; T.G. provided key samples from Il-6-/- mice; and M.-P.R. and H.C. led and supervised the project through all stages, helped in data analyses, and wrote the manuscript with suggestions and comments from all authors.

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Induction of activin B by inflammatory stimuli up-regulates expression of the iron-regulatory peptide hepcidin through Smad1/5/8 signaling

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