Short Research Report

# Combination use of sildenafil and simvastatin increases BMPR-II signal transduction in rats with monocrotaline-mediated pulmonary hypertension

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Dysfunctional bone morphogenetic protein (BMP) signaling has been found in patients with pulmonary arterial hypertension (PAH); however, the exact role of BMP signaling in the treatment of PAH remains unknown. The BMP receptor type II (BMPR-II) is a member of the TGF-β family of signaling molecules. Functional receptors are heterodimers composed of a BMPR-II subunit and a serine–threonine kinase type I subunit, of which there are three members: BMPR-Ia, BMPR-Ib and Alk2.[1,2] Both BMPR-II and BMPR-Ia/Ib are highly expressed in the pulmonary vascular smooth muscle and endothelium. The discovery of heterozygous mutations of the BMPR-II gene (*BMPR2*) in patients with hereditary (or familial) PAH and patients with idiopathic PAH [3,4] represented a significant advance in the understanding of the genetic contributions to PAH.

The two main pathways downstream of BMP signaling are the Smad-dependent pathway, which uses Smad-signaling proteins, and the Smad-independent pathway, which involves p38, MAPK, ERK and JNK proteins.[1,5,6] In the Smad pathway, activation of the receptor complex by ligand binding results in the recruitment and phosphorylation of regulatory Smad proteins (Smads-1, 5 or 8). These Smad proteins recruit Smad-4, and the resulting complex is translocated to the nucleus, where it regulates transcription of target genes containing the Smad-binding sequence. Although BMP signaling may provide a common pathway in PAH pathogenesis, it is unclear whether current treatments targeting different pathways lead to an increase in BMP signal transduction in the lung tissues. A treatment that targets a common underlying cause of PAH, such as BMP dysfunction, may prove efficacious.

Both sildenafil, a phosphodiesterase-5 inhibitor, and simvastatin, a cholesterol-lowering drug, have therapeutic effects on PAH. In our previous studies, we investigated three different therapeutic regimens for treating pulmonary hypertension (PH) in rats injected with monocrotaline (MCT) using sildenafil, simvastatin and a combined sildenafil and simvastatin treatment. MCT injections cause a significant increase in the mean pulmonary arterial pressure (PAP) and pulmonary vascular medial thickening. The time course for the MCT-induced increase in mean PAP is associated with the MCT-induced increase in pulmonary vascular wall thickness, occurring after 2 weeks of the injection of MCT.[7] Although all treatment regimens have an effect on right ventricular systolic pressure (RVSP) in rats with MCT-mediated PH, the combined drug treatment of both sildenafil and simvastatin is more effective in reducing RVSP than either drug alone.[7]

The purpose of this study was to investigate the changes in BMP signaling, including both Smad and ERK downstream

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pathways, in rats with MCT-induced PH treated with sildenafil and simvastatin.

We first examined whether key BMP molecules (BMPR-II, BMPR-Ia and BMP-2) were changed in lung tissues over a 4-week time course in rats subcutaneously injected with MCT (50 mg/kg or 25 ml/kg of 2% MCT). Interestingly, animals at both weeks 1 and 2 (after injection of MCT) had significantly increased levels of BMPR-II (3.655±1.008 in week 1 group and 2.164±0.508 in week 2 group compared to 1.874±0.121 in the saline injected control), BMPR-Ia (1.451±0.334 in week 1 group and 1.082±0.106 in week 2 group compared to 0.625±0.188 in the saline injected control animals), and BMP-2 (1.500±0.243 in the week 1 group and 1.428±0.056 in week 2 group vs. 0.696±0.068 in the saline injected control group); however, by the third week, BMPR-II had declined to levels below those of the control group and, by the fourth week, BMP-2 had also declined to below the control levels [Figure 1]. Our observations indicate that MCT injection caused a transient increase in the mRNA and protein expression of BMP-2, BMPR-II and BMPR-Ia, which correlates with a transient increase in Smad1 phosphorylation.[7] The changes in BMP signaling molecules correlate well with the observed MCT-induced PH at 3 and 4 weeks.[7] Thus, both hemodynamic indicators of PAH and underlying molecular abnormalities (e.g., BMP-signaling molecules and Smad) correlate with each other in rats during the development of MCT-induced PH.

Then, we examined the effects of sildenafil, simvastatin and combination treatment on BMPR-Ia, BMPR-II and BMP-2 mRNA levels in our MCT model of PH. Compared with rats in the sham group (normotensive saline-injected rats), lung tissue BMPR-Ia, BMPR-II and BMP-2 mRNA levels detected by real-time reverse transcriptase polymerase chain reaction (RT-PCR) in the MCT-injected control group decreased significantly (BMPR-Ia: 0.50±0.09 compared with 0.39±0.11; BMPR-II: 1.75±0.28 compared with 0.40±0.02; BMP-2: 0.68±0.02 compared with 0.37±0.05 arbitrary unit) [Figure 2A(a–c)]. Either sildenafil or simvastatin treatment alone prevented decreases in BMPR-Ia, BMPR-II and BMP-2 mRNA levels, but the combination therapy was more effective at preventing the decrease in BMPR-II and BMPR-Ia [Figure 2B(b and c)]. The level of BMP-2 in the combination group, while being higher than that in the MCT-injected control animals, was not significantly different from either treatment alone [Figure 2B(a)].

BMP signaling, including BMP-2 and its receptors, plays an important role in maintaining the normal structure of the pulmonary vasculature through its regulation of apoptosis and suppression of cell proliferation.[8-10] In the pulmonary vasculature, BMP-2 is predominantly expressed in endothelial cells, with a low level of expression in vascular smooth muscle cells, whereas BMPR-II localizes to endothelial cells, smooth muscle cells and adventitial fibroblasts.[8] In both apical and basal membranes of the arteriolar endothelium, BMPR-II colocalizes with caveolin-1,[11] indicating that BMPR-II may have a hemodynamic regulatory role because caveolae also contains hemodynamically relevant signaling molecules, including eNOS,[12,13] the serotonin transporter,[14-16] transient receptor potential (TRP) channels[17,18] and endothelin receptors.[19,20]

In our study, the expression of BMP-2, BMPR-Ia and BMPR-II mRNAs during the 4-week course of MCT-induced PH increased significantly in the first week after MCT injection. At week 2, these levels were still above baseline, although they had started to decline from week 1. This initial increase was followed by a significant decrease 3–4 weeks after MCT injection. We did not study BMP signaling or pulmonary artery smooth muscle cell (PASMC) proliferation in MCT-treated rats beyond 4 weeks after injection; however, within the 4-week time course, we observed an important difference

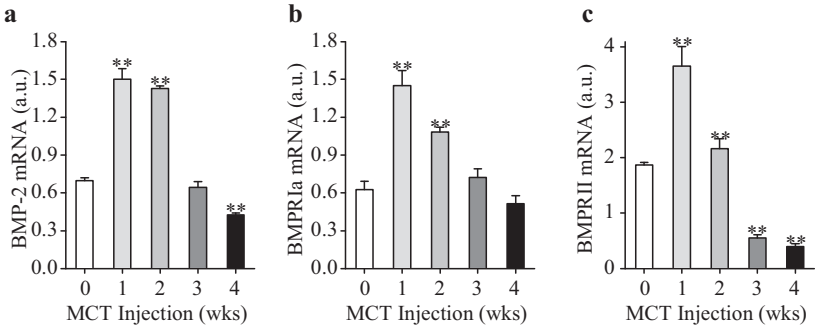


**Figure 1:** Bone morphogenetic protein (BMP) signaling proteins in rat lung initially increase and then decrease after monocrotaline (MCT) injection. mRNA was collected from whole lung from rats injected with MCT at the indicated time points after injection. The week 0 group was injected with saline and RNA was isolated the next day. mRNA was used in reverse transcriptase-polymerase chain reaction to quantify BMP-2 (a), BMPR-Ia (b) and BMPR-II (c) mRNA levels. All BMP signaling molecule mRNA levels were normalized against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. Data are presented in arbitrary units (a.u.) as mean±SD; $n$=8/group; **$P$<0.01 vs. 0 week control group

between MCT-induced PAH and clinical PAH caused by defective BMP signaling. In many patients, dysfunctional BMP signaling due to BMPR-II mutations and/or downregulated BMPR-Ia causes PAH.[21-24] However, in the MCT model, there was an initial increase in BMP signaling molecules as previously noted. The subsequent decline in these correlated with the development of elevated RVSP, right ventricular hypertrophy (RVH) and remodeling.[7] An initial increase in BMP signaling molecules in models of PAH is not without precedent, however, as hypoxia induces an initial rise (~3 weeks) in BMP4 in mouse lung followed by a decrease in BMP4 mRNA after week 3.[25] It may be that initial MCT-induced endothelial injury causes the subsequent rise in BMP signaling. Lung tissue p-Smad1, a downstream signaling molecule involved in proapoptotic BMPR-II signaling, paralleled the changes of BMP2, BMPR-Ia and BMPR-II mRNAs, consistent with the observation of previous studies.[26,27] This pattern suggests that acute injury to the pulmonary vascular endothelium may stimulate BMPR-II, BMPR-Ia and BMP-2 expression in lung vascular endothelial cells, whereas chronic injury results in a decline of these molecules.

BMPR-II signaling in pulmonary artery endothelial cells (PAEC) promotes cell survival and protects against apoptosis, whereas it is proapoptotic (through Smad signaling) in PASMC.[10,28] It is therefore tempting to speculate that PAEC respond to MCT injury initially by increasing BMP signaling to promote endothelial protection; the subsequent MCT-induced decline in BMP-signaling molecules may then tip the balance toward an increase in PASMC proliferation, which overtakes the already injured endothelium, thus leading to muscularization and PH by week 3 after MCT injection. Indeed, the pulmonary vascular remodeling associated with clinical PAH is believed to result largely from increased proliferation and decreased apoptosis of PASMC that could be caused by defects in the proapoptotic BMPR-II/Smad signaling pathway.[6,21-23,28,29]

Our results suggest that targeting BMPR-II deficiencies in PAH may provide a useful therapeutic approach to treat the disease. Current PAH medications do not target the BMP pathway, but a more complete understanding of BMPR-II signaling in the MCT model of PH, such as provided here, will prove useful in future investigations aimed at developing interventions that target this pathway.

The levels of lung tissue BMPR-II, BMPR-Ia and BMP-2 mRNA and p-Smad1 protein were much higher in both the sildenafil and the simvastatin groups compared with

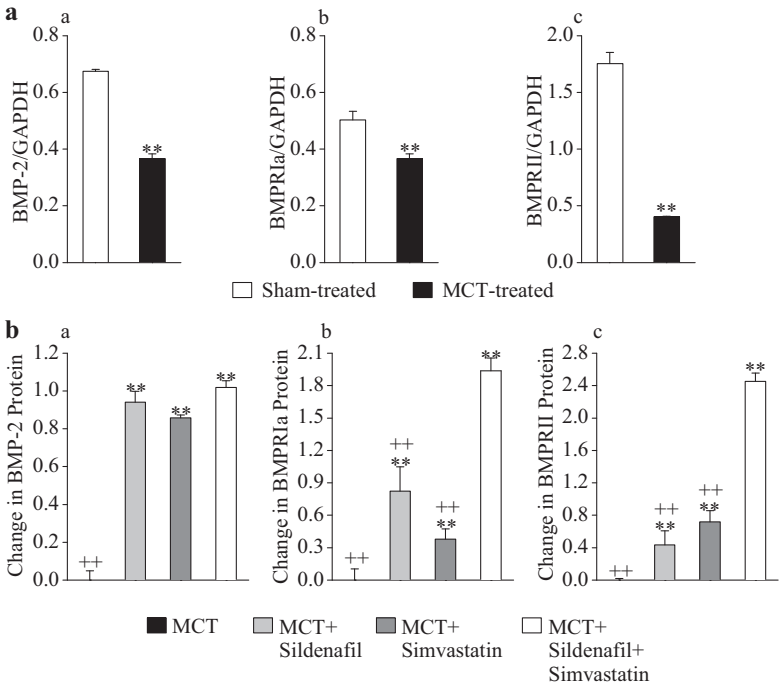


**Figure 2:** Combined sildenafil and simvastatin treatment prevents the monocrotaline (MCT)-induced decrease in bone morphogenetic protein (BMP)-signal pathway molecules to a greater extent than either drug alone. mRNA was collected from whole lung tissue from saline-injected sham treated rats, MCT-injected control rats and rats treated with sildenafil, simvastatin or both. mRNA levels of BMP-2 (A*a*), BMPR-Ia (A*b*) and BMPR-II (A*c*) were determined using reverse transcriptase-polymerase chain reaction and normalized to GAPDH. Increases in BMP-2 (*a*), BMPR-Ia (*b*) and BMPR-II (*c*) are shown in (B) as the change compared with the levels in the MCT-injected control group. Data are presented in arbitrary units (A) as mean±SD; $n$=8/group; $^{**}P<0.01$ vs. sham-injected (A) or vs. MCT-injected control (B); $^{++}P<0.01$ vs. combination group (B)

the MCT-injected control group, indicating that either drug alone inhibits the decrease in the expression of the BMP signaling molecules. The inhibition of the loss of BMPR-II signaling may be an important mechanism underlying the prevention of PASMC overproliferation and pulmonary vascular remodeling in the sildenafil or simvastatin treatment groups. The low levels of matrix metalloproteinases (MMPs) may be due to the concomitant increase in BMP-signal transduction, as the reduction in BMP signaling has been shown to reduce MMP activity.[30] Furthermore, reductions in BMP signaling and/or MMP activity confer resistance to apoptosis,[30] indicating that sildenafil and simvastatin may reduce PASMC proliferation at least in part by maintaining BMP signaling molecules.

Because combination treatment was able to prevent the decrease in lung tissue BMPR-II and BMPR-Ia to a greater extent than either treatment alone, it is likely that the additive effect of combination treatment in preventing the development of PH involves BMP signaling.

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