BMP-7 attenuates adverse cardiac remodeling mediated through M2 macrophages in prediabetic cardiomyopathy

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Submitted 28 May 2014; accepted in final form 25 June 2014

Urbina P, Singla DK. BMP-7 attenuates adverse cardiac remodeling mediated through M2 macrophages in prediabetic cardiomyopathy. Am J Physiol Heart Circ Physiol 307: H762–H772, 2014. First published July 3, 2014; doi:10.1152/ajpheart.00367.2014.—The main objective of this study was to determine whether or not monocyte infiltration occurs in the prediabetic (PD) heart and its role in PD cardiomyopathy. We hypothesized that the PD heart is significantly populated with monocytes and that bone morphogenetic protein (BMP)-7, a novel mediator of monocyte polarization, activates infiltrated monocytes into anti-inflammatory M2 macrophages, thereby inhibiting apoptosis and fibrosis and improving cardiac function. C57Bl6 mice were assigned to control, PD, or PD + BMP-7 groups. PD and PD + BMP-7 groups were administered streptozotocin (50 mg/kg), whereas control animals received sodium citrate buffer. Afterward, the PD + BMP-7 group was administered BMP-7 (200 μg/kg) for 3 days. Our data showed significantly increased infiltrated monocytes and associated pro-inflammatory cytokines, adverse cardiac remodeling, and heart dysfunction in the PD group (P < 0.05). Interestingly, M2 macrophage differentiation and associated anti-inflammatory cytokines were enhanced and there were reduced adverse cardiac remodeling and improved cardiac function in the PD + BMP-7 group (P < 0.05). In conclusion, our data suggest that PD cardiomyopathy is associated with increased monocyte infiltration and released proinflammatory cytokines, which contributes to adverse cardiac remodeling and cardiac dysfunction. Moreover, we report that BMP-7 possesses novel therapeutic potential in its ability to differentiate monocytes into M2 macrophages and confer cardiac protection in the PD heart.

macrophages; monocytes; prediabetic cardiomyopathy; heart; apoptosis; bone morphogenetic protein-7

DIABETES is a group of metabolic disorders that results in chronic hyperglycemia. Symptoms include abnormal metabolism of lipids, carbohydrates, and protein and may also involve impaired insulin secretion (1). Two major classifications of diabetes exist: type 1 diabetes mellitus and type 2 diabetes mellitus. Type 1 diabetes mellitus is regarded as an autoimmune condition that involves the destruction of pancreatic β-cells and may lead to total insulin deficiency (1). Type 2 diabetes mellitus accounts for the majority of diabetic patients in the United States (4) and manifests as insulin resistance despite normal pancreatic β-cell function. More recently, the Word Health Organization introduced prediabetes (PD) to include patients with impaired fasting glucose and impaired glucose tolerance (3). Diabetes, regardless of the type, increases the risk for conditions such as cardiovascular disease (9) and nephropathy (31). Recent studies have shown that even subclinical states of hyperglycemia can lead to adverse complications.

Moreover, the exact source of increased immunoregulation and associated oxidative stress and inflammation in diabetes is complex and not well understood. It has recently been reported that monocytes are the first cell subtype to infiltrate the pancreas in rodent models of PD (8). Moreover, increased immune cell infiltration and monocyctic activity have been reported in diabetic patients (2, 8). However, to the best of our knowledge, there are no studies that have investigated whether diabetic cardiomyopathy is also associated with increased numbers of infiltrated monocytes or their role in the disease.

In this regard, we hypothesized that increased monocyte infiltration would be present along with an increased inflammatory immune response, which contributes to the adverse cardiac remodeling, in streptozotocin (STZ)-induced cardiomyopathy. In addition, the present study was also undertaken to extrapolate the role of bone morphogenetic protein (BMP)-7 in the polarization of proinflammatory monocytes into anti-inflammatory M2 macrophages and the associated inhibition of adverse cardiac remodeling in the PD heart. Our data showed that there were increased numbers of infiltrated monocytes, enhanced proinflammatory cytokine expression, and increased adverse cardiac remodeling in the PD group. Importantly, we suggest that treatment with BMP-7 differentiated monocytes into an enhanced number of M2 macrophages and stimulated the secretion of anti-inflammatory cytokines as well as inhibited oxidative stress in the PD heart. Moreover, our data indicate attenuated cardiomyocyte apoptosis, fibrosis, and improved cardiac function after BMP-7 treatment.

MATERIALS AND METHODS

STZ-induced PD in mice. The Institutional Animal Care Committee of the University of Central Florida reviewed and approved all mice and animal protocols used within the present study as per National Institutes of Health (NIH) guidelines. C57Bl/6 mice (8–10 wk old) were maintained in a controlled environment and given all appropriate nutritional support ad libitum. Mice were separated into three groups consisting of control, PD, and PD + BMP-7 (n = 7–10 animals/group). Animals were administered 50 mg/kg STZ via intraperitoneal injections for 2 days (days 1 and 2) in PD and PD + BMP-7 groups. For the PD + BMP-7 group, animals were additionally treated with an intravenous injection of 200 μg/kg BMP-7, as previously reported, on days 3–5 (26). Moreover, control animals were administered sodium citrate buffer injections on days 1 and 2. Twenty-one days following the last STZ injection, animals were euthanized humanely with 4% isofluorane for 10 min followed by cervical dislocation.

Tissue processing. Hearts from each group were harvested, washed with PBS, and stored in either RNA Later or formalin solutions for future experiments. Formalin-fixed hearts were processed with the Leica TP1020 system and embedded in paraffin wax with a Tissue...
Glucose levels and tolerance. Blood glucose levels were measured 7 and 21 days after the last STZ injection using an OneTouch Ultra Mini glucose meter. Twenty days after STZ, all groups were subjected to a glucose tolerance test. In brief, mice were fasted for 6 h, and their initial blood glucose was measured. An intraperitoneal injection of glucose (1 g/kg) was administered to each mouse, and blood glucose levels were checked via tail vein puncture every 30 min for a total of 120 min postglucose injection.

Immunohistochemistry to identify monocytes, macrophages, and pro- and anti-inflammatory cytokines. Heart sections were deparaffinized and rehydrated as previously described (30). Sections were processed for heat-induced epitope retrieval for 20 min and then blocked with 10% normal goat serum (Vector Labs) for 1 h. Primary antibodies, such as CD14 to identify monocytes, CD206 to identify M2 macrophages, IL-6 and TNF-α to identify proinflammatory cytokines, and IL-10 to identify anti-inflammatory cytokines, were incubated overnight at 4°C. Tissues were washed and incubated with the appropriate secondary antibodies, including goat anti-rabbit Alexa 488, goat anti-rabbit Alexa 568, or goat anti-rat Alexa 594, for 1 h at room temperature. Sections were counterstained with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Labs), and slides were coverslipped. Four images per section were taken for data quantification using an Olympic IX-70 fluorescent microscope, and representative images were captured using confocal microscopy. Cells positive for CD14 and CD206 were counted, divided by the total number of nuclei, and expressed as the percentage of positive cells per section. IL-10, IL-6, and TNF-α intensities were measured, and the average corrected integrated density was calculated for each animal using ImageJ software (NIH).

Determination of pro- and anti-inflammatory cytokines by ELISA. Whole blood was collected from the jugular vein, and serum was separated by centrifugation at 13,000 rpm for 10 min. Commercially available ELISA kits were purchased from Raybiotech. The instructions provided in the kits were strictly followed and used to determine levels of proinflammatory cytokines (TNF-α and IL-6) and anti-inflammatory cytokines [IL-10 and IL-1 receptor antagonist (IL-1RA)].

Determination of oxidative stress by Western blot analysis and dihydroethidium staining. SDS-PAGE and Western blot analysis were used to analyze MnSOD expression and performed as described elsewhere. For dihydroethidium (DHE) staining, heart sections were stained with 1 μM/ml DHE for 25 min as previously described (23). DHE intensity was then measured in 4 random images/section, and the average corrected integrated density was calculated for each section using ImageJ software.

TUNEL staining. TUNEL staining was performed as we have previously reported (23). In brief, heart sections were deparaffinized, rehydrated, and incubated with proteinase K for antigen retrieval. TUNEL staining was performed using a cell death detection kit (Roche) following the manufacturer’s protocol. Sections were then mounted and coverslipped, and 4 images/section were quantified using ImageJ software. TUNEL-positive (red) cells that merged with nuclei (blue) were counted, divided by total nuclei, and multiplied by 100.

Determination of apoptotic proteins by Western blot analysis. Heart samples were prepared, supernatants were collected, and standard SDS-PAGE was performed. β-Actin was used as a loading control. Proteins were transferred onto polyvinylidene difluoride membranes and blocked with 5% milk. Membranes were incubated with primary antibodies [phosphorylated (p-)Akt, total Akt, p-phosphatase and tensin homolog (pPTEN), and total PTEN] for 1 h at room temperature or overnight at 4°C. Membranes were then washed and incubated with secondary antibody (goat anti-rabbit horseradish peroxidase) for 1 h at room temperature. Membranes were then washed, incubated with an enhanced chemiluminescent substrate for 2 min, and exposed at various times. Blots were scanned, and band intensities were measured using ImageJ software.

Masson’s trichrome staining. Sections were stained with Masson’s trichrome as previously described (30). Using ImageJ software, collagen deposition (blue) was measured to quantify the fibrotic area (in mm²). Interstitial fibrosis was measured by quantifying collagen in 6 areas/section, whereas vascular fibrosis was quantified as vessel fibrosis/tot al vessel area × 100 in 6 vessels/section.

Determination of cardiac function. Three weeks after day 5, two-dimensional transthoracic echocardiography was performed with a 5500 Ultrasound System. Using a 15-6L Hockey stick transducer, M-mode images of the left ventricle (LV) were captured and used to calculate end-diastolic volume, end-systolic volume, ejection fraction

Fig. 1. Bone morphogenetic protein (BMP)-7 attenuates prediabetic (PD) hyperglycemia. A and B: Representative blood glucose data at 7 days (A) and 21 days (B) after the last streptozotocin injection. C: Representative graph of glucose tolerance test results at 0, 30, 60, 90, and 120 min postglucose administration. n = 7–10 animals/group. *P < 0.05 vs. the control group; #P < 0.05 vs. the PD group.
(in %), LV internal dimension at diastole, LV internal dimension at systole, and fractional shortening (in %). After the heart function assessment, mice were euthanized with 4% isoflurane followed by cervical dislocation.

**Statistical analysis.** Statistical analyses of all data were performed using one-way ANOVA followed by the Tukey test. Data are presented as means ± SE with P values of <0.05 considered statistically significant.

**RESULTS**

**Increased glucose levels in PD animals.** After STZ injections, glucose levels and tolerance were assessed to ascertain that PD mice had been generated. Blood glucose levels were significantly elevated in the PD group on days 7 and 21 after the last STZ injection compared with control mice (P < 0.05; Fig. 1, A and B). Interestingly, the addition of BMP-7 significantly attenuated the hyperglycemia observed in the PD group (P < 0.05; Fig. 1, A and B). During the glucose tolerance test, the ability of PD mice to clear glucose from blood was significantly impaired relative to the control group at all time points assessed after glucose administration (P < 0.05; Fig. 1C). Although not statistically significant, a trend of decreased hyperglycemia was recorded in the PD + BMP-7 group at 30

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**Fig. 2.** BMP-7 ameliorates inflammation by regulating cytokine expression. **A**: representative photomicrographs of tissue sections stained for CD14, a marker for monocytes, in red (a, f, and k), sarcomeric α-actin in green (b, g, and l), 4′,6-diamidino-2-phenylindole (DAPI) in blue (c, h, and m), and merged images (d, i, and n). White boxes are enlarged in merged images in e, j, and o. Scale bar = 100 μm. n = 4–5 per group. **B**: quantitative analysis of percent monocytes. **C**: representative photomicrographs of tissue sections stained for TNF-α, a proinflammatory cytokine, in red (a, f, and k), sarcomeric α-actin in green (b, g, and l), DAPI in blue (c, h, and m), and merged images (d, i, and n). White boxes are enlarged in merged images in e, j, and o. Scale bar = 100 μm. n = 4–5 per group. **D**: quantitative analysis of TNF-α immunostaining. A.U., arbitrary units. **E**: quantitative analysis of the TNF-α ELISA performed on serum samples. n = 5–7 samples/group. *P < 0.05 vs. the control group; #P < 0.05 vs. the PD group.

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*AJP-Heart Circ Physiol* • doi:10.1152/ajpheart.00367.2014 • www.ajpheart.org
and 60 min postglucose treatment relative to the PD group (Fig. 1C). However, at 90 and 120 min, PD + BMP-7 mice had significantly reduced blood glucose compared with PD mice ($P < 0.05$; Fig. 1C).

**Increased monocyte infiltration and inflammatory cytokines in PD mouse hearts.** To determine whether inflammatory cells have a direct relationship with the PD heart, infiltrated monocytes were quantified. Figure 2A shows representative photomicrographs of heart sections stained with CD14, a marker for monocytes, in red ($a$, $f$, and $k$), cardiac myocytes in green ($b$, $g$, and $l$), nuclei in blue ($c$, $h$, and $m$), merged images ($d$, $i$, and $n$), and enhanced merged images ($e$, $j$, and $o$). Quantitative analyses suggested a significant upregulation of monocytes in the PD group relative to the control group ($P < 0.05$; Fig. 2B). Conversely, monocytes were significantly reduced upon BMP-7 treatment compared with the PD group (PD group vs. BMP-7 group; 0.655 ± 0.185% vs. PD group: 1.759 ± 0.238% for CD14-positive cells/total nuclei; Fig. 2B). Additionally, no significant differences in the monocytic population were observed between control and PD + BMP-7 groups (Fig. 2B).

Next, the consequences of monocyte infiltration and associated proinflammatory cytokines were evaluated. Figure 2C shows representative photomicrographs of heart sections from each group stained with TNF-α ($a$, $f$, and $k$), sarcomeric α-actin ($b$, $g$, and $l$), and DAPI ($c$, $h$, and $m$). Quantification of stained tissues indicated significantly augmented TNF-α levels in the PD group relative to the control group ($P < 0.05$; Fig. 2D). However, treatment with BMP-7 attenuated levels of TNF-α compared with the PD group [PD + BMP-7 group: 3.023 ± 0.367 AU, PD group: 5.495 ± 0.367 AU, $P < 0.05$; Fig. 2D]. Although not statistically significant, similar TNF-α expression patterns resulted via TNF-α ELISA analyses using isolated serum (Fig. 2E).

Levels of the proinflammatory cytokine IL-6 were also measured. Figure 3A shows representative images of heart sections from each group stained with IL-6, sarcomeric α-actin, and DAPI. Quantitative analysis of PD tissues showed upregulated IL-6 levels compared with control tissues ($P < 0.05$; Fig. 3B). Conversely, significantly decreased IL-6 levels in PD + BMP-7 hearts relative to PD hearts were observed (PD + BMP-7 group: 2.984 ± 0.577 AU vs. PD group: 5.495 ± 0.367 AU, $P < 0.05$; Fig. 3B). In addition, an ELISA for IL-6 on serum samples from control and experimental groups was performed, and the results indicated increased levels of IL-6 in the PD group compared with the control group, whereas the PD + BMP-7 group had significantly decreased levels compared with the PD group ($P < 0.05$; Fig. 3C). Notably, no statistical deviation in IL-6 expression was noted between control and PD + BMP-7 groups (Fig. 3, B and C).

**BMP-7 augments M2 macrophages and reduces inflammation in the PD heart.** To elucidate the mechanisms by which BMP-7 promotes beneficial effects in the PD heart, M2 macrophage and associated anti-inflammatory cytokine expression were evaluated. Representative images of heart sections stained with CD206, an M2 macrophage marker, sarcomeric α-actin, and DAPI are shown in Fig. 4A. Importantly, no differences in M2 macrophage concentration were observed between control and PD groups. However, the results showed significantly elevated M2 macrophages in PD + BMP-7 hearts relative to PD hearts, suggesting that BMP-7 may play a role in the monocytic to M2 macrophage polarization (PD + BMP-7 group: 2.005 ± 0.131% vs. PD group: 1.253 ± 0.175% for CD206-positive cells/total nuclei, $P < 0.05$; Fig. 4B).

Previous data have suggested M2 macrophages secrete anti-inflammatory cytokines (16, 18). To this end, levels of IL-1RA and IL-10, critical mediators of inflammation and immuno-
regulation, were evaluated. Quantitative analysis of the IL-1RA concentration in serum samples insinuated a significant decrease in the PD group ($P < 0.05$; Fig. 4C), which was considerably increased upon BMP-7 treatment ($P < 0.05$; Fig. 4C). IL-10 analyses performed on heart sections stained with IL-10, sarcomeric $\alpha$-actin, and DAPI (Fig. 4D) suggested an observable decrease in IL-10 levels in the PD group relative to the control group, which was significantly increased upon treatment with BMP-7 (Fig. 4E). To corroborate immunocytochemistry data, an ELISA for IL-10 was performed on serum samples. Coinciding with previous data, significantly reduced levels of IL-10 in the PD group compared with the control group and dramatically elevated IL-10 concentrations upon BMP-7 treatment were noted ($P < 0.05$; Fig. 4F).

**BMP-7 ameliorates cardiac oxidative stress by upregulating MnSOD.** A previous study (33) has shown that BMP-7 may act as an antioxidant by upregulating levels of MnSOD. To determine the effects of BMP-7 on oxidative stress in the PD heart,
superoxide anion production was assessed. Figure 5A shows representative photomicrographs of heart tissue stained with DHE in red (a, d, and g), DAPI in blue (b, e, and h), and merged images in pink (c, f, and i). Analysis revealed that PD hearts had significantly increased ROS relative to control hearts ($P < 0.001$; Fig. 5B). Conversely, BMP-7 administration dramatically reduced levels of ROS compared with the PD group (PD + BMP-7 group: 3.667 ± 0.333 AU vs. PD group: 8.167 ± 0.872 AU, $P < 0.001$; Fig. 5B). MnSOD expression was also quantified through Western blot analysis with representative blots and β-actin controls shown in Fig. 4C. Densitometric analysis indicated decreased expression of MnSOD in the PD group relative to the control group ($P < 0.05$; Fig. 5C). However, treatment with BMP-7 blunted the diminished MnSOD expression compared with the PD group (PD + BMP-7 group: 0.888 ± 0.0608 AU vs. PD group: 0.630 ± 0.0550, $P < 0.05$; Fig. 5C).

**BMP-7 inhibits apoptosis in PD mice by regulating PTEN and Akt.** To determine the effects of BMP-7 treatment on cardiomyocyte apoptosis in vivo, TUNEL staining was performed. Figure 6A shows representative photomicrographs of heart sections from each group stained with TUNEL in red (a, d, and g), total nuclei in blue (b, e, and h), and merged images in pink (c, f, and i). The data indicated increased apoptosis in PD hearts relative to control hearts ($P < 0.001$; Fig. 6B). Moreover, analysis revealed a significantly decreased percentage of apoptotic nuclei in the PD + BMP-7 group relative to the PD group (PD + BMP-7 group: 0.286 ± 0.0442% vs. PD group: 0.505 ± 0.0369% for TUNEL-positive nuclei/total nuclei, $P < 0.001$; Fig. 6B). To demonstrate that apoptosis occurred in the cardiomyocytes, sections were stained with TUNEL (red), sarcomeric α-actin (green), caspase-3 (pink), and DAPI (blue), and representative images from the PD group are shown in Fig. 6C.

To elucidate the mechanisms of the blunted apoptosis noted in the PD + BMP-7 heart, the expression of PTEN, an inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt activation, and Akt, a prosurvival and cell cycle regulator, were evaluated. Densitometric analysis of p-PTEN Western blot bands revealed a significant increase in the expression of activated PTEN in the PD group relative to the control group ($P < 0.05$; Fig. 6D). Notably, a significant decrease in p-PTEN expression was observed in the PD + BMP-7 group relative to the PD group (PD + BMP-7 group: 0.784 ± 0.123 AU vs. PD group: 1.305 ± 0.134 AU, $P < 0.05$; Fig. 6D). Inversely proportional to the p-PTEN data, significantly diminished levels of p-AKT in the PD group relative to the control group ($P < 0.05$) and considerably upregulated levels of the survival protein in the PD + BMP-7 group compared with the PD group were found (PD + BMP-7 group: 0.715 ± 0.0784 AU vs. PD group: 0.500 ± 0.107 AU, $P < 0.05$; Fig. 6E).

**BMP-7 ameliorates cardiac remodeling in PD mice.** To assess the influence of BMP-7 on fibrosis formation in the PD heart, heart tissue from each of the study groups was analyzed for collagen deposition using Masson’s trichrome staining. Figure 7A shows representative photomicrographs from control and experimental groups demonstrating interstitial fibrosis (a–c) and vascular fibrosis (d–f). Quantitative analyses suggested that interstitial fibrosis was dramatically increased in the PD group relative to the control group ($P <
However, after BMP-7 treatment, interstitial fibrosis was significantly blunted relative to the PD group (PD/BMP-7 group: 0.110 ± 0.0222 mm² vs. PD group: 0.170 ± 0.0159 mm², P < 0.05; Fig. 7B). Concurrent with the interstitial fibrosis data, vascular fibrosis in the PD group was significantly elevated relative to the control group (P < 0.001; Fig. 7C), whereas the addition of BMP-7 significantly abrogated vascular collagen deposition in the PD group (P < 0.05; Fig. 7C).

**BMP-7 improves cardiac function in PD mice.** Three weeks after day 5, mice underwent transthoracic echocardiography to assess cardiac function. The raw echocardiogram data obtained are shown in Fig. 8A. The data suggested impaired LV function in the PD group as demonstrated by deviated LV internal dimension at diastole, LV internal dimension at systole, end-diastolic volume, end-systolic volume, ejection fraction, and fractional shortening compared with the control group (P < 0.001; Fig. 8B). More importantly, a

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**Fig. 6.** BMP-7 attenuates apoptosis in the PD heart by regulating phosphatase and tensin homolog (PTEN) and Akt. **A**: representative photomicrographs of heart sections demonstrating TUNEL-positive cells (apoptotic nuclei) stained in red (a, d, and g), DAPI in blue (b, e, and h), and merged images (c, f, and i). Scale bar = 100 µm. n = 5–6 samples/group. **B**: quantitative analysis of percent apoptotic nuclei. **C**: representative photomicrographs demonstrating that apoptosis occurs in cardiomyocytes with TUNEL-positive cells in red (a), sarcomeric α-actin in green (b), caspase-3 in purple (c), DAPI in blue (d), merged image (e), and enlarged merged image (f). **D**: representative bands and quantitative Western blot analysis of phosphorylated (p-)PTEN and total PTEN expression. n = 5–6 samples/group. **E**: representative bands and quantitative Western blot analysis of p-Akt and total β-actin expression. n = 5–6 samples/group. *P < 0.05 vs. the control group; #P < 0.05 vs. the PD group.

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**Exogenous BMP-7 improves cardiac function in PD mice.** Three weeks after day 5, mice underwent transthoracic echocardiography to assess cardiac function. The raw echocardiogram data obtained are shown in Fig. 8A. The data suggested impaired LV function in the PD group as demonstrated by deviated LV internal dimension at diastole, LV internal dimension at systole, end-diastolic volume, end-systolic volume, ejection fraction, and fractional shortening compared with the control group (P < 0.001; Fig. 8B). More importantly, a
significant improvement in cardiac function was achieved after BMP-7 administration as indicated by augmented fractional shortening and ejection fraction relative to the PD group ($P < 0.05$; Fig. 8).

**DISCUSSION**

Monocytes, during pathological circumstances, migrate from the circulation and infiltrate tissues, where they differentiate into macrophages. Monocyte to macrophage polarization and differentiation yields two distinct phenotypes, including M1 and M2 macrophages. M1 macrophages are proinflammatory and heighten the inflammatory response, whereas M2 macrophages are anti-inflammatory and express cytokines that mitigate inflammation (16). Several clinical studies (2, 22, 24) have shown increased levels of monocytes and proinflammatory cytokines, TNF-$\alpha$ and IL-6, in PD patients. Additional evidence further suggests that proinflammatory cytokines promote cardiomyocyte apoptosis (11), which inevitably leads to cardiac remodeling and depressed cardiac function. However, no animal model has yet been used to examine monocyte infiltration and associated inflammation in the PD heart, nor has any attempt been made to control monocyte polarization. Of particular interest, BMP-7, a growth factor belonging to the transforming growth factor-$\beta$ superfamily, has been recently reported to alleviate inflammation after administration (6, 13). However, whether BMP-7 plays a role in monocyte polarization and differentiation and the subsequent effects on adverse cardiac remodeling in the PD heart remains elusive. To this end, a PD mouse model was generated using multiple low-dose injections of STZ (50 mg/kg) as previously reported (25, 34), and the impact of BMP-7 on inflammation, apoptosis, fibrosis, and LV output in the diabetic heart was investigated.

Concurrent with previous studies (2, 8) showing increased monocyte infiltration and monocyte activity in patients with PD, monocyte infiltration was significantly increased in the hearts of PD mice, as evidenced by CD14 staining. Such findings, indicating increased cardiac monocyctic populations, are unique in that no study has previously identified these specific cell types infiltrating the myocardium and residing in the vicinity of cardiomyocytes. Additionally, a correlation between monocyte infiltration and levels of proinflammatory cytokines was confirmed in PD mice by the presence of increased levels of TNF-$\alpha$ and IL-6 in heart tissue as well as in serum samples. These data are in agreement with clinical reports (22, 24) that have shown elevated levels of proinflammatory cytokines in serum samples of PD patients. Upon treatment with BMP-7, the cardiac monocytes in PD mice were dramatically decreased, which was observed with a concomitant decrease in levels of the proinflammatory cytokines TNF-$\alpha$ and IL-6 (10). The anti-inflammatory effects of BMP-7 found in the present study correlate with a report by Gould et al. (13), which showed that treatment with BMP-7 inhibited proinflammatory cytokines in proximal tubular epithelial cells. The evidence provided suggest potential cardioprotective effects of BMP-7 against inflammation by directing the secretion of anti-inflammatory cytokines.

The data suggest that the ratio of M1 to M2 macrophage polarization after monocyte differentiation may determine the severity and progression of inflammation (12, 20, 29). M2 macrophages are a class of macrophages that reduce inflammation by secreting IL-10, an anti-inflammatory cytokine (16). Specifically, IL-10 has been reported to modulate inflammation by regulating the secretion pattern of proinflammatory cytokines from inflammatory cells (21). In the present study, we provide evidence showing that infiltrated monocytes, in the presence of BMP-7, polarize into beneficial M2 macrophages. Anti-inflammatory effects of BMP-7 have been previously reported (13). In a previous study (26), we showed that monocytes express receptors for BMP-7 and, in the presence of BMP-7, differentiate into beneficial M2 macrophages in vitro.
Previous reports (27, 32) have supported that BMP-7 promotes the differentiation of mesenchymal stem cells and white adipocytes into brown adipose tissue. Parallel to increased M2 macrophage concentrations, BMP-7 administration significantly enhanced IL-10 secretion. It has previously been reported that IL-10 can mediate TNF-α-induced cardiomyocyte apoptosis (11). As we also observed downregulated levels of TNF-α upon BMP-7 treatment, it is plausible to presume BMP-7 inhibits TNF-α-induced apoptosis in PD cardiomyopathy by upregulating IL-10. In addition, increased levels of another anti-inflammatory cytokine, IL-1RA, were observed in the BMP-7 treatment group. Collectively, the assimilated data suggest that although monocytic infiltration is inevitable in the PD heart, BMP-7 confers cardioprotection by directing monocyte differentiation into M2 macrophages and promoting the secretion of anti-inflammatory mediators.

It is widely accepted that diabetes results in cardiac oxidative stress. In fact, a previous study (23) has indicated elevated levels of ROS in the STZ-induced diabetic heart. Similarly, increased levels of ROS and blunted expression of the antioxidant MnSOD were detected in PD hearts relative to control hearts within the present study. Notably, we showed that treatment with BMP-7 increased cardiac MnSOD expression and reduced oxidative stress in the PD heart. Our results align with those of a study by Xu et al. (33), which demonstrated that cardiomyocytes transfected with BMP-7 had increased levels of MnSOD. Whether the diminished oxidative stress noted in the PD + BMP-7 group is consequent with attenuated ROS levels and upregulated antioxidants alone remains obscure. However, our data suggest that BMP-7-conferred PD cardioprotection is a multifactorial chain of events.

After inflammation and oxidative assault, adverse cardiac remodeling, including cardiomyocyte apoptosis, has been reported in disease-state myocardium. Specifically, clinical and animals studies have reported increased cardiomyocyte apoptosis in diabetic patients (7) as well as in STZ models of diabetes (17). In corroboration with these previously published reports, our study showed increased cardiomyocyte apoptosis in the PD heart. Moreover, BMP-7 administration significantly inhibited cardiomyocyte apoptosis in the PD heart, which correlates with a previous study (14) suggesting BMPs inhibit apoptosis of cardiomyocytes in vitro.

To elucidate the mechanisms of cardiomyocyte apoptosis in the PD heart, expression levels of activated PTEN and Akt were quantified. PTEN is a tumor suppressor that regulates the prosurvival protein Akt through inhibition of its upstream activator, PI3K. Densitometric analyses indicated elevated p-PTEN expression in PD mouse hearts relative to control mouse.
hearts with a concomitant downregulation in p-AKT. Our data are concurrent with a previous report (15) that showed a downregulation of p-AKT in STZ models of diabetes. Moreover, a previous study (28) reported that overexpression of PTEN encourages cardiomyocyte apoptosis. Importantly, as shown here for the first time, BMP-7 inhibits the expression of cardiac p-PTEN while significantly upregulating p-Akt in the PD heart. Collectively, our data suggest the presence of enhanced apoptosis in the monocytic-infiltrated PD myocardium is consequent to augmentation of normal PI3K/Akt signaling, which was ameliorated with BMP-7.

As well established within the research community, significant cardiac apoptosis promotes collagen deposition and fibrosis formation in an attempt to rescue the indigenous architecture and integrity of the heart. Quantitative analysis revealed significantly elevated interstitial and vascular fibrosis in PD hearts relative to control hearts. The aforementioned data are in conjunction with a previous report (9) that suggested that cardiac remodeling begins as early as the PD stage. Of particular interest, BMP-7 treatment resulted in decreased cardiac remodeling compared with the PD group. Antifibrotic effects of BMP-7 have been previously reported in the diabetic kidney (5). However, the present study is the first to report the antifibrotic effects of BMP-7 in the PD heart.

A previous study (19) has implied that cardiac dysfunction can occur during PD. Our data correlate with this report and demonstrated decreased cardiac function in the PD group, which was significantly improved upon treatment with BMP-7. Although causes of cardiac dysfunction are complex, it is conceivable that the decreased apoptosis and fibrosis presented in this study contributed to the improvement in cardiac function in the BMP-7 treatment group.

In conclusion, the data presented in this study show, for the first time, that the PD myocardium is infiltrated with monocytes and subsequent secreted proinflammatory cytokines. Notably, in the presence of BMP-7, monocytes polarize into beneficial M2 macrophages, thereby inhibiting inflammation and protecting the PD heart from cardiomyocyte apoptosis, fibrosis, and cardiac dysfunction. We strongly suggest that the present study opens many new avenues for research to elucidate the following questions that remain. First, does monocyte infiltration and the subsequent proinflammatory cytokines release play a key role in PD conditions for the development and progression of diabetic cardiomyopathy? Second, are the inflammatory factors released by infiltrated monocytes the only players in inflammation leading to diabetic cardiac dysfunction, or do other inflammatory cytokines released by fibroblasts contribute to adverse outcomes? Three, under BMP-7, M2 macrophage populations are enhanced in the diabetic heart; is this consequent to direct monocyte to M2 macrophage polarization, or is it consequent to an M1/M2 paradigm shift? Finally, is the adverse cardiac remodeling inhibition after BMP-7 administration a chain reaction of events (i.e., a decrease of monocytes results in fewer proinflammatory cytokines, which blunt oxidative stress, apoptosis, and fibrosis), or does BMP-7 act on each property independently?

**Study limitations.** Within the context of the present study, the data suggest decreased glucose levels and enhanced M2 macrophage polarization after BMP-7 treatment in PD mice with consequential augmented cardiac architecture and function. However, the present study did not include the use of a glycemic control group in which hyperglycemia was exogenously regulated in STZ-treated mice. With this in mind, whether M2 macrophage polarization is directly consequential to BMP-7 treatment or a result of improved glucose homeostasis conferred by BMP-7 remains unknown. The question may be addressed by including a glycemic control group in a followup investigation using metformin or insulin to regulate STZ-induced glycemic dysregulation and may identify the exact mechanisms by which the monocyte to M2 polarization occurs after BMP-7 treatment in the PD heart.

**GRANTS**

This work was supported in part by National Heart, Lung, and Blood Institute Grants RO1-HL-090646, HL-117241, and 1-R01-HL117241-01A1 (to D. K. Singla).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: P.U. performed experiments; P.U. analyzed data; P.U. prepared figures; P.U. drafted manuscript; D.S. conception and design of research; D.S. interpreted results of experiments; D.S. approved final version of manuscript.

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