BMP-2 regulates cardiomyocyte contractility in a phosphatidylinositol 3 kinase-dependent manner

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Abstract Bone morphogenetic protein-2 (BMP-2) regulates development of heart during vertebrate embryogenesis. In vitro BMP-2 induces differentiation of precardiac cells into mature cardiomyocytes by inducing the expression of cardiac-specific genes. However, the role of BMP-2 and its signaling in other cardiac functions have not been studied. We examined the action of phosphatidylinositol (PI) 3 kinase in isolated adult rat cardiomyocytes. Incubation of rat ventricular cardiomyocytes with BMP-2 increased the PI 3 kinase activity. Ly294002, a pharmacological inhibitor of PI 3 kinase, blocked BMP-2-induced PI 3 kinase activity completely. To investigate the contractility of isolated cardiomyocytes, fractional shortening was examined. BMP-2 significantly increased the percent fractional shortening of the cardiomyocytes. Inhibition of PI 3 kinase activity completely abolished this action of BMP-2. These data indicate that PI 3 kinase regulates BMP-2-induced myocyte contractility. To further confirm this observation, we used adenovirus-mediated gene transfer to express a constitutively active myristoylated catalytic subunit of PI 3 kinase in rat cardiomyocytes. Infection of cardiomyocytes with the adenovirus vector increased the expression of constitutively active PI 3 kinase within 24 h. Expression of constitutively active PI 3 kinase significantly increased cardiomyocyte contractility. Together, these data show for the first time that the growth and differentiation factor, BMP-2, stimulates cardiomyocyte contractility. Also we provide the first evidence that BMP-2-induced PI 3 kinase activity regulates this cardiomyocyte function.

Key words: Cardiomyocyte contraction; Bone morphogenetic protein-2; Phosphatidylinositol 3 kinase

1. Introduction

Bone morphogenetic proteins (BMPs) constitute a subclass of the TGFβ super family of proteins and were originally identified in the extracts of demineralized bone [1]. BMP-2, a member of this family of protein, similar to its *Drosophila* homolog *dpp*, is an important growth and differentiation factor, which regulates differentiation of cardiogenic mesoderm in vertebrates [2]. Homozygous inactivation of BMP-2 allele in mouse results in embryonic lethality due to abnormal heart development [3]. Similar to the functional role of BMP-2 in vivo, it has been demonstrated that this growth and differentiation factor induces differentiation of P19 teratocarcinoma cells into mature cardiomyocytes with concomitant expression of cardiac-specific transcription factors [4].

BMP-2 exerts its biological effects via type I and type II serine threonine kinase receptors [5,6]. Binding of BMP-2 to the receptor induces oligomerization of the receptor complexes resulting in increased serine threonine kinase activity of the type I receptor, which recruits and phosphorylates the BMP receptor-specific R-Smads, Smad 1, Smad 5 and Smad 8 [5–7]. Upon phosphorylation, the R-Smad heterodimerizes with co-Smad, Smad 4 and translocates to the nucleus, where it associates with tissue-specific transcription factors and co-activators and acts as a transcription factor to induce specific genes required for the biological activity of BMP-2 [5–9]. BMP-2-soaked beads when implanted into non-precardiac mesoderms in chicks induced cardiac-specific transcription factors Nkx2.5 and GATA-4 [10]. Furthermore, induction of these transcription factors was also observed during BMP-2-induced differentiation of precardiac cells into mature cardiomyocytes [4]. It is established that major signal transduction pathways used by BMPs to induce tissue-specific genes are mediated by Smads [8,11]. However, BMP-2 has been reported to activate mitogen-activated protein kinase (MAPK) pathways including Erk1/2 and p38 MAPK [12]. We have recently shown that in preosteoblasts, BMP-2 stimulates phosphatidylinositol (PI) 3 kinase, as one of the contributors of many signaling cascades, that regulates differentiation of these cells into mature osteoblasts [13].

Heart failure is a result of multiple positive and negative signaling pathways, which regulate physiologically complex biological responses including myocytes contractility [14]. Thus one of the major causes of heart dysfunction is the loss of responsiveness of myocytes to contractility [15]. Although a role of BMP-2 and BMP-specific Smads in cardiac development in vertebrate and cardiomyocyte differentiation in vitro has been established, its role in other cardiomyocyte function has not been investigated. In this study, we show that BMP-2 increases PI 3 kinase activity in isolated rat ventricular cardiomyocytes. We demonstrate, for the first time, that BMP-2 stimulates myocyte fractional shortening. Further-

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more, we provide the first evidence that PI 3 kinase regulates BMP-2-induced myocyte shortening.

2. Materials and methods

2.1. Materials

Tissue culture reagents were obtained from Life Technologies, Inc. Recombinant BMP-2 was obtained from Genetics Institute, Cambridge, MA, USA. NP-40, Na_3VO_4, phenylmethylsulfonylfluoride and actin antibody were purchased from Sigma. Aprotinin was obtained from Bayer. Antibody against the p85 subunit and p110 subunit of PI 3 kinase were obtained from Santa Cruz Biotechnology. Ad Myr-p110 adenovirus vector expressing constitutively active p110 catalytic subunit of PI 3 kinase was a kind gift from Dr. Wataru Ogawa, Kobe University, Japan.

2.2. Cell culture

Calcium-tolerant myocytes were isolated from male WKY rats (~200 g) using the method of Piper et al. with modifications [16–18]. Briefly, after deep anesthesia hearts were rapidly removed and mounted via the aorta onto a cannula attached to a Langendorff-type apparatus allowing retrograde perfusion of the coronary arteries. Hearts were perfused for 5 min with 37°C sterile-filtered calcium-free Krebs–Ringers–bicarbonate buffer (KRB; 110 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose and 30 mM 2,3-butanedione monoxime) at 80 mm Hg. Hearts were then perfused for 15–20 min with KRB enzyme solution containing 0.5 mg/ml type II collagenase, 25 mM $CaCl_2$ and 1 mg/ml fatty acid-free albumin. After digestion, the ventricles were trimmed free and minced in KRB enzyme solution containing 1 mg/ml albumin. Tissues were then filtered through a nylon mesh and sedimented by centrifugation at 25×*g* for 90 s. Cells were resuspended in 10 ml pre-warmed, gas-equilibrated KRB containing 25 μM calcium, and allowed to sediment by gravity for 15 min. This step was repeated with KRB containing 50, 100, 250 and 500 μM calcium. Cells were then incubated on 100 mm polystyrene culture dishes for 2 h at 37°C with 5% CO_2 atmosphere with a 1:1 mixture of KRB with 500 μM calcium and ACM medium (M199 medium with 0.5% bovine serum albumin (BSA)). Non-adherent cells were removed, washed and replated in laminin-coated T_{25} culture flasks with ACM medium containing 4% fetal bovine serum. The myocytes were then replated at 250 000 cells/T_{25} flask in fresh M199 medium containing 0.5% BSA.

2.3. Adenovirus infection

Ventricular cardiomyocytes were infected with Ad Myr-p110 at 50 MOI (multiplicity of infection), essentially as described [13,19].

2.4. Immunoprecipitation, immunoblotting and PI 3 kinase assay

Cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 0.05% aprotinin and 1% NP-40). Cleared cell lysates were prepared by centrifugation at 10 000×*g* for 30 min at 4°C to separate the debris. Protein concentration was determined in the lysate. An equal amount of protein was immunoprecipitated with p85 regulatory subunit of PI 3 kinase as described [13,19]. The immunoprecipitates were used in immunocomplex-associated PI 3 kinase assay using PI as substrate in the presence of [γ-^{32}P]ATP. The reaction products were separated by thin layer chromatography. ^{32}P-labeled PI 3-phosphate was visualized by autoradiography as described [20,21]. Immunoblotting of the lysates was performed using appropriate antibodies as described previously [13,19].

2.5. Measurement of myocyte shortening

Contractile properties of isolated ventricular myocytes were assessed by measuring fractional shortening of single cell cardiomyocyte. The coverslips with attached cells were placed in a temperature-controlled chamber at 37°C and superfused with buffer containing 100 ng/ml BMP-2. Myocytes were field-stimulated with 5 ms pulses at 0.5 Hz. Cardiomyocyte shortening and relengthening was measured on the stage of an inverted phase-contrast microscope (Nikon Eclipse TE 200) using an optical video system in which the myocyte analog motion signal was digitized and analyzed by a computer. Measurements were performed on three to five individual cardiomyocytes in each experimental group and represented as mean percent fractional shortening.

2.6. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical significance was assessed by Student's unpaired *t*-test. Significance was determined as probability (*P*) value less than 0.05.

3. Results

3.1. BMP-2 stimulates PI 3 kinase activity in cardiomyocytes

To investigate the role of BMP-2 in cardiomyocyte function, we isolated cardiomyocytes from an adult rat. These freshly prepared cells maintain normal signaling responses including contraction characteristics upon electrical stimuli, thereby demonstrating Ca^{2+}-dependent excitation–contraction coupling [16,22]. To examine PI 3 kinase activity, ventricular myocytes were incubated with BMP-2. Cell lysates were immunoprecipitated with an antibody that recognizes the regulatory p85 subunit of PI 3 kinase. The immunoprecipitates were used in immunocomplex PI 3 kinase assay. Appreciable basal PI 3 kinase activity was observed in these ventricular myocytes (Fig. 1, lane 1). However, BMP-2 significantly increased the PI 3 kinase activity (Fig. 1, compare lane 2 with lane 1). Treatment of ventricular cardiomyocytes with the pharmacological inhibitor of PI 3 kinase, Ly294002, followed by incubation with BMP-2 completely blocked BMP-2-induced PI 3 kinase activity (Fig. 1, compare lane 4 with lane 2).

3.2. BMP-2 regulates cardiomyocyte contractility

One of the physiological responses of cardiomyocytes is to exhibit shortening and relengthening upon activation. In many cardiovascular diseases, myocyte dysfunction is due to lack of shortening. We examined the effect of BMP-2 on shortening of isolated ventricular cardiomyocytes. The cells were incubated with BMP-2 and cardiomyocyte shortening was measured using a phase-contrast microscope attached to

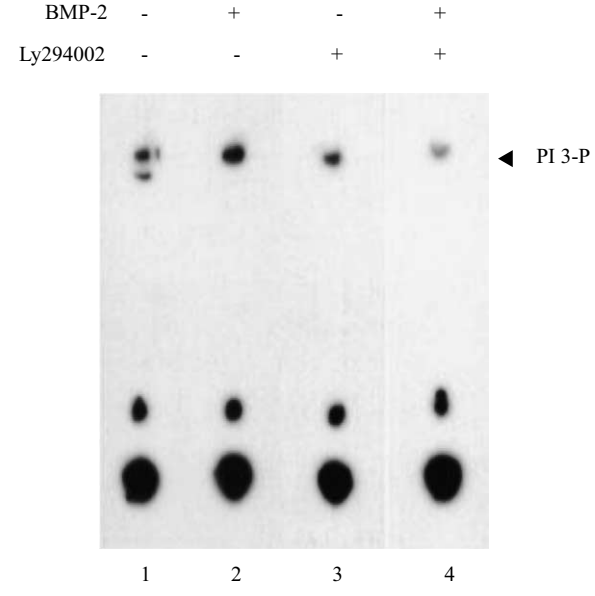

Fig. 1. Effect of BMP-2 on PI 3 kinase activity. Rat ventricular myocytes were prepared as described in Section 2. Cardiomyocytes were treated with 12.5 μM Ly294002 for 1 h before incubation with BMP-2 for 15 min. Cleared cell lysates were immunoprecipitated with anti-p85 regulatory subunit antibody of PI 3 kinase. Immunocomplex PI 3 kinase assay was performed as described in Section 2. The arrow indicates the position of PI 3-phosphate.

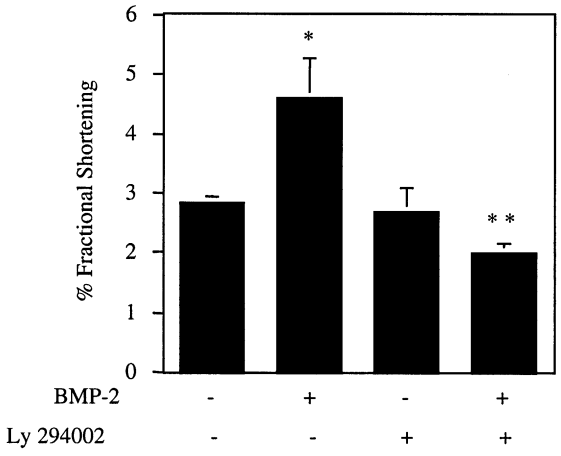

Fig. 2. Inhibition of PI 3 kinase blocks fractional shortening of cardiomyocytes. Rat ventricular myocytes were treated with Ly294002 before incubating with BMP-2. The myocyte shortening was measured as described in Section 2. *$P < 0.05$ vs. BMP-2 unstimulated. **$P < 0.05$ vs. BMP-2 alone.

an optical video system in which the cardiomyocyte motion was analyzed by a computer. BMP-2 significantly increased the fractional shortening (Fig. 2). These data for the first time demonstrate that along with the established cardiogenic role of BMP-2, this growth and differentiation factor also plays an important role in myocyte shortening.

3.3. PI 3 kinase regulates cardiomyocyte shortening

To examine the role of PI 3 kinase in BMP-2-induced cardiomyocyte shortening, we used the PI 3 kinase inhibitor Ly294002, which abolished BMP-2-induced PI 3 kinase activity completely (Fig. 1). Isolated rat cardiomyocytes were treated with Ly294002 before incubation with BMP-2. The fractional shortening was determined in these cells. Inhibition of PI 3 kinase significantly prevented BMP-2-induced fractional shortening (Fig. 2). These data indicate that PI 3 kinase regulates BMP-2-induced shortening of cardiomyocytes. To confirm the involvement of PI 3 kinase, we used myristoylated p110 catalytic subunit of PI 3 kinase (Myr-p110), which acts as a constitutively active kinase. An adenovirus vector expressing Myr-p110 (Ad Myr-p110) was used to infect rat ventricular cardiomyocytes for 24 h. Immunoblot analysis of cell lysates using anti-p110 antibody showed high basal expression of the PI 3 kinase catalytic subunit (Fig. 3A, lane 1). However, infection of Ad Myr-p110 yielded appreciable expression of the constitutively active catalytic subunit at 24 h (Fig. 3A, compare lane 2 with lane 1). Myocyte shortening was measured in these adenovirus vector-infected primary cardiomyocytes. Expression of the constitutively active PI 3 kinase catalytic subunit significantly increased fractional shortening (Fig. 3B). These data provide the first evidence that PI 3 kinase regulates BMP-2-induced cardiomyocyte contractility.

4. Discussion

A significant finding of this study is that BMP-2 activates PI 3 kinase activity in cardiomyocytes. We demonstrate for the first time that the cardiogenic growth and differentiation factor BMP-2 stimulates cardiomyocyte shortening. Furthermore, we provide the first evidence that PI 3 kinase signaling regulates BMP-2-induced shortening of cardiomyocytes.

One major biological function of BMP-2 in vertebrates is cardiogenesis during embryonic development [3]. Also BMP-2 acts as survival factor for neonatal cardiac myocytes via Smad 1, the downstream target of BMP receptor [23]. This action of BMP-2 was ascribed to the antiapoptotic function BMP-2 [23]. Apart from this, any other function of BMP-2 in cardiac function has not been described. Here we demonstrate for the first time that BMP-2 increases cardiomyocyte shortening (Fig. 2).

PI 3 kinase functions as a focal point in receptor and non-receptor tyrosine kinase- and G-protein-coupled receptor-mediated signal transduction resulting in various cell functions, which include proliferation, secretion, migration, survival, differentiation and membrane ruffling [24,25]. Also PI 3 kinase has been implicated in several TGFβ-induced biological activities, which include epithelial and endothelial cell survival and in epithelial to mesenchymal transition [26–28]. These findings indicate that activation of serine threonine kinase receptor also utilizes this central lipid kinase pathway. Indeed we have recently shown that BMP-2 receptor stimulation results in activation of PI 3 kinase in preosteoblasts. Also we showed that PI 3 kinase signaling regulates differentiation of osteoblasts [13]. Now we show that activation of BMP-2 receptor serine threonine kinase in cardiomyocytes activates PI 3 kinase (Fig. 1).

A role of PI 3 kinase has been implicated in cardiac hyper-

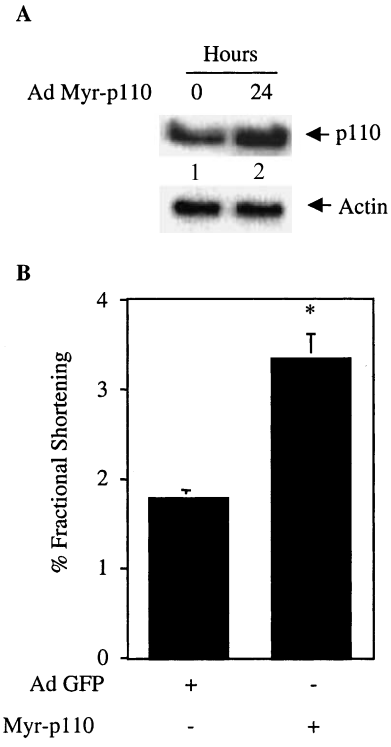

Fig. 3. Constitutively active PI 3 kinase regulates cardiomyocyte contractility. A: Expression of constitutively active PI 3 kinase in cardiomyocytes. Ventricular cardiomyocytes were infected with 50 MOI of Ad Myr-p110 for 24 h as described [13,19]. The cleared cell lysates were immunoblotted with anti-p110 antibody. The bottom panel shows immunoblot analysis of the samples with actin antibody. B: Constitutively active PI 3 kinase increases fractional shortening of the cardiomyocytes. Ventricular cardiomyocytes were infected with Ad Myr-p110 or with a control virus Ad GFP for 24 h. The shortening of the cardiomyocytes was measured as described in Section 2. *$P < 0.05$ vs. Ad GFP-infected cells.

trophy induced by various agonists [29–32]. Moreover, PI 3 kinaseγ, the specific isotype activated by G-protein-coupled receptors, is activated in pressure overload-induced hypertrophy in vivo, indicating a role of this isotype in agonist-induced cardiac hypertrophy [33]. More recently Crackower et al. demonstrated that PI 3 kinaseγ negatively regulates myocytes contractility in the absence of exogenous agonists [34]. However, we present the first evidence that PI 3 kinase positively regulates BMP-2-induced shortening of cardiomyocytes (Figs. 2 and 3). Multiple targets of PI 3 kinase have been identified including phosphoinositide-dependent kinase 1, Akt, MEK and novel and atypical forms of protein kinase C [19,35–39]. In cardiomyocytes the target of PI 3 kinase that modulates shortening has not yet been identified.

The osteogenic function of BMP-2 in bone fracture and osteoporosis has been proposed. During the course of many heart diseases, progressive decrease in myocyte contractility ultimately leads to chronic heart failure. Our finding that BMP-2 stimulates myocytes shortening indicates that this growth and differentiation factor may be used as therapeutic molecule in restoring cardiomyocyte function where mechanical contractility is the cause of reduced heart function.

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