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Original article

Cross-talk between bone morphogenetic protein 2 and leukemia inhibitory factor through ERK 1/2 and Smad1 in protection against doxorubicin-induced injury of cardiomyocytes

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Abstract

The survival of cardiomyocytes is regulated by growth factors and cytokines such as bone morphogenetic protein (BMP) 2 and leukemia inhibitory factor (LIF). BMP2 and LIF induce distinct signal transduction pathways that each activate a different transcription factor [Smad1 and signal transducing activating transcriptional factor (Stat) 3, respectively] and common signal pathway [mitogen-activated protein kinase (MAPK)]. We previously demonstrated that BMP2 and LIF protect cardiomyocytes via Smad1 and STAT3 signaling pathways, respectively. On the other hand, these signals are known to act in synergy via synergistic integration of signaling pathways. Here, we examined interaction between BMP2 and LIF in primary cultured neonatal rat cardiomyocytes. LIF sustained phosphorylation/activation of Smad1 by BMP2. The role of extracellular signal-regulated kinase (ERK) 1/2 cascade activated by LIF was highlighted by the use of a MAPK/ERK kinase (MEK) 1/2 inhibitor, U0126, or overexpression of dominant-negative form of MEK1 that abolished sustained phosphorylation of Smad1 and cell survival effect induced by co-stimulation of LIF with BMP2, while BMP2 alone did not activate ERK1/2. Conversely, overexpression of the constitutive-active form of MEK1 increased BMP2-induced phosphoration of Smad1 without additional LIF. Moreover, BMP2 and LIF synergistically induced bcl-xL mRNA in doxorubicin (DOX)-injured cardiomyocytes. These findings suggest that the ERK1/2 pathway downstream of LIF is involved in sustained phosphorylation/activation of Smad1 by BMP2 and provide a possible mechanism for cooperation between intracellular signals activated by LIF and BMP2 in protection against DOX-induced injury of cardiomyocytes.

Keywords: Bone morphogenetic protein 2 (BMP2); Leukemia inhibitory factor (LIF); Smad1; Signal transducing activating transcriptional factor (Stat) 3; Mitogen-activated protein kinase (MAPK); doxorubicin

1. Introduction

Cardiovascular receptor signaling involves a maze of effects comprising several receptor pathways that are tightly coordinated and perfectly synchronized to ensure harmony, integrity and continuity of this vital function. The signaling pathways involved in the regulation of cardiac function are extremely diverse and range from direct programs, such as the adrenoreceptor and angiotensin pathways, to multistep ones, such as the protein kinase A and Ras/mitogen-activated protein kinase (MAPK) pathways. The most important question remains how the various pathways communicate with one another to execute their functions. A malfunction or alteration in the transduction of any one of these signaling pathways may positively or adversely affect the signaling of another in their regulation of cardiac function.

Recent evidence that apoptosis of cardiomyocytes is a feature of several myocardial disease states, including ischemic heart disease and congestive heart failure, has raised hopes that inhibition of cardiomyocyte apoptosis can prevent the loss of contractile cells and thus provide a new target in a multimodal

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therapeutic approach to cardiac disease [1]. We and other groups have reported that bone morphogenetic protein 2 (BMP2), leukemia inhibitory factor (LIF), insulin-like growth factor-1, neuregulin, and cardiotrophin-1 (CT-1) reduce myocyte apoptosis after ischemia, serum withdrawal, myocyte stretch, and treatment with the cardiotoxic chemotherapeutic drug doxorubicin (DOX) [2–6]. These extracellular factors that promote cardiac survival often bind to receptors at the cell surface. Signals are then transduced to the nucleus where they activate specific transcription factors that elicit changes in the anti-apoptotic pattern of gene transcription. There is cross-talk among these linear, intracellular signal transduction pathways, but how and where they intersect and whether this cross-talk results in enhanced or reduced signals is not known. To create new therapeutic strategies for heart failure, it is important to investigate synergy and its mechanisms involving various cardiac survival factors.

BMPs and related members of the transforming growth factor (TGF)-superfamily signal through heterotetrameric serine-threonine kinase receptors [7,8]. Activated BMP receptors phosphorylate transcription factors Smad1, 5, or 8, which in turn associate with a common mediator, Smad4. The resultant heteromeric Smad complexes then translocate into the nucleus to regulate transcription [7,8]. We have demonstrated that BMP2 promotes survival of neonatal rat cardiomyocytes in vitro [2]. This survival effect is accompanied by a marked reduction in the proportion of apoptotic cells in BMP2-treated cultures. We have also described the survival effect of BMP2 involving an increase in Bcl-xL via a Smad1 signaling pathway [2,9].

Interleukin (IL) 6 and related cytokines IL11, LIF, ciliary neurotrophic factor, oncostatin M, and CT-1, share the membrane glycoprotein (gp) 130 as a receptor component critical for signal transduction [10]. These IL6-type cytokines trigger the dimerization of gp130, activating associated cytoplasmic tyrosine kinases in the Janus kinase family and a downstream transcription factor, signal transducing activating transcriptional factor (Stat) 3 [10]. A growing body of evidence indicates that cytokines of the IL6 family play critical roles in the development of heart failure [11]. LIF activates a distinct form of cardiomyocyte hypertrophy, which at the cellular and morphological levels is characteristic of volume overload [12]. This hypertrophic phenotype induced by LIF is characterized by an increase in myocyte length, reflecting the addition of sarcomeric units in series and resulting in ventricular dilatation. Intriguingly, LIF has additional protective effects on myocytes [3].

Interestingly, these BMP2 and LIF pathways have been found to act in synergy on primary fetal neural progenitor cells to induce astrocytes, a mechanism which involves the formation of a complex between Smad1 and Stat3, bridged by p300 [13]. Therefore, in the present study, we examined whether cross-talk between BMP2 and LIF in protection against injury of cardiomyocytes. LIF and BMP2 were found to act in synergy on cardiomyocytes to protect against DOX-induced injury. Sustained phosphorylation/activation of Smad1 by LIF-activated ERK1/2 might be involved in the cooperative signaling of LIF and BMP2 and subsequent induction of cell survival effect on cardiomyocytes.

2. Materials and methods

2.1. Reagents

Recombinant human BMP2 (80 mg/ml) from Yamanouchi Co. Ltd. (Tokyo, Japan) and recombinant human LIF (10^6 U/ml) from CHEMOCON International, Inc. (Temecula, CA) were used in the present study. DOX was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo Japan). Anti-Stat1, anti-Stat3, anti-ERK1/2, anti-p38 MAPK and anti-c-Jun N-terminal kinase (JNK) 1/2 antibodies were purchased from Cell Signaling Technology (Santa Cruz, CA), anti-α-tubulin monoclonal antibody from Calbiochem (San Diego, CA), phospho-Smad1 (Ser463/465), Stat1, Stat3, p38 MAPK, ERK1/2 and Akt from New England Biolabs (Beverly, MA) and phospho-JNK1/2 antibodies from Promega (Madison, WI). All other chemicals were reagents of molecular biology grade obtained from standard commercial sources.

2.2. Cell culture

Primary cultures of neonatal cardiomyocytes were prepared from the ventricles of 1–2-day-old Wistar rats obtained from Kiwa Dobutsu (Wakayama, Japan) as described previously [2].

2.3. Western blotting analysis

Neonatal rat cardiomyocytes were cultured at a cell density of 5×10^5 cells/ml in six-well dishes in duplicate. After being cultured with M-199 with 10% fetal calf serum (FCS) for 24 h, the medium was changed to M-199 with 1% FCS, and was incubated for 12 h. Thereafter, cardiomyocytes were cultured without serum for another 12 h without or 36 h with adenovirus vector. The cells were then stimulated with BMP2 or/and LIF. Ten microgram of proteins were separated in 10% SDS-polyacrylamide gel and electrophoretically transferred on to a polyvinylidene difluoride membrane with transfer buffer (25 nM Tris, 190 mM glycine, and 20% methanol). Membrane was blocked with 5% skimmed milk and probed with antibodies at a 1:1000 dilution for 1 h. The ECL system was used for detection.

2.4. Northern blotting analysis

Neonatal rat cardiomyocytes were cultured at a density of 1×10^6 cells/ml in 6 cm dishes in duplicate. After being cultured with M-199 with 10% FCS for 24 h, the medium was changed to M-199 with 1% FCS, and was incubated for 12 h. Thereafter, cardiomyocytes were cultured without serum for another 12 h without or 36 h with adenovirus vector. The cells were then stimulated with BMP2 or/and LIF. Northern blotting was performed as previously described [9]. Mouse smad6 cDNA was kindly donated by Dr. K. Miyazono (Tokyo University, Japan), and mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA by Dr. K.R. Chien (University of California, San Diego CA, USA). Ten micrograms of total RNA was size-fractionated by 1.5% formaldehyde–agarose gel electrophoresis and blotted onto a nylon membrane (Hybond N^+, Amersham Biosciences, Inc.) in 20 × SSC. After prehybridization in a hybridization buffer (QuikHyb Hybridization Solution,