Journal of Molecular and Cellular Cardiology 49 (2010) 655–663

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

EMMPRIN activates multiple transcription factors in cardiomyocytes, and induces interleukin-18 expression via Rac1-dependent PI3K/Akt/IKK/NF-κB and MKK7/JNK/AP-1 signaling

Balachandar Venkatesan [a,b], Anthony J. Valente [c], Sumanth D. Prabhu [d,e], Prakashsrinivasan Shanmugam [b], Patrice Delafontaine [b], Bysani Chandrasekar [a,b,*]

[a] Research Service, Southeast Louisiana Veterans Health Care System, New Orleans, LA 70161, USA
[b] Heart and Vascular Institute, Tulane University School of Medicine, New Orleans, LA 70112, USA
[c] Department of Medicine, University of Texas Health Science Center, San Antonio, TX 78229, USA
[d] Institute of Molecular Cardiology, Department of Medicine, University of Louisville, Louisville, KY 40292, USA
[e] Medical Service, Louisville Veterans Affairs Medical Center, Louisville, KY 40206, USA

ARTICLE INFO

Article history:
Received 6 April 2010
Received in revised form 17 May 2010
Accepted 20 May 2010
Available online 9 June 2010

Keywords:
Proinflammatory cytokines
Matrix metalloproteinases
Myocardial remodeling
Cardiac hypertrophy
Myocardial failure

ABSTRACT

The transmembrane glycoprotein extracellular matrix metalloproteinase inducer (EMMPRIN), and the pleiotropic proinflammatory cytokine interleukin (IL)-18, play critical roles in myocardial remodeling, by inducing matrix degrading metalloproteinases (MMPs). Previously we showed that IL-18 induces EMMPRIN expression in cardiomyocytes via MyD88/IRAK4/TRAF6/JNK-dependent Sp1 activation. Here in reciprocal studies we demonstrate that EMMPRIN is a potent inducer of IL-18 transcription, protein expression and protein secretion in primary mouse cardiomyocytes. We show for the first time that EMMPRIN stimulates the activation of NF-κB, AP-1, CREB, and ATF-2 in cardiomyocytes, and induces IL-18 expression via Rac1-dependent PI3K/Akt/IKK/NF-κB and MKK7/JNK/AP-1 signaling. Moreover, EMMPRIN induces robust time-dependent induction of various MMP mRNAs. EMMPRIN also induces the mRNA of TIMPs 1 and 3, but in a delayed fashion. These results suggest that IL-18-induced EMMPRIN expression may favor net MMP expression and ECM destruction, and thus identify both as potential therapeutic targets in countering adverse myocardial remodeling.

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1. Introduction

A balance between extracellular matrix (ECM) deposition and matrix destruction is important for maintaining normal myocardial architecture and integrity. This balance is regulated by matrix degrading metalloproteinases (MMPs) and their inhibitors the tissue inhibitors of matrix metalloproteinases (TIMPs). Under pathological conditions, increased expression of MMPs can induce excess ECM breakdown, and lead to adverse myocardial remodeling. In addition to various proinflammatory cytokines expressed during myocardial injury and inflammation, EMMPRIN (extracellular matrix metalloproteinase inducer) has also been shown to induce the expression of diverse MMPs in cell type-specific manner [1,2]. EMMPRIN was first identified in tumor cells, where its high level of expression is thought to induce MMPs in peritumoral or stromal cells, thus effecting ECM destruction and tumor metastasis [1,2]. Later, it was discovered that EMMPRIN is actually expressed at low levels in various organs under normal physiological conditions, and its induction in pathological conditions is associated with adverse tissue remodeling [1–3].

EMMPRIN is thought to exert its biological effects in a number of ways. As an integral membrane protein, intercellular homophilic EMMPRIN–EMMPRIN interaction has been shown to induce MMPs expression in adjacent cells [4]. Further, the presence of leucine zipper-like sequences, and a positively charged glutamic acid in its hydrophobic transmembranous region implies that EMMPRIN may also interact with non-EMMPRIN transmembrane proteins and activate diverse cellular signaling pathways [1,3]. Following its cleavage from cell surface by MMPs, soluble EMMPRIN may act distally in a paracrine manner [5]. Its critical role in numerous physiological functions is further emphasized in EMMPRIN-null mice, which show various developmental defects, including poor implantation of the embryos, sterility, blindness, and memory and sensory defects [6,7].

Recently, it was demonstrated that EMMPRIN levels are elevated in monocytes in patients with acute myocardial infarction [8]. Further, treatment of monocytes isolated from normal subjects with soluble EMMPRIN activated NF-κB and induced κB-responsive cytokine gene

* Corresponding author. Heart and Vascular Institute, Tulane University School of Medicine, 1430 Tulane Avenue, SL-48, New Orleans, LA 70112, USA. Tel.: +1 504 988 3034; fax: +1 504 988 4237.
E-mail address: bchandra@tulane.edu (B. Chandrasekar).

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doi:10.1016/j.yjmcc.2010.05.007

Table 1
Primers used in the Protein/DNA Array 1.

Transcription factor	Lanes on array	DNA sequence
AP-1 (1)	A1, A2	CGCTTGATGACTCAGCCGGAA
AP-1 (2)	A17, A18	GATCAGCTTGATGATGAGTCAGCCCG
AP-2 (1)	A3, A4	GATCGAACTGACCGCCCGCGGCCCGT
CREB	C1, C2	AGAGATTGCCTGACGTCAGAGAGCTAG
NF-κB	G7, G8	AGTTGAGGGGACTTTCCCAGGC

expression [9], implying that monocyte-derived EMMPRIN could be involved in adverse remodeling via induction of MMPs and cytokines in myocardial constituent cells. In fact, cardiomyocytes and smooth muscle cells also express EMMPRIN [10,11]. Of note, EMMPRIN expression levels are increased in hypertrophied left ventricular tissue in patients undergoing aortic valve replacement [12], and in patients with end-stage cardiomyopathy [1,13]. Importantly, transgenic overexpression of EMMPRIN in a cardiac-specific manner results in adverse myocardial remodeling and failure [14]. These transgenic mice express increased levels of myocardial MMP-2 and membrane type-1 MMP (MT1-MMP or MMP14), and show fibrosis and adverse LV remodeling [14]. EMMPRIN has also been shown to induce the expression of MMPs 1 and 9 in fibroblasts [14], and all of these MMPs play a role in adverse remodeling. Interestingly, it is reported that EMMPRIN fails to modulate tissue inhibitors of MMPs (TIMPs; [2]), thus its upregulation would favor unopposed MMP expression, activation, and ECM destruction.

The proinflammatory cytokine IL-18, that belongs to the IL-1 family of pleiotropic cytokines, is synthesized as an inactive, non-glycosylated precursor. Activated proteolytically by caspase-1, IL-18 binds to cell surface receptors comprised of α and β transmembrane protein subunits, which heterodimerize upon IL-18 binding, and activate an intricate network of intracellular signaling pathways [15]. Encoded by a separate gene, the IL-18 binding protein (IL-18BP) lacks the extracellular ligand-binding domain of the IL-18 receptor, but binds IL-18 with high specificity and affinity, and neutralizes its biological activity [15]. All four major myocardial constituent cells express IL-18, but respond to IL-18 in a cell type-specific manner. In endothelial cells, it induces the expression of cytokines, and stimulates apoptosis via NF-κB-dependent PTEN activation [16]. It stimulates SMC migration in part via MMP9 [17], and its administration induces fibrotic changes in fibroblasts [18]. It exerts pro-growth effects in cardiomyocytes [19]. Together, these *in vitro* results suggest that IL-18 is a pleiotropic cytokine with pro-apoptotic, pro-mitogenic, pro-hypertrophic, and proinflammatory effects. *In vivo*, it is upregulated in various models of myocardial inflammation and injury. We have previously demonstrated that neutralization of IL-18 attenuates experimental myocardial ischemia/reperfusion injury [20], and lack of IL-18 expression in IL-18 null mice inhibits pressure overload-induced myocardial hypertrophy [21]. More recently, administration of mesenchymal stem cells expressing IL-18 binding protein, the natural inhibitor of IL-18, has been shown to improve myocardial protection following ischemia and infarction [22]. More critically, its expression is increased in patients with myocardial infarction, hypertrophy and failure [23]. These studies imply that IL-18 plays a causal role in various models of cardiac injury and inflammation. Since both EMMPRIN and IL-18 expression is elevated in myocardial injury, hypertrophy, and failure, and may induce cytokine and MMP expression, and mediate adverse remodeling, we investigated whether EMMPRIN induces IL-18, as well as the expression of various MMPs and TIMPs in cardiomyocytes and determined the underlying molecular mechanisms.

2. Materials and methods

2.1. Adult and neonatal mouse cardiomyocytes

Calcium-tolerant adult mouse cardiomyocytes (ACM) were isolated from 3 month-old male C57Bl/6 mice by a modified Langendorff perfusion and collagenase digestion technique, and have been previously described [24]. Since ACM are extremely difficult to transfect using standard transfection protocols, we used neonatal mouse cardiomyocytes (NMCM), which show relatively higher transfection efficiency (~33% with only 9% cell death as determined using the pEGFP-N1 vector; Clontech, Mountain View, CA) to investigate the regulation of the *IL18* 5′ *cis* regulatory region. NMCM were isolated from 1- to 3-day-old neonatal mice (C57Bl/6 background) as previously described [20].

2.2. Adeno and lentiviral transduction

Cardiomyocytes were infected at ambient temperature with adenoviruses (Supplementary file) in PBS at the indicated multiplicities of infection (MOI; [10,20,24]). After 1 h, the adenovirus was replaced with culture media supplemented with 0.5% BSA. Assays were carried out 24 h later. The transfection efficiency with the adenoviral vectors was near 100%, and infection with the adenoviral vectors at indicated MOI had no significant effect on cardiomyocyte shape, adherence, and viability.

2.3. Cell death detection ELISA

Cardiomyocytes exposed to EMMPRIN for 24 h were harvested and analyzed for mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by ELISA (Cell Death Detection ELISA^{PLUS} kit, Roche Applied Science). Doxorubicin, a potent anti-neoplastic drug that induces cardiomyocyte death, served as a positive control (1 μM for 24 h; [24]).

2.4. Transcription factor activation

2.4.1. Nuclear extracts

Nuclear extracts were prepared using the Panomics Nuclear Extraction Kit according to the manufacturer's instructions (#AY2002; Panomics/Affymetrix, Freemont, CA; [20]).

2.4.2. Protein–DNA interaction array

Protein–DNA interactions were analyzed using the TranSignal Protein/DNA Array 1 (PD array 1, Panomics/Affymetrix) essentially as described by Imam et al. ([25]; A detailed description of the methodology and the TFs assayed are provided in the Supplementary file and Fig. S2).

Regulation of EMMPRIN-mediated transcription factor activation was confirmed by a highly sensitive signaling profiling ELISA (TransFactor kits (BD Mercury TransFactor Profiling Assay). This assay profiles NF-κB-p65, p50, c-Rel, cFos, FosB, cJun, JunD, CREB, ATF2, Sp-1, and STAT1, and has a 10-fold higher sensitivity than traditional electrophoretic mobility shift assays with fewer false negative results (sensitivity for NF-κB >0.3 nM; [26]). Cardiomyocytes were treated with EMMPRIN for 2 h, and 25 μg of nuclear extracts was used. After the addition of chromogen, absorbance at 650 nm was measured in a SPECTRAMax Plus spectrophotometer.

The formation of NF-κB protein–DNA complexes and the subunit composition were also analyzed by an ELISA (TransAM™ TF ELISA kits; #43296; Active Motif, Carlsbad, CA; [10,20,24]). Activation of NF-κB was also confirmed by a reporter assay using adenoviral transduction of an NF-κB reporter vector (Ad.NF-κB-Luc, MOI 50) as described previously [20]. Ad.AP-1-Luc was also previously described. Ad.MCS-Luc (MOI 50) served as negative control, and Ad.β-gal (MOI 50) served as an internal control. β-Galactosidase activity in cell extracts was determined using a luminescent β-galactosidase detection kit II (BD Biosciences), and the results are expressed as the ratio of firefly luciferase to β-galactosidase activity measured in relative light units. Activation of NF-κB was further confirmed by immunoblotting using anti-p65 antibodies. The binding of AP-1 and NF-κB to the *IL18* 5′ *cis* regulatory region *in vivo* was investigated by ChIP assays [20].

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