International Journal of Cardiology 168 (2013) 3770–3778

Contents lists available at ScienceDirect

International Journal of Cardiology

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

Inhibition of NADPH oxidase 4-related signaling by sodium hydrosulfide attenuates myocardial fibrotic response

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ARTICLE INFO

Article history:
Received 18 June 2012
Received in revised form 29 April 2013
Accepted 15 June 2013
Available online 2 July 2013

Keywords:
Hydrogen sulfide
Cardiac fibroblast
Fibrosis
NADPH oxidase 4

ABSTRACT

Background: Myocardial fibrosis plays a pivotal role in the development of heart failure. Hydrogen sulfide (H_2S) is an endogenous gasotransmitter with potent cardioprotective properties; however, whether H_2S is involved in fibrotic process remains unknown. This study aimed to explore the role of H_2S in the process of cardiac fibrosis and the underlying mechanisms.

Methods: Myocardial infarction (MI) was established in rats by ligation of coronary artery. Activation of rat neonatal cardiac fibroblasts was induced by angiotensin II (Ang II). Fibrotic responses in ischemic myocardium and in Ang II-stimulated cardiac fibroblasts were examined. The effects of sodium hydrosulfide (NaHS, an exogenous H_2S donor) on NADPH oxidase 4 (Nox4), reactive oxygen species (ROS) production, extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, heme oxygenase-1 (HO-1), and cystathionine γ-lyase (CSE) were tested to elucidate the protective mechanisms of H_2S on fibrotic response.

Results: NaHS treatment inhibited Ang II-induced expression of α-smooth muscle actin, connective tissue growth factor (CTGF), and type I collagen and upregulated expression of HO-1 in cardiac fibroblasts. Ang II-induced Nox4 expression in cardiac fibroblasts was quenched by NaHS and this was associated with a decreased ROS production and reduced ERK1/2 phosphorylation and CTGF expression. *In vivo* studies using MI model indicated that NaHS administration attenuated Nox4 expression and fibrotic response. Moreover, NaHS therapy also prevented cardiac inflammatory response accompanied by increases in HO-1 and CSE expression.

Conclusions: The beneficial effect of H_2S, at least in part, was associated with a decrease of Nox4-ROS-ERK1/2 signaling axis and an increase in HO-1 expression.

1. Introduction

Fibrotic remodeling of cardiac tissue is associated with a poor prognosis of cardiomyopathy leading to heart failure, which results in an elevated risk of morbidity and mortality [1]. Among the multiple causes of heart failure, myocardial ischemia is perhaps the most common. Myocardial fibrosis occurs in response to cardiac stress (*e.g.* myocardial infarction (MI)) and neurohormonal activation (*e.g.* angiotensin II (Ang II)), and may induce profibrotic growth factors release and lead to cardiac fibroblast proliferation and activation [2,3]. Cardiac fibroblasts are responsible for synthesis of structural extracellular matrix [4]. Activated cardiac fibroblasts adopt a myofibroblast phenotype, characterized by expression of α-smooth muscle actin (α-SMA) and excessive deposition of extracellular matrix under pathological conditions [5,6]. Collagen is the major determinant of myocardial structural integrity. This pathological extracellular matrix accumulation in the non-ischemic myocardium can lead to myocardial fibrosis and ventricular dysfunction, and ultimately heart failure [7]. Therefore, pharmacological approaches for intervening in cardiac fibroblast activation may be therapeutic intervention strategies for cardiac pathologies characterized by adverse fibrotic remodeling [8].

Cardiac fibroblasts change their phenotype and differentiate into myofibroblasts in response to growth factor, cytokine, or mechanical stimuli [5]. Ang II is a key mediator of cardiac fibroblast activation and exerts a number of actions in the pathological process of myocardial remodeling and failure [4]. Recent studies have demonstrated a potential link between Ang II-associated fibrotic response and NADPH oxidases (Nox) in cardiac fibroblasts [9]. Nox-dependent reactive

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0167-5273/$ – see front matter
http://dx.doi.org/10.1016/j.ijcard.2013.06.007

Table 1
Primer sequences used in this study.

Genes		Sequences
Rat iNOS	Sense	5′-CTCACTGTGGCTGTGGTCACCTA-3′
	Antisense	5′-GGGTCTTCGGGCTTCAGGTTA-3′
Rat ICAM-1	Sense	5′-ACCAGACCCTGGAGATGGAGA-3′
	Antisense	5′-ACCGTGGGCTTCACACTTCA-3′
Rat VCAM-1	Sense	5′-GGATGCCGGAGTATACGAGTGTG-3′
	Antisense	5′-CAATGGCGGGTATTACCAAGGA-3′
Rat TNF-α	Sense	5′-ATACACTGGCCCGAGGCAAC-3′
	Antisense	5′-CCACATCTCGGATCATGCTTTC-3′
Rat GAPDH	Sense	5′-GGCACAGTCAAGGCTGAGAATG-3′
	Antisense	5′-ATGGTGGTGAAGACGCCAGTA-3′

oxygen species (ROS) production is activated in response to an array of extracellular stimuli, including Ang II [10]. Among these isoforms, Nox4 represents a major source of ROS in heart failure and activated cardiac fibroblast [11,12], which in turn modulate activation of redox-dependent signaling cascades and transcription of important target genes, including collagen and connective tissue growth factor (CTGF) [13]. CTGF, a potent profibrotic factor, is positively correlated with excessive interstitial fibrosis and heart failure and implicated in the Ang II-induced pathologic fibrosis process [14]. Therefore, inhibition of Nox4-dependent ROS may modulate the profibrotic effects of Ang II.

Recently, hydrogen sulfide (H_2S) was proposed as a physiological gasotransmitter with a diverse physiological profile [15,16]. In mammalian cells, endogenous H_2S was formed from cysteine and homocysteine by enzymes cystathionine γ-lyase (CSE), cystathionine β-synthetase, and 3-mercaptopyruvate sulfurtransferase [16], while CSE was mainly expressed in heart and smooth muscle and the most relevant enzyme for the cardiovascular system [16,17]. H_2S exerted a wide range of physiological functions in cardiovascular system, including anti-inflammation, cardioprotection, vessel relaxation, and so on [18–21]. Reduction of plasma and tissue H_2S levels was observed in various cardiovascular disease states, including MI [17,22–24]. Several groups, including our own, have demonstrated that administration of H_2S played an important role in the process of fibrotic myocardial remodeling in the injured and failing heart [22,25]. H_2S possessed a diverse physiological profile that contributes to its cardioprotective actions, such as anti-oxidation, anti-inflammation, and so on [16]. Recently, some groups reported H_2S or H_2S-donating drugs served as a potent inhibitor of Nox expression and activity [26–28], which played a pivotal role in the development of cardiac remodeling and dysfunction associated with heart failure [10]. These findings suggested that administration of H_2S or the modulation of endogenous H_2S production may be of importance in the process of myocardial remodeling.

The present study provided the first evidence that exogenous H_2S inhibited Ang II-mediated fibrotic responses in cardiac fibroblast involvement of repressing Nox4-ROS-ERK1/2 signaling pathway. We also demonstrated that the inhibition of Nox4 expression by exogenous H_2S therapy was associated with suppressed inflammatory and profibrotic responses in acute ischemic myocardium induced by MI.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and fetal calf serum were from Invitrogen (Carlsbad, California). PD98059 (an extracellular-signal regulated kinase 1/2 (ERK1/2) inhibitor) was purchased from Calbiochem (Darmstadt, Germany). Ang II was from Tocris Bioscience (Avonmouth, UK). Sodium hydrosulfide (NaHS, an exogenous H_2S donor) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), if not otherwise stated.

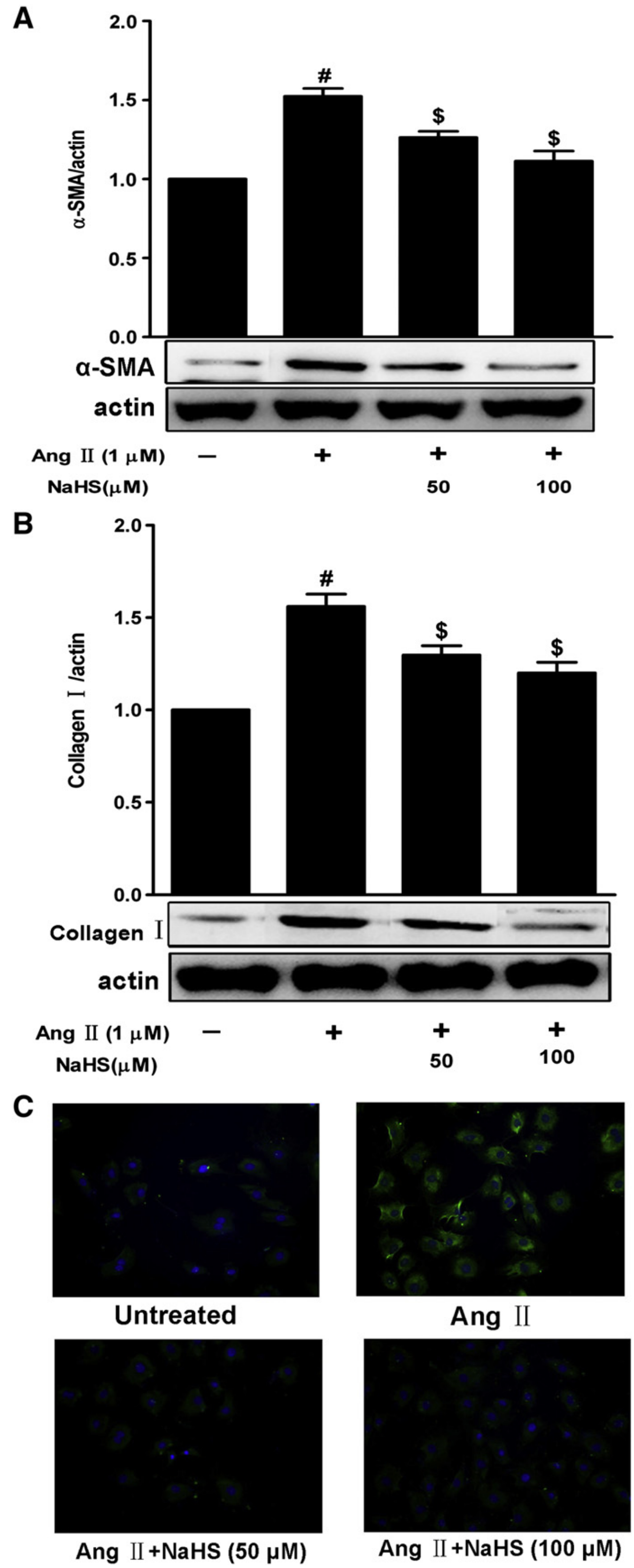

Fig. 1. NaHS inhibited Ang II-induced α-SMA and type I collagen expression in cardiac fibroblasts. After NaHS (50 or 100 μM) pretreatment, cells were stimulated with or without Ang II (1 μM) for 24 h. Western blot and densitometric analysis for α-SMA (A) and type I collagen (B) expression, respectively, β-Actin was used as loading control; representative photomicrographs showing type I collagen (C) determined by fluorescence microscope (magnification, 200×). Green, type I collagen; blue, nucleus. #$P < 0.05$ compared with unstimulated cells, $$P < 0.05$ compared with Ang II-stimulated cells; data were from at least three independent experiments, each performed in duplicate.