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Research paper

Hypoxia-induced SOCS3 is limiting STAT3 phosphorylation and NF-κB activation in congenital heart disease

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ABSTRACT

Suppressor of cytokine signaling 3 (SOCS3) is a critical attenuator of the JAK-STAT signaling pathway, and it is involved in mediating the intensity and duration of STAT3 activation in the process of myocardial protection. Nuclear factor-κB (NF-κB) has emerged as a decisive transcription factor in cardiac myocyte compensatory responses to stress that enhance survival. However, the expression, activation and regulation of this signaling molecule in response to hypoxic stress have not been elucidated. We investigated 40 infants with cyanotic or acyanotic cardiac defects, as well as H9c2 embryonic rat cardiomyocytes, to examine the effect of hypoxia on the expression or activation of SOCS3, STAT3 and NF-κB *in vivo* and *in vitro*. We found an increase in endogenous cardiac SOCS3, p-STAT3 and AC-RelA activation in the myocardium of infants with cyanotic cardiac defects. In hypoxic cultivated H9c2 cells, SOCS3, STAT3 and AC-RelA activity slowly increased and then reached a stable expression. We evaluated the interaction of SOCS3 with STAT3 and NF-κB by transfecting the SOCS3 plasmid to hypoxic cultured H9c2 cells. Forced expression of SOCS3 suppressed tyrosine phosphorylation of STAT3 and transcription of the *C-myc* and *interleukin-6* genes. AC-RelA activation was also suppressed by over expression of SOCS3. These findings suggest that the mechanism of a positive transactivation loop that maintains higher levels of NF-κB and p-STAT3 and the negative feedback factor SOCS3, which maintains balanced NF-κB and p-STAT3 activities, is important in the process of myocardial adaptation to chronic hypoxia. SOCS3 is a rapid hypoxia inducible gene and acts to inhibit activation of the cellular signaling pathway in a classical negative feedback loop. Upregulated SOCS3 might play an important role in cardiocytes during chronic hypoxia as SOCS3 regulates cell signaling crosstalking between NF-κB and p-STAT3 under stressful conditions.

1. Introduction

Cytokines related to interleukin-6 (IL-6) comprise a family of substances known to play cytoprotective and growth-promoting roles in many different cell types [1]. IL-6 related cytokines potently promote tyrosine-phosphorylation of Janus kinases (JAKs) and cytoplasmic latent transcription factors of the signal transducers and activators (STATs) family. STAT proteins regulate the expression of genes encoding proteins involved in angiogenesis, inflammation, apoptosis, extracellular matrix composition and cellular signaling [2]. The JAK-STAT signaling pathway mediates cytoprotective effects in cardiomyocytes [3]. Eight members have been identified as suppressors of the cytokine signaling (SOCS) family (CIS and SOCS1 to SOCS7), and not only act as direct negative feedback regulators of JAK-STAT signaling, but are also involved in the fine tuning of the myocardial adaptation response and in crosstalk of the complicated cytokine signal network in myocytes [4,5].

Accumulating evidence indicates that activation and expression of the IL-6-JAK-STAT signaling pathway are facilitated by hypoxia [6–9]. Among these signaling molecules, STAT3 can be activated by ischemic-oxidative stress and this signaling pathway exerts cardioprotection in the ischemic heart [10,11]. Blockade of the STAT3 pathway enhances myocardial injury after infarction [12]. Evidence from cardiomyocyte-restricted ablation of STAT3 mice further indicates that STAT3 protects the heart from ischemic injury by suppressing cardiomyocyte apoptosis, inducing local growth factor production [10]. As a product of the STAT3-inducible gene, SOCS3 is a major feedback regulator of STAT3, and it blocks STAT3 activation by the gp130 receptor [13]. Through its N-terminal kinase

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inhibitory domain and SH2 domain, SOCS3 can bind to various kinases including JAK2 kinase, and inhibit their activity [14]. Hypoxia has profound effects on the expression of SOCS3 in pulmonary arterial smooth muscle cell (PASMC) [15]. SOCS3 can also be induced and can inhibit signaling by a wide-spectrum of growth factors and cytokines including Toll-like receptor (TLR) agonists (such as lipopolysaccharide and CpG-DNA), IL-10, IL-6 and other gp130 signaling cytokines, leptin and interferon-γ [16–21]. These properties suggest that SOCS3 may broadly regulate cytokine signaling and might be a chief factor in these classical signal loops. Moreover, it seems that SOCS3 expression determines the character of the response of cardiac myocytes to the IL-6-type cytokines and also impacts on other signaling pathways of other agonists. While many studies have evaluated the protective effects of STAT3 and regulatory effects of SOCS3 for ischemia/reperfusion injury of heart [10,11,22–24], it is unknown whether SOCS3 can be directly induced by hypoxia in cardiac myocytes. Additionally, little is known about the expression, activation and regulation of these signaling molecules in myocytes exposed to chronic low ambient oxygen levels.

NF-κB is a ubiquitous transcription factor and its activation involves the regulation of a large variety of genes [25–27]. NF-κB consists of five Rel-related proteins such as p50, p52 (NF-κB2), p65 (RelA), c-Rel and RelB. The prototypical NF-κB complex is a RelA/p50 heterodimer, which is important for NF-κB-mediated anti-apoptotic effects [28]. Recent studies have demonstrated that the amplitude and half-life of nuclear NF-κB are influenced by acetylation of RelA [29], which requires prior RelA phosphorylation [30]. In particular, endogenous RelA is acetylated in a signal-coupled manner following stimulation [29,31,32]. Reversible acetylation of RelA is essential for the duration of NF-κB activity.

Signaling pathways that mediate protection from apoptosis involve activation of the transcription factor NF-κB, which in turn induces the expression of proteins that possess cytoprotective roles [33]. NF-κB is induced by hypoxic stimulation and it regulates the secretion of IL-6 in cardiac myocytes, and may be the primary positive regulator of transcriptional activation of the IL-6-JAK-STAT signaling pathway during hypoxia [34]. STAT3 is a transcription factor that can promote oncogenesis [35], and it is commonly activated in cancer [36] as well as in tumor-associated myeloid cells [37]. STAT3 and NF-κB stimulate a highly overlapping repertoire of pro-survival, proliferative, and pro-angiogenic genes [36]. Crosstalk between STAT3 and NF-κB has been demonstrated at multiple levels, including activation of STAT3 by NF-κB-regulated factors such as IL-6 [38] and Cox-2 [39], possible inhibition of IκB kinase (IKK) activity in normal immune cells by STAT3 [40], and nuclear translocation of unphosphorylated NF-κB by unphosphorylated STAT3[41]. A recent study further demonstrated that activated STAT3 prolongs NF-κB nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF-κB nuclear export [42]. Therefore, the STAT3/NF-κB interaction is important for tumor cells to adapt to a hypoxic tumor microenvironment and to mediate antiapoptotic effects.

The present study was undertaken to test the following hypotheses: in chronic hypoxia cardiac myocytes: 1) the IL-6-STAT3 and NF-κB signaling pathways are sustained and stably activated; 2) STAT3/NF-κB interaction occurs; and 3) expression of SOCS3 is induced by hypoxia, and it suppresses STAT3 phosphorylation and mediates NF-κB activation.

We examined the expression and activation of IL-6, STAT3 and NF-κB in the myocardium of infants with cyanotic cardiac defects, as well as in cultured cardiac myocytes subjected to chronic hypoxia. We evaluated the interaction of SOCS3 with STAT3 and NF-κB by transfecting the SOCS3 plasmid to hypoxic cultured H9c2 cells.

The purposes of this study were to determine the crosstalk between NF-κB and STAT3 signaling and the effect of SOCS3 on this interaction on myocardial adaptation to chronic hypoxia.

2. Materials and methods

2.1. Antibodies

Polyclonal rabbit anti-human IL-6 was purchased from Beijing Boaosen Biotechnology Ltd (Boaosen Biotechnology, Beijing, China), and polyclonal rabbit anti-human NF-κB p65(acetyl K310) and SOCS3 IgG were purchased from Abcam (UK). Polyclonal rabbit anti-human STAT3, phosphor-Tyr-705-STAT3 IgG, polyclonal rabbit anti-human phosphor-Ser-276-NF-κB p65 IgG, GAPDH and β-actin were all from Cell Signaling Technology, Inc. (USA). These polyclonal rabbit anti-human antibodies have been shown to cross-react with murine IL-6, NF-κB p65 (acetyl K310), SOCS3, STAT3, phosphor-Tyr-705-STAT3 and phosphor-Ser-276-NF-κB p65 in our laboratory (data not shown) and were used in immunohistochemistry and western blot experiments. Polyclonal goat anti-rabbit IgG-horseradish peroxidase (ZSGB-BIO, Beijing, China) was used as a secondary antibody for immunohistochemistry.

2.2. Study subjects

2.2.1. Patients

Forty infants, aged 3.2 to 60.0 months (mean = 9.4 months), admitted to the Institute of Cardiovascular Surgery, Xinqiao Hospital of the Third Military Medical University with congenital heart disease were eligible for inclusion in the study. Eighteen infants had cyanotic defects and twenty-two had acyanotic cardiac defects. The local ethics review board of the Third Military Medical University Affiliated Hospital approved the study protocol and the informed consent form. The investigation conformed to the principles outlined in the Declaration of Helsinki. Written consents were obtained from parents of all participants.

2.2.2. Cardiac operation and sampling of myocardial biopsies

Drugs given for premedication and conventional general anesthesia in all cases included midazolam, fentanyl sulfate and vecuronium bromide. After institution of a hypothermic cardiopulmonary bypass with a flow index of 2.5 $L\,min^{-1}\,m^{-2}$ body surface area for 20–30 min, the aorta was cross-clamped and cardiac arrest instituted by intra aortal injection of a 4 °C cardioplegic solution (St. Thomas solution, 20 $ml\,kg^{-1}$ body weight), which was re-aspirated through the right atrium. Immediately after cardiac arrest, a biopsy was taken from the right ventricular outflow tract, under the condition of deep hypothermia (20–26 °C) and low flow bypass (1.0 $L\,min^{-1}\,m^{-2}$ body surface area). Myocardial samples taken for western blots, EMSA and Real-time PCR were immediately snap frozen in liquid nitrogen and stored at −80 °C until analysis. Samples taken for immunohistochemistry were fixed in 10% formaldehyde solution, embedded in paraffin and cut into 4 μm thick sections.

2.3. Cell culture

Embryonic rat-heart-derived H9c2 cells were purchased from American Type Culture Collection (ATCC, CRL-1466, passage number 4–18) and were maintained in growth medium composed of Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum. The cells were placed in an *Invivo*200 cultivator (Ruskin Technology Ltd, UK) at 37 °C and exposed to 1.0% O_2 and 5% CO_2 for durations of 6, 12, 24, 48, and 72 h. The medium was changed with pre-equilibration in hypoxia every 48 h. After different