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Original Contribution

Nox 2 stimulates muscle differentiation via NF-κB/iNOS pathway

Yu Ji Piao[a,b], Yong Hak Seo[a,b], Feng Hong[a,b], Jin Hwan Kim[a,b], Young-Joo Kim[a,b], Myung Ho Kang[a,b], Bum Shik Kim[c], Sangmee Ahn Jo[d], Inho Jo[d], Dae-Myung Jue[e], Insug Kang[a,b], Joohun Ha[a,b], Sung Soo Kim[a,b,*]

[a]*Department of Biochemistry and Molecular Biology, School of Medicine, Kyung Hee University, #1, Hoegi-dong, Dongdaemoon-gu, Seoul 130-701, Korea*

[b]*Medical Science and Engineering Reseach Center for Bioreaction to Reactive Oxygen Species, School of Medicine, Kyung Hee University, Seoul 130-701, Korea*

[c]*Department of Thoracic Surgery, School of Medicine, Kyung Hee University, Seoul 130-701, Korea*

[d]*National Institute of Health, Seoul 122-701, Korea*

[e]*Department of Biochemistry, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea*

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Abstract

The NF-κB/iNOS pathway stimulates muscle differentiation downstream of the PI 3-kinase/p38 MAPK pathway and diverse antioxidants block muscle differentiation. Therefore, we here investigated whether Nox 2 links those two myogenic pathways in H9c2 and C_2C_{12} myoblasts. Compared with the proliferation stage, ROS generation was enhanced from the early stage of differentiation and gradually increased as differentiation progressed. Antioxidants suppressed the activated NF-κB/iNOS pathway during muscle differentiation. Nox 2 activity was also increased during muscle differentiation. Treatment with DPI and apocynin, two inhibitors of NADPH oxidase, and suppression of Nox 2 expression using siRNA, but not Nox 1, inhibited NADPH oxidase activity, muscle differentiation, and the NF-κB/iNOS pathway. Inhibition of PI 3-kinase and p38 MAPK suppressed the Nox 2/NF-κB/iNOS pathway. Nitric oxide restored muscle differentiation blocked by treatment with antioxidants or suppression of the Nox 2/NF-κB/iNOS pathway. In conclusion, Nox 2 stimulates muscle differentiation downstream of the PI 3-kinase/p38 MAPK pathway by activating the NF-κB/iNOS pathway via ROS generation.

Keywords: Muscle differentiation; Nox 2; Reactive oxygen species (ROS); PI 3-kinase; p38 MAPK; NF-κB; iNOS; Nitric oxide; Free radicals

Introduction

Muscle differentiation is a highly ordered multistep process that is largely regulated by two groups of myogenic transcription factors, the basic helix-loop-helix muscle regulatory factors (MyoD, Myf5, myogenin, and MRF4) and the myocyte enhancer-binding factor 2 proteins [1]. Insulin-like growth factors [IGF-1 and 2, IGFs][1] stimulate muscle differentiation by activating several signaling pathways [2,3]. During the last few years, it has been known that IGFs stimulate the myogenic process via phosphatidylinositol 3-kinase (PI 3-kinase), p38 mitogen-activated protein kinase (p38 MAPK), and the mammalian target of rapamycin [4–6]. In contrast, the Raf/MEK/ERK pathway has been

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DPI, diphenyleniodonium; DFOM, deferoxamine mesylate; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DM, differentiation medium; dnIκB-α, dominant negative construct IκB-α (S32/36A); EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; IGF, insulin-like growth factor; iNOS, inducible nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl mster; MHC, myosin heavy chain; NF-κB, nuclear factor-κB; NO, nitric oxide; PI 3-kinase, phosphatidylinositol 3-kinase; p38 MAPK, p38 mitogen-activated protein kinase; PDTC, pyrrolidinedithiocarbamate acid; PM, proliferation medium; PMSF, phenymethylsulfonyl fluoride; ROS, reactive oxygen species; siRNA, small interfering RNA; SNP, sodium nitroprusside; S-2-AEITU, *S*-2-aminoethyl isothiourea; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid.

* Corresponding author. Department of Biochemistry and Molecular Biology, School of Medicine, Kyung Hee University, #1, Hoegi-dong, Dongdaemoon-gu, Seoul 130-701, Korea. Fax: +82 2 959 8168.

E-mail address: sgskim@khu.ac.kr (S.S. Kim).

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shown to stimulate cell proliferation rather than muscle differentiation [7,8].

Reactive oxygen species (ROS) consist of diverse chemically reactive molecules derived from oxygen, the majority of which include superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2). Through the inherent and indiscriminating reactivity to the main biomolecules, i.e, DNA, proteins, lipids, and carbohydrates, ROS can be cytotoxic when present at high and/or sustained levels [9]. However, ROS at low concentrations have been shown to function as physiological intracellular signaling mediators. In fact, it has been shown that a variety of extracellular stimuli including peptide growth factors, cytokines, and receptor agonists propagate mitogenic and antiapoptotic signals by increasing the intracellular concentration of ROS. In addition, inhibition of ROS generation or treatment with antioxidants completely blocks ligand-dependent signaling transduction [10–12]. At molecular levels, ROS exert their effects by reversibly oxidizing proteins on thiol groups. Redox-sensitive proteins are targets of specific oxidation by various oxidants. Protein tyrosine phosphatases have been found to be one of them. Extensive investigation revealed that their inhibition by ROS helps receptor tyrosine kinases propagate their signals downstream, and activate diverse transcription factors (NF-κB and AP-1) and signaling molecules such as PI 3-kinase, Akt, MAPK, and phospholipase A_2 [13–17].

NADPH oxidase catalyzes the single-electron reduction of oxygen using NADH or NADPH as the electron donor to generate superoxide anion. The phagocytic (prototype) NADPH oxidase is a membrane-associated multicomponent enzyme consisting of two membrane proteins, $gp91^{phox}$ and $p22^{phox}$, that together form the flavocytochrome b558, and three cytosolic proteins, $p47^{phox}$, $p67^{phox}$, and Rac. Upon stimulation, the cytosolic component $p47^{phox}$ is phosphorylated, and the entire cytosolic complex translocates to the plasma membrane forming the active NADPH oxidase by associating with cytochrome b558. The activated phagocytic NADPH oxidase produces a large amount of superoxide and plays a pivotal role in host defense against microbial infection. Recently, two families of the phagocytic $gp91^{phox}$ homologues, namely NADPH oxidase (Nox) and dual oxidase (Duox), have been found in a variety of nonphagocytic cells. The Nox family consists of Nox 1, Nox 2 (phagocytic $gp91^{phox}$), Nox 3, Nox 4, and Nox 5. The Duox family members are Duox 1 and Duox 2. The Nox and Duox families exhibit unique patterns of cellular expression. Stimulation with various growth factors in nonphagocytic cells results in a rapid oxidative burst with the generation of ROS from the NADPH oxidase complex [18]. The ROS levels in nonphagocytic cells are orders of magnitude lower than those in immune cells. In addition, H_2O_2 represents an ideal signaling molecule due to rapid synthesis and diffusibility. Signaling pathways for Nox 2 activation have been most extensively investigated and several kinases that activate Nox 2 have been identified. For example, there has been accumulating evidence that protein kinase C (PKC), ERK, and p38 MAPK activate NADPH oxidase in neutrophils [19,20]. PI 3-kinase has also been identified to activate NADPH oxidase upstream of Akt, ERK, and p38 MAPK [21–24].

We previously showed that ROS are essential mediators in muscle differentiation [25]. Also, it has been shown that p38 MAPK, but not ERK, stimulates muscle differentiation in the downstream of PI 3-kinase [26,27]. Also, there is abundant evidence that nuclear factor-κB (NF-κB) activity is extremely sensitive to cellular redox status. In addition, NF-κB activation by PI 3-kinase has been suggested to stimulate the myogenic process by increasing the expression of inducible nitric oxide synthase (iNOS) in rat, human, and chick embryonic cultured myoblasts [28,29]. Therefore, we here investigated (1) if ROS stimulate muscle differentiation via NF-κB activation/iNOS expression, (2) if NADPH oxidase is involved in ROS production and NF-κB activation/iNOS expression, (3) which Nox isozymes are expressed in myoblasts and play a major role in muscle differentiation, (4) if NADPH oxidase is activated by the PI 3-kinase/p38 MAPK pathway during muscle differentiation.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), DMEM/F-12, fetal bovine serum, donor calf serum, and G418 were purchased from GIBCO-BRL (Grand Island, NY). Diphenyleniodonium (DPI), deferoxamine mesylate (DFOM), 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), N^G-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), and NADPH were from Sigma (St. Louis, MO). *S*-2-Aminoethyl isothiourea (S-2-AEITU), pyrrolidinedithiocarbamate acid (PDTC), apocynin, SB203580, and LY294002 were from Tocris Cookson, Ltd. (Bristol, UK). The NF-κB probe for electrophoretic mobility-shift assays was from Santa Cruz Biotechnology (Santa Cruz, CA). [γ- ^{32}p]ATP was purchased from Amersham Biosciences (Little Chalfont, UK). Anti-Rac antibody was from Upstate Biotechnology (Lake Placid, NY). Antibodies specific to myosin heavy chain (MHC) and myogenin, $p47^{phox}$ protein, Nox 1, Nox 2, Nox 4, IκB-α, phospho-IκB-α (Ser^{32}), and iNOS were from Santa Cruz Biotechnology (Santa Cruz). Antibodies specific to p38 MAPK and phospho-p38 MAPK (Thr^{180}/Tyr^{82}) were from Cell Signal Technology (Beverly, MA).

Cell culture

H9c2 rat cardic and C_2C_{12} mouse myoblasts were grown in 10-cm-diameter dishes in DMEM/F-12 containing 10% (v/v) donor calf serum and DMEM supplemented with 10%