NADPH oxidases in the cardiovascular system

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2014

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Chapter 3

p47^{phox}-dependent reactive oxygen species stimulate nuclear translocation of the transcription factor FOXO1 in ischemia-challenged H9c2 cells

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Submitted for publication

Abstract

Reactive oxygen species (ROS) control forkhead box o (FOXO) transcription factors by influencing their nuclear translocation. However, knowledge of the cellular source(s) of ROS involved herein remains scarce. Recently, we have shown $p47^{phox}$-dependent activation of ROS-producing NADPH oxidase (NOX) at the nuclear pore in H9c2 rat cardiomyoblasts in response to ischemia. This localizes NOX perfectly to affect proteins translocating in or out of the nucleus, including transcription factors. Therefore, in this study involvement of $p47^{phox}$-dependent production of ROS in the nuclear translocation of FOXO1 was analyzed in H9c2 cells under ischemia.
The effect of ischemia on H9c2 cardiomyoblasts was mimicked by metabolic inhibition. Nuclear translocation of FOXO1 was determined via quantitative digital imaging fluorescence and Western blot analysis. Subsequently, the effect of inhibiting of $p47^{phox}$-dependent ROS production via short hairpin RNA (shRNA) transfection on FOXO1 translocation was analyzed by digital-imaging microscopy.
Ischemia induced a significant translocation of FOXO1 into the nucleus. Transfection with $p47^{phox}$-shRNA successfully inhibited $p47^{phox}$ expression, reduced the generation of ROS at the nucleus and inhibited the nuclear translocation of FOXO1. Our data for the first time show that ischemia-induced nuclear import of FOXO1 in H9c2 depends critically on $p47^{phox}$-mediated ROS production.

Introduction

It is well known that mammalian cells produce reactive oxygen species (ROS) in response to a multitude of (patho)physiological conditions and thereby regulate cell fate decisions. In the last two decades it has become recognized that these ROS, rather than being by-products of signaling events, act as second messengers in cellular signaling through the reversible oxidation of cysteine residues in signal transduction proteins, which alters their activity.[1] These signal transduction proteins include protein kinases, phosphatases, and transcription factors. Through redox modification of these proteins, ROS are involved in the regulation of gene expression and so influence such diverse processes as cell proliferation, migration, and apoptosis.[1]

A number of redox-sensitive transcription factors have been described that are either activated or inactivated through redox modifications, including NF-κB, *c-Jun* and Nrf2.[2-5] Another group of transcription factors that has been shown to be under redox control is the forkhead box o (FOXO) family of transcription factors,[6] four of which (FOXO1, FOXO3a, FOXO4 and FOXO6) are expressed in humans.[7] FOXO transcription factors are involved in a number of cellular functions, including cell-cycle arrest, apoptosis, ROS scavenging and DNA repair and as such are important in cell fate decisions.[7]

The functional control of FOXO is complex and includes phosphorylation-dephosphorylation and redox signaling.[6/8] A major aspect of FOXO control is the regulation of its subcellular localization. In response to insulin and growth factor signaling functional FOXO, that resides in the nucleus, is deactivated through phosphorylation by the PI3K/Akt pathway, leading to release from its DNA binding sites and export from the nucleus into the cytoplasm.[8] However, oxidative stress can directly oppose the PI3K/Akt pathway and promote nuclear translocation of FOXO.[9] It was shown recently in cardiomyocytes that hypoxia/reoxygenation and oxidative stress induced nuclear translocation of FOXO1, where it promoted cell survival.[10] In C2 C12 murine myocytes, ROS scavenger NAC counteracted insulin-like growth factor 1 (IGF-1)-induced FOXO1 phosphorylation.[11]

Although it has been established that ROS are involved in FOXO control, knowledge of the cellular source(s) of ROS involved remains scarce. As ROS-producing enzymes, the family of multi-component NADPH oxidases (NOX) appear to be important in redox signaling[12] and may be linked to FOXO. In human pulmonary artery smooth muscle cells (SMC) NOX4 positively regulated FOXO3a as NOX4 over-expression increased- and NOX4 knock-down decreased FOXO3a activity.[13] Whereas in hepatic stellate cells isolated from NOX1 null-mutant mice showed significant reduction in AKT and FOXO4 phosphorylation compared to cells isolated from wild-type mice.[14] These data link NOXes to FOXO. We have shown in H9C2 rat cardiomyoblasts that the NOX subunits NOX2, $p22^{phox}$ and $p47^{phox}$ are targeted to the nuclear pore in response to ischemia and homocysteinemia, and locally produce ROS, which depended critically on the presence of $p47^{phox}$.[15/16] Although it is not fully understood how NOX-derived ROS achieve signal specificity, subcellular localization and activation of NOX in specific subcellular compartments may be a means to achieve signal specificity. The activation at the nuclear pore perfectly places NOX to regulate transport of proteins in or out of the nucleus, including transcription factors.

Therefore, in this study involvement of $p47^{phox}$-dependent production of ROS in subcellular translocation of FOXO1 was analyzed in H9C2 cells in response to ischemia.

Materials and Methods

Cell culture

Rat cardiomyoblasts (H9C2 cells; ATCC, Manassas, VA, USA) were cultured in culture medium: Dulbecco's modified eagles medium (DMEM, Cambrex Corporation, East Rutherford, NJ, USA) containing 10% (v/v) heat inactivated fetal calf serum (FCS, Bio-Whittaker, Walkersville, MD, USA), 100 IU/mL penicillin (Yamanouchi Europe BV, Meppel, The Netherlands), 100 ug/mL streptomycin (Radiopharma Fisiopharme, Palomonte, Italy) and 2 mmol/L L-glutamine (Invitrogen, Carlsbad, CA, USA). H9C2 cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. To mimic ischemia H9C2 cells were incubated for 4 hours in a metabolic inhibition buffer (0.9 mmol/L $CaCl_2H_2O$, 106 mmol/L NaCl, 3.8 mmol/L $NaHCO_3$, 4.4 mmol/L KCL, 1 mmol/L $MgCl_2H_2O$, pH 6.6), containing 20 mmol/L (2-deoxy)glucose to impair glycolysis, and 5 mmol/L NaCN to block the mitochondrial electron transport chain.[17]

Quantitative digital imaging fluorescence microscopy

H9C2 cells were cultured in 4-well chamber slides (Nalge Nunc International, Naperville, IL, USA). After treatment, cells were fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.2% Triton for 10 minutes and subsequently washed with 0.05% (v/v) PBS/Tween20. The cells were then incubated with the primary and secondary antibodies: rabbit-anti-human FOXO1 (1:100, Cell Signaling Technology Inc, Danvers, USA), goat-anti-human $p47^{phox}$ (1:50, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and rabbit anti-nitrotyrosine (1:50, Invitrogen, Eugene, OR, USA) for 1 hour at room temperature and then overnight at 4°C. After a wash with 0.05% (v/v) Tween20 in PBS, the cells were incubated with the secondary antibodies: donkey-anti-rabbit-Cy5 (1:40, Alexa Fluor 647, Invitrogen) or donkey-anti-goat-Cy3 (1:40, Alexa Fluor 568, Invitrogen) for 30 minutes at room temperature in the dark. All antibodies were diluted in Normal Antibody Diluent (Immunologic, Duiven, The Netherlands). After a wash in 0.05% (v/v) Tween20 in PBS, the slides were covered using 4',6-Diamidino-2-Phenylindole (DAPI)-containing mounting medium (Vector Laboratories Inc, Burlingame, CA, USA). Controls using only the secondary antibodies all showed no staining (data not shown).

The slides were analyzed with a 3I Marianas™ digital-imaging microscopy workstation (Zeiss Axiovert 200M inverted microscope; Carl Zeiss, Sliedrecht, The Netherlands) equipped with a nanostepper motor (Z-axis 10 nm) multiple brightfield and darkfield imaging modalities and a thermo-electrically cooled EMCCD camera (QuantEM: 512C, 512×512 pixels; Photometrics, Tucson, AZ, USA). Data acquisition as well as data processing was performed using Slidebook™ software (version 4.2; Intelligent Imaging Innovations, Denver, CO, USA).

shRNA transfection

Rat $p47^{phox}$-specific sequence 5'-CCCATCATCCTTCAGACCTAT-3' targeting nucleotides 477-497 of the rat *Ncf1* gene encoding $p47^{phox}$ (defined by NCBI Refseq NM_053734.2) or the non-targeting sequence 5'-CAACAAGATGAAGAGCACCAA-3' (as a negative control) were cloned into the pLKO.1 shRNA expression vector.[18] pLKO.1 plasmids express 52 basepair shRNA molecules with 21-nucleotide mRNA specificity, driven by the efficient, ubiquitously active U6 snRNA promoter. Cells were transiently transfected with Lipofectamine (Invitrogen), according to the manufactures' protocol. Briefly, 0.8 µg DNA was mixed with 2.0 µL Lipofectamine in 500 µL DMEM without serum and incubated for 20 minutes at RT, after which the transfection mix was applied to 90% confluent cells. 72 hours after transfection, cells were incubated with metabolic inhibition buffer for 4 hours.

RNA isolation and RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen), according to the manufacturers' instructions. RNA quality was ensured via analysis on the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). RNA extracts were DNase-treated (Promega, Mannheim, Germany) prior to cDNA synthesis using specific reverse primers (see below). Quantitative RT-PCR was performed as described previously[19] using the following primers for $p47^{phox}$: forward 5'-CCATTGAGGCCGGTGAGAT-3' (nucleotides 190-208 of NM_053734.2) and reverse 5'-TGCACGCTGCCCATCATA-3' (nucleotides 255-272) and the probe FAM-5'-CATCCCTCACCTCCCAGCTCCCA-3'-TAMRA (nucleotides 227-249) using Universal PCR Master Mix (Life Technologies Europe BV, Bleiswijk, The Netherlands).

Western blotting

After treatment, the nucleus and cytosol fractions of H9C2 cells were separated using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo scientific, Rockford, IL, USA) according to the manufacturers protocol. Equal amounts (20 µg) of protein were dissolved in Laemmli sodium dodecyl sulfate (SDS) sample buffer, stirred and heated at 95°C for 10 minutes. Proteins were separated using 10% (w/v) SDS polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and immunoblotted with rabbit-anti-human FOXO1 (1:1000, Cell Signaling Technology Inc.) or goat-anti-human $p47^{phox}$ (1:500, Abnova, Heidelberg, Germany) for 1 hour at room temperature, followed by incubation overnight at 4°C. After a wash the blots were incubated with mouse-anti-rabbit-HRP or swine-anti-goat-HRP (1:500, Dako, Glostrup, Denmark) for 30 minutes at room temperature, washed again and visualized by enhanced chemiluminescence (ECL; 1:40, Amersham Biosciences AB, Uppsala, Sweden). Protein bands were quantified using a charge-coupled device camera (Fuji Science Imaging Systems, Düsseldorf, Germany) in combination with AIDA Image Analyzer software (Isotopenmessgeräte, Staubenhardt, Germany).

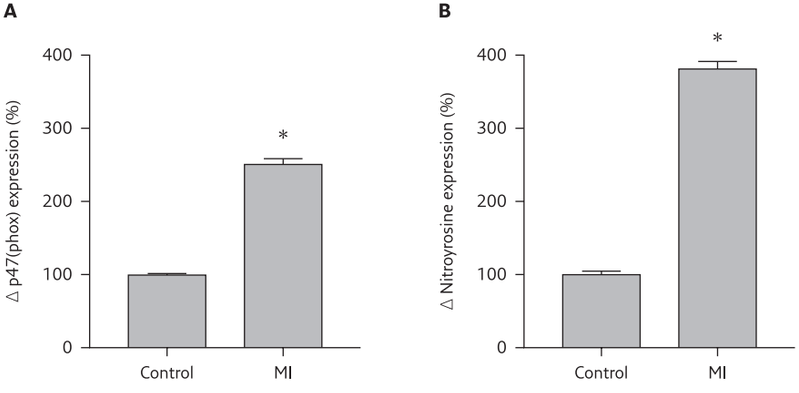
Statistics

The GraphPad Prism program (Windows version 5, GraphPad Software, San Diego California USA) was used for statistical analysis. To evaluate whether observed differences were significant, T-tests or One-way ANOVA with post hoc Bonferroni tests were used when appropriate. All values are expressed as mean ± standard error of the mean (SEM). A p-value (two sided) of 0.05 or less was considered to be significant.

Results

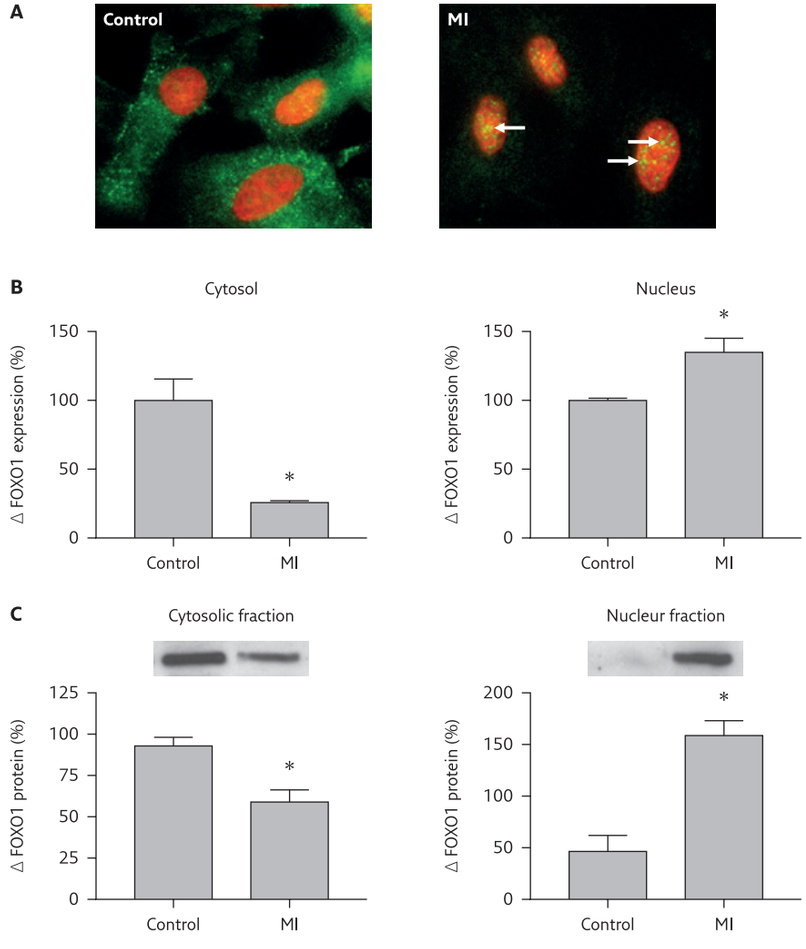
Ischemia induces nuclear translocation of FOXO1

The ischemia-induced nuclear translocation of $p47^{phox}$ and local ROS production that we have reported previously[15] was verified via fluorescent digital-imaging microscopy of H9c2 rat cardiomyoblasts. Metabolic inhibition induced a significant 152±8% increase in nuclear $p47^{phox}$ expression (figure 1A, $p<0.0001$) and a significant 382±10% increase in expression of nitrotyrosine, an indirect marker for ROS production (figure 1B, $p<0.0001$). These results thus confirm ischemia-induced nuclear $p47^{phox}$ expression and local ROS production.



1. Ischemia induces increased nuclear expression of $p47^{phox}$ and nitrotyrosine | Quantitative fluorescent digital imaging analysis of $p47^{phox}$ (A) and nitrotyrosine expression (B) in the nucleus of H9c2 cells subjected to control conditions or 4 hours of metabolic inhibition (MI). The expressions are depicted as the difference (△) in percentage compared to control cells (n=4).

Subsequently, the effect of ischemia on the subcellular localization of FOXO1 in H9c2 cells was analyzed using quantitative digital imaging fluorescence microscopy. In control cells FOXO1 expression was found mainly in the cytosol with occasional focal expression in the nucleus (figure 2A, left panel), while in cells subjected to metabolic inhibition most FOXO1 was detected in the nucleus (figure 2A, right panel). Metabolic inhibition induced a significant 75±2% decrease in cytosolic FOXO1 expression ($p<0.0005$) and a significant 35±20% increase in nuclear FOXO1 ($p<0.004$) compared to control cells (figure 2B).



2. Ischemia induces nuclear translocation of FOXO1 | Analysis of the subcellular localization of FOXO1 in H9c2 cells subjected to control conditions or 4 hours of metabolic inhibition (MI), using quantitative fluorescent digital imaging microscopy and Western blot. (A) Example of the subcellular localization of FOXO1 (green signal). Nuclei were stained with DAPI (red signal). Arrows indicate dispersed expression of FOXO1 in the nucleus (right picture). Original magnification 40x. (B) Quantification of fluorescent digital imaging analysis of FOXO1 expression in the cytosol (*p=0.0005) and the nucleus (*p=0.004), depicted as the difference (△) in percentage compared to control cells (n=7). (C) Quantification of Western blot analysis of FOXO1 in the cytosolic (*p<0.01) and nuclear (*p<0.01) fractions, shown as the relative difference (△) compared to control cells (n=4).

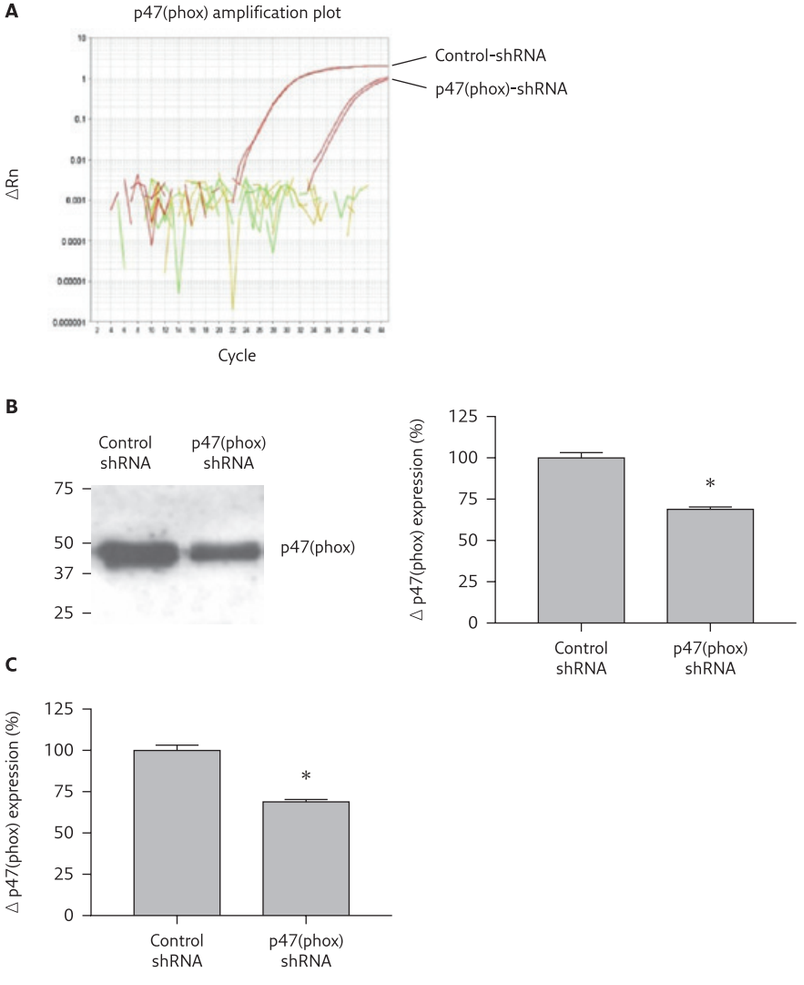
In addition, ischemia-induced subcellular expression of FOXO1 in H9c2 cells was validated via Western blot analysis of isolated nuclear and cytosol fractions. The high level of FOXO1 in the cytosol fraction of control cells was significantly decreased by 37±8% after metabolic inhibition (figure 2C, $p<0.01$). In contrast, only little FOXO1 protein was found in the nuclear fraction of control cells minor expression was found, while metabolic inhibition significantly increased nuclear FOXO1 by 71±16% (figure 2C, $p<0.01$). To ensure successful separation of the nuclear and cytosol fractions, immunoblotting for Lamin B1, an intermediate-filament protein of the nuclear lamina, was performed. Lamin B was detected only in the nuclear fractions (data not shown).

We concluded that ischemia induced nuclear translocation of the transcription factor FOXO1 in H9c2 cells.

Inhibition of p47^{phox}-mediated ROS production reduces nuclear FOXO1 expression

To study the effect of p47^{phox}-mediated ROS production on the subcellular localization of FOXO1, shRNA knockdown of p47^{phox} was used. The effectiveness of the p47^{phox}-specific shRNA on the expression of p47^{phox} mRNA, p47^{phox} protein and nuclear expression of p47^{phox} in H9c2 cells subjected to metabolic inhibition were validated via quantitative real time (qRT)-PCR, Western blot and quantitative digital imaging fluorescence microscopy respectively.

Transfection of H9c2 cells with p47^{phox}-shRNA resulted in a down-regulation of p47^{phox} mRNA (figure 3A), as well as a significant decrease in p47^{phox} protein (figure 3B, $p<0.04$) compared to cells transfected with control shRNA. In addition, p47^{phox}-shRNA significantly reduced the nuclear expression of p47^{phox} protein (figure 3C, $p<0.001$). These results show successful knockdown of p47^{phox} by p47^{phox}-shRNA.

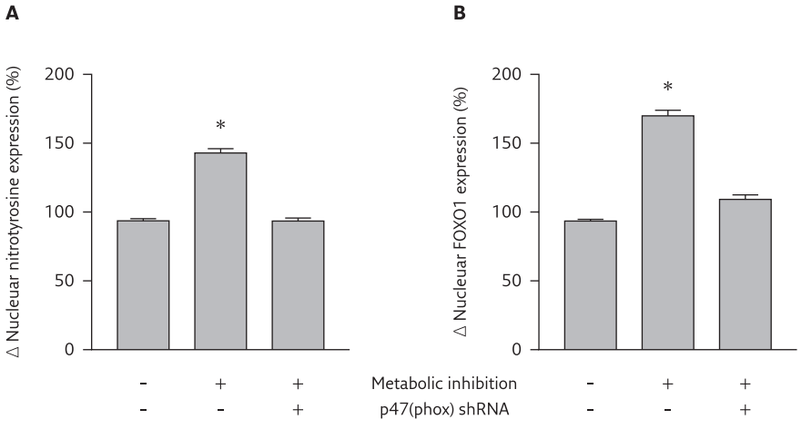


3. Transfection of p47^{phox}-shRNA inhibits p47^{phox} expression | Efficacy validation of p47^{phox}-specific shRNA transfection of H9c2 cells on the expression of p47^{phox}. (A) Quantitative RT-PCR analysis of cellular p47^{phox} mRNA levels in cells transfected with either p47^{phox}-shRNA or control-shRNA and subsequently subjected to 4 hours of metabolic inhibition (n=3), (B) Western blot analysis of cellular p47^{phox} protein levels in cells transfected with either p47^{phox}-shRNA or control-shRNA and subsequently subjected to 4 hours of metabolic inhibition (n=3) and (C) Quantification of fluorescent digital imaging microscopy of nuclear p47^{phox} expression in cells transfected with either p47^{phox}-shRNA or control-shRNA and subsequently subjected to 4 hours of metabolic inhibition (n=3), $p<0.001$.

In addition, the shRNA knock-down of nuclear $p47^{phox}$ expression coincided with a significant reduction of nuclear ROS levels as the nuclear nitrotyrosine expression levels induced by metabolic inhibition were 53±3% lower after $p47^{phox}$ knock-down compared to metabolic inhibition alone (figure 4A, $p<0.001$). These nuclear nitrotyrosine expression levels after $p47^{phox}$ knockdown were comparable to those in control cells.

Interestingly, $p47^{phox}$-shRNA induced a significant 65±4% reduction in metabolic inhibition-induced nuclear FOXO1 expression compared to metabolic inhibition alone (figure 4B, $p<0.001$), resulting in nuclear FOXO1 expression levels comparable to control cells.

These results point to $p47^{phox}$-mediated ROS production as a crucial mediator of FOXO1 translocation to the nucleus in ischemic H9c2 cells.



4. $p47^{phox}$ knock-down reduces nuclear ROS and reduces nuclear translocation of FOXO1 | Quantitative fluorescent digital imaging analysis of nitrotyrosine (A) and FOXO1 expression (B) in the nucleus of H9c2 cells subjected to control conditions or to 4 hours of metabolic inhibition (MI) either or not preceded by transfection with $p47^{phox}$-specific shRNA (shRNA). The expressions are depicted as the difference (△) in percentage compared to control cells (n=4). (A) ∗$p<0.001$, (B) ∗$p<0.001$.

Discussion

The family of FOXO transcription factors has been shown to be crucially involved in cell-fate decisions and is significantly involved in a number of cardiovascular, oncological and neurological disorders. It has been well established that ROS are involved in their control, although information on the cellular source(s) of ROS involved is scarce. Recently we have shown $p47^{phox}$-dependent ROS production at the nuclear pore in ischemic H9c2 cells,[15] a hypothetically ideal localization to affect proteins translocating in or out of the nucleus. In the current study we for the first time show that ischemia-induced nuclear translocation of FOXO1 depends critically on $p47^{phox}$-dependent ROS production in H9c2 cells.

Although NOX proteins have been shown to be important in mediating redox-sensitive signal transduction, so far only two recently published studies provide evidence for a direct link between NOXes and FOXO.[13/14] In human pulmonary artery SMC NOX4 over-expression increased FOXO3a activity and NOX4 knock-down decreased FOXO3a activity,[13] whereas NOX1 knock-out significantly reduced FOXO4 phosphorylation in mice hepatic stellate cells,[14] suggesting that different NOX isoforms can be involved in FOXO control. Indeed, the $p47^{phox}$-induced nuclear translocation of FOXO1 we show now implicates a role for the NOX2 isoform herein, as $p47^{phox}$ predominantly activates NOX2 and we earlier found colocalization of $p47^{phox}$ with NOX2 and ROS in ischemic H9c2 cells.[15] However, although NOX4 is not activated by $p47^{phox}$, we cannot exclude the possibility of involvement of the NOX1 isoform, since it was shown in mouse endothelial cells *in-vivo* that in addition to the NOX organizing protein 1 (NOXO1), $p47^{phox}$ can also activate NOX1.[20] Furthermore, it has been shown that H9c2 cells express NOX1.[21]

In human monocytes cultured under hyperglycemic conditions Yun *et al.* observed an increase in overall $p47^{phox}$ expression, coinciding with a decrease in overall FOXO3a expression.[22] However, since only their respective expression levels were analyzed, this study did not provide evidence for a direct link between $p47^{phox}$ and FOXO3a. Similarly, in NIT-1 mouse pancreatic β-cells cultured in the presence of free fatty acids, increased cellular ROS levels coincided with nuclear FOXO1 translocation.[23] However, although it was determined that siRNA-induced knock-down of NOX2 did reduce ROS, the effect thereof on FOXO1 translocation was not analyzed. Remarkably, whereas NOX4 in SMCs[13] and $p47^{phox}$ in H9c2 cells (in the current study) were shown to induce FOXO target gene activation and nuclear translocation respectively, indicative of FOXO activation, NOX1[14] was suggested to increase FOXO4 phosphorylation, indicative of inactivation. This suggests that different NOX isoforms may have opposing effects on FOXO activity. Whether these various NOX isoforms control different FOXO family members or to what extent species, cell type,

or (patho)physiological stimulus determines their involvement and the outcome of their activity remains to be determined.

Controversy exists regarding the role of FOXO1 in response to ischemia. Nuclear translocation and activation of FOXO1 in response to ischemia or ischemia/reperfusion has been shown before in mouse liver,[24], in mouse and rat cardiomyocytes,[10/25/26] in gerbil and mouse neurons[27] and in H9c2 cells.[28] In these studies FOXO1 was shown to have both a pro-apoptotic[24/26/27] and/or an anti-apoptotic (cell protective) effect.[10/25/28] We have shown previously that $p47^{phox}$/NOX2-induced ROS production at the nucleus in H9c2 cells in response to ischemia contributes to apoptosis induction,[15/29] suggesting that the $p47^{phox}$-induced nuclear translocation of FOXO1 we show now could also contribute to apoptosis. Although the possibility of $p47^{phox}$ involvement in both pro- and anti-apoptotic signaling simultaneously cannot be ruled out.

The molecular mechanism of FOXO1 nuclear translocation regulation by the ROS production induced by $p47^{phox}$ is not yet clear. ROS have been shown to regulate FOXO1 activity directly, as well as indirectly through protein kinases that affect FOXO1. For instance, activation of FOXO4 was shown to depend on oxidation of a specific highly conserved cysteine residue in FOXO4 (Cys477) in human HEK293t cells,[30] indicating that ROS can affect FOXO transcription factors directly. On the other hand, in mouse fibroblast and myocyte cell lines and in human HEK293t cells, H_2O_2-induced nuclear translocation and transcriptional activation of FOXO4 depended on its phosphorylation by *c-Jun* N-terminal kinase (JNK),[31] whereas in rat granular neurons Ste20-like kinase 1 (MST1) induced phosphorylation and activation of FOXO3 in response to H_2O_2 treatment.[32]

In conclusion, in this study we show for the first time that in H9c2 ischemia-induced translocation of FOXO1 to the nucleus cells critically depends on $p47^{phox}$-dependent ROS production.

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