Chapter 2

NOX2, p22\textsuperscript{phox} and p47\textsuperscript{phox} are targeted to the nuclear pore complex in ischemic cardiomyocytes colocalizing with local reactive oxygen species

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Abstract

NADPH oxidases play an essential role in reactive oxygen species (ROS)-based signaling in the heart. Previously, we have demonstrated that (peri)nuclear expression of the catalytic NADPH oxidase subunit NOX2 in stressed cardiomyocytes, e.g. under ischemia or high concentrations of homocysteine, is an important step in the induction of apoptosis in these cells. Here this ischemia-induced nuclear targeting and activation of NOX2 was specified in cardiomyocytes.

The effect of ischemia, mimicked by metabolic inhibition, on nuclear localization of NOX2 and the NADPH oxidase subunits p22phox and p47phox, was analyzed in rat neonatal cardiomyoblasts (H9c2 cells) using Western blot, immuno-electron microscopy and digital-imaging microscopy.

NOX2 expression significantly increased in nuclear fractions of ischemic H9c2 cells. In addition, in these cells NOX2 was found to colocalize in the nuclear envelope with nuclear pore complexes, p22phox, p47phox and nitrotyrosine residues, a marker for the generation of ROS. Inhibition of NADPH oxidase activity, with apocynin and DPI, significantly reduced (peri)nuclear expression of nitrotyrosine.

We for the first time show that NOX2, p22phox and p47phox are targeted to and produce ROS at the nuclear pore complex in ischemic cardiomyocytes.

Introduction

Reactive oxygen species (ROS) are oxygen-containing molecules with one or more unpaired electrons that can be highly reactive with other molecules. For many years ROS were viewed as the inevitable but unwanted by-product of an aerobic existence that inflict cellular damage by reacting with macromolecules such as DNA, lipids and proteins. However, it is now known that ROS at lower concentrations function as signaling molecules that react with cysteine residues on certain proteins and thereby alter their functional state. Through this so called redox signaling, ROS are involved in the regulation of diverse physiological processes such as cell proliferation, migration, gene expression and apoptosis.

The multi-component NADPH oxidase has been shown to play an essential role in redox-dependent signaling. Because ROS are diffusible and short-lived molecules, tight regulation of the activation and localization of NADPH oxidase is essential for mediating redox signaling at the right place and time. The mechanism of activation of NADPH oxidase is well characterized in neutrophilic granulocytes and is known to occur through a complex series of interactions with several subunits/activating proteins which in resting cells reside in the cytosol. The flavocytochrome b23 is the
central membrane-associated component and is composed of the catalytic subunit gp91
phox (NOX2) non-covalently bound to p22phox that provides membrane stabilization and a docking site for the cytosolic subunit/activating protein p47phox. As such, at the onset of the respiratory burst in intact neutrophilic granulocytes, p47phox is phosphorylated and translocates to the cytoplasmic region of p22phox. Like p22phox, NOX2 also contains binding sites for phosphorylated p47phox to form the active enzyme. While the activation process of NADPH oxidase has been elucidated in detail in neutrophilic granulocytes, the precise structure and mechanisms of activation/targeting of NADPH oxidase in cardiomyocytes is less known. Our group has shown that ischemia or high concentrations of homocysteine induced (peri)nuclear targeting of neutrophilic granulocytes, the precise structure and mechanisms of activation/

Western blotting

H9c2 cells were grown to a confluency of 70-90%. After treatment the cells were lysed in buffer and nuclear and cytosolic membranes were separated using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo scientific, Rockford, USA). Samples were dissolved in Laemmli sodium dodecyl sulfate (SDS) sample buffer, stirred and heated at 95°C for 10 minutes. The samples were subjected to SDS polyacrylamide 10% gel electrophoresis, transferred onto nitrocellulose membranes and immunoblotted with mouse anti-NOX2, rabbit anti-Lamin b1, goat anti-rabbit HRP (1:500, Dako, Glostrup, Denmark) or goat anti-rabbit HRP (1:500, Dako) for 30 minutes at RT. Blots were visualized by enhanced chemiluminescence (1:40, Amersham Biosciences AB, Uppsala, Sweden) and quantified with a charge-coupled device camera (Fuji Science Imaging Systems, Düsseldorf, Germany) in combination with AIDA Image Analyzer software (Isotopenmessgeräte, Staubenhardt, Germany). To ensure successful separation of these fractions, immunoblotting for nuclear protein Lamin b1, an intermediate-filament protein of the nuclear lamina, was performed. Lamin b1 was detected only in the nuclear fractions (data not shown).

Immuno-Electron Microscopy

Ischemically challenged H9c2 cells were fixed for 2 hours in 2% paraformaldehyde with 0.2% glutaraldehyde in 0.1M phosphate buffer (50 mM PIPES, 2 mM MgCl2, 10 mM EGTA, pH 6.9) and then processed for ultrathin cryosectioning as previously described. Briefly, 50-nm cryosections were cut at -120°C using diamond knives in a cryoultramicrotome (Leica Aktiengesellschaft, Vienna, Austria) and transferred with a mixture of sucrose and methylcellulose onto formvar-coated copper grids. The grids were placed on 35 mm petri dishes containing 2% gelatine.

Materials and Methods

Cell-culture and metabolic inhibition

Rat cardiomyoblasts (H9c2 cells), derived from embryonic rat hearts, were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA) and cultured in culture medium: dulbecco’s modified eagles medium (DMEM, Cambrex Corporation, East Rutherford, NJ, USA) with addition of 10% (v/v) heat inactivated fetal calf serum (FCS, BioWhittaker, Walkersville, MD, ASU), 100 IU/ml penicillin (Yamanouchi Europe BV, Meppel, The Netherlands), 100 μg/ml streptomycin (Radiopharma Fisiopharme, Palomonte, Italy) and 2 mM L-glutamine (Invitrogen Corporation, Carlsbad, CA, USA). H9c2 cells were cultured at a 5% CO2 atmosphere at 37°C. To mimic ischemia H9c2 cells were incubated for 2 hours with a metabolic inhibition buffer (0.9 mM CaCl2, 106 mM NaCl, 3.8 mM NaHCO3, 4.4 mM KCl, 1 mM MgCl2, H2O, pH 6.6), including 20 mM 2-deoxyglucose to impair glycolysis, and 5 mM NaCN to impair the mitochondrial electron transport chain.
Digital-Imaging Microscopy

Two days before metabolic inhibition 19c2 cells were passaged onto sterile Lab-Tek II 4-well chamber CC:2 glass slides (Nalge Nunc International, Naperville, IL, USA). Apocynin (100 μM, Sigma-Aldrich, St. Louis, MO, USA) and diphenylene iodonium (DPI; 10 μM, Sigma-Aldrich) were used to inhibit NADPH oxidase activity. After treatment, cells were fixed with 4% paraformaldehyde for 10 minutes at 37°C and permeabilized with 0.2% Triton for 10 minutes at RT. The cells were subsequently incubated with the primary antibodies for 1 hour at RT followed by incubation overnight at 4°C. The primary antibodies were rabbit anti-gp91phox (NOX2; 1:50, Upstate, North Billerica, MA, USA), mouse anti-p22phox,10 (1:25), goat anti-p47phox (1:50, Santa Cruz Biotechnology Inc, Heidelberg, Germany), rabbit anti-nitrotyrosine, as an indirect marker of ROS generation20 (1:50, Invitrogen, Carlsbad, California, USA) and mouse anti-nucleoporins (monoclonal 414,11 NUP62, NUP153, NUP214 and NUP358, protein complexes associated with the nuclear pore complex;21-25). The cells were then incubated with the secondary antibodies Alexa Fluor 568-labeled donkey anti-goat (Invitrogen, 1:40), Alexa Fluor 488-labeled donkey anti-mouse (Invitrogen, 1:40), and Alexa Fluor 647-labeled donkey anti-rabbit (Invitrogen, 1:40) for 30 min at RT in the dark. Negative controls with only the secondary antibody were included to assess nonspecific binding. All negative controls showed no staining (data not shown). Before visualization, HardSet mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI; H-1500, Vector Laboratories Inc, Burlingame, CA, USA) was added and the cells were covered. 2D/3D stack optical sections were acquired and analyzed with a 31 Marianas digital-imaging microscopy workstation (Zeiss Axiovert 200M inverted microscope; Carl Zeiss, Sliedrecht, The Netherlands) equipped with a nanostepper motor (2-axis increments: 10 nm) and a thermo-electrically cooled EM CCD camera (Quantum: 512x512 pixels; Photometrics, Tucson, AZ, USA). Exposures, objectives and pixel binning were automatically recorded with each 3D stack/2D image and stored in memory (Dell Dimension workstation: 3.0 GHz Xenon dual processor, 4 GB RAM). The microscope, camera and all other aspects of data acquisition as well as data processing were controlled by Slidebook software (version 4.2; Intelligent Imaging Innovations, Denver, CO, USA).

Statistics

The GraphPad Prism program (Windows version 5) 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. 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

Nuclear NOX2 expression is upregulated in ischemic cardiomyocytes

Previously we observed using fluorescent microscopy that NOX2 targeted to the nucleus in cardiomyocytes under ischemic insult. Now, we quantified NOX2 in nuclear fraction of non-ischemic (control) and ischemic cardiomyocytes using Western blot analysis. NOX2 protein was detected at approximately 60 kDa (figure 1). In the nuclear fraction of control cardiomyocytes a low basal level of NOX2 was found (figure 1A). Ischemia significantly increased the amount of NOX2 in the nuclear fraction 1.87±0.06 fold (p<0.001). Similarly, in the cytosol/membrane fraction a low basal level of NOX2 was found in control cardiomyocytes that significantly increased 1.37±0.05 fold after ischemia (p<0.01) (figure 1B).

1. Nuclear NOX2 expression is upregulated in ischemic cardiomyocytes | Western blot analysis of NOX2 expression in nuclear (a) and cytosol/membrane (b) fractions of control and ischemic H9c2 cells. 30 μg per lane of protein was added. The graphs represent the signal intensities relative to those in control cells (n=3).
NOX2 colocalizes with the nuclear pore complex and ROS at the nuclear envelope in ischemic cardiomyocytes

The induced nuclear localization of NOX2 in ischemic cardiomyocytes was further specified using immuno-electron microscopy. In ischemic H9C2 cells, NOX2 was found in and in close proximity of the nuclear envelope (figure 2, arrows).

Because external and internal membranes of the nuclear envelope are fused at the site of nuclear pore complexes (NPCs),22/23 that are involved in coordinating the delivery of genetic information to the cytoplasmic protein synthesis machinery and other nucleocytoplasmic exchange,24 we next analyzed NOX2 localization in relation to the NPC using digital-imaging microscopy (figure 3). In ischemic H9C2 cells 3D stack images showed (peri)nuclear NOX2 expression (figure 3-IA/IIA, red signal). NOX2 was found to colocalize with NUP (figure 3-III, green signal) as shown in figure 3-IC (yellow signal, arrows). NOX2 was found dispersed (peri)nuclear and also colocalized with local ROS (figure 3-IIIb, green signal) as shown in figure 3-IIIc (yellow signal, arrows). In control cells 3D stack images showed a very low basal (peri)nuclear presence of NOX2 (figure 3-IE), while no (peri)nuclear nitrotyrosine was found (figure 3-IIIE).

2. Localization of NOX2 at the nuclear envelope in ischemic cardiomyocytes | Immuno-electron microscopy showing the association of NOX2 with the nuclear envelope in ischemic H9C2 cells. The nuclear envelope (NE) is separating the nucleus (N) from the cytosol (C). Gold particles labeling NOX2 (arrows) are located in close proximity of the nuclear envelope.

3. NOX2 colocalizes with ROS production and the nuclear pore complex in ischemic cardiomyocytes | Nuclear localization of NOX2 in H9C2 cells as shown by digital-imaging microscopy. Cells were stained for NOX2 (red: I and II), NUP (green: I) or nitrotyrosine (green: II). DNA was stained by dapi (blue). Pictures I demonstrate that under ischemia (peri)nuclear NOX2 focially coincides with NUP (ID) or ROS (IID), visible as yellow signal (arrows). Pictures I demonstrate that under control very low basal (peri)nuclear NOX2 is present (IIE), while no (peri)nuclear nitrotyrosine was found (IIIE). Original magnification 63x (n=4).
The NADPH oxidase subunits \( p47^{\text{phox}} \) and \( p22^{\text{phox}} \) colocalize with NOX2 at the nuclear pore complex in ischemic cardiomyocytes

To analyze whether the NADPH oxidase subunits \( p47^{\text{phox}} \) (needed for activation and targeting of NADPH oxidase)\(^{25}\) and \( p22^{\text{phox}} \) (needed for membrane stabilization and docking site for NADPH oxidase)\(^{7}\) colocalize with NOX2 at the NPC in ischemic cardiomyocytes, these subunits were also analyzed using digital-imaging microscopy. In ischemic h9c2 cells (peri)nuclear NOX2 (figure 4-ia, blue signal) colocalized with both \( p47^{\text{phox}} \) (figure 4-ib, red signal) and \( p22^{\text{phox}} \) (figure 4-ic, green signal) as shown in figure 4-id (white signal, arrows). This colocalization of NOX2 with \( p47^{\text{phox}} \) was found to be homogenous, while \( p22^{\text{phox}} \) was found dispersed (figure 4-id). This (peri) nuclear expression of \( p47^{\text{phox}} \) (figure 4-ia, red signal) and \( p22^{\text{phox}} \) (figure 4-ic, green signal) also colocalized with NUP (figure 4-ia, blue signal) as shown in figure 4-id (white signal, arrows), indicating that in addition to NOX2, these NADPH oxidase subunits are also expressed at the NPC in ischemic cardiomyocytes. In the (peri)nuclear region of control cells, 3D stack images showed a low basal presence \( p47^{\text{phox}} \), while \( p22^{\text{phox}} \) was virtually absent (figure 4-iie).

4. The NADPH oxidase subunits \( p47^{\text{phox}} \) and \( p22^{\text{phox}} \) colocalize with NOX2 at the nuclear pore complex in ischemic cardiomyocytes | Nuclear localization of NOX2 and the NADPH oxidase subunits \( p47^{\text{phox}} \) and \( p22^{\text{phox}} \) in h9c2 cells as shown by digital-imaging microscopy. Cells were stained for \( p47^{\text{phox}} \) (red: i and ii), \( p22^{\text{phox}} \) (green: i and ii), NOX2 (blue: i) or NUP (blue: ii). Picture d depicts that under ischemia (peri)nuclear \( p47^{\text{phox}} \) and \( p22^{\text{phox}} \) colocalize with NOX2 (i0) or NUP (i0), visible as white signal (arrows). Pictures e demonstrate that under control low basal \( p47^{\text{phox}} \) is present, while \( p22^{\text{phox}} \) was virtually absent (iie). Original magnification \( \delta 3x \ (n=4) \).
Inhibition of NADPH oxidase reduced ischemia-induced (peri)nuclear ROS production

To assess whether the observed nuclear ROS production was due to NADPH oxidase activity, the effects of the NADPH oxidase inhibitors apocynin and DPI on ischemia-induced nitrotyrosine expression were analyzed using digital-imaging microscopy.

As expected, ischemia significantly increased the presence of nitrotyrosine in (peri)nuclear regions with 88.8±5% (p<0.001) compared to control cells (figure 5). Apocynin and DPI significantly reduced the presence of nitrotyrosine in (peri)nuclear regions compared to ischemia with 101.2±3% and 82.9±3%, respectively (p<0.001). Both these inhibitors reduced the (peri)nuclear levels of nitrotyrosine to those found in control cells, indicating that the increase in (peri)nuclear ROS under ischemia is predominantly due to NADPH oxidase activity.

Discussion

In previous work we have demonstrated in cardiomyocytes that NOX2 is upregulated under ischemia or high concentrations of homocysteine, and is translocated to (peri)nuclear regions, colocalizing with apoptosis. We, to the best of our knowledge, are the first to show now that NOX2, p22phox and p47phox colocalize with the NPC in ischemic H9c2 cells, colocalizing with local ROS production.

Cell signaling is mediated by specific and reversible modifications of proteins and other biomolecules that participate in specific cascades of signal transduction. Most well-known among these modifications is the phosphorylation and dephosphorylation of molecules. However, in recent years several other types of modifications have been shown to also be of importance. Most prominent among these newly discovered modifications are the oxidative modifications that are at the base of redox signaling.

While there are several groups of enzymes that generate ROS for signal transduction, the NADPH oxidases are considered unique in that they generate ROS in a highly regulated manner. Unlike kinases and phosphatases the NADPH oxidases have no target-specific protein surface that mediates signaling specificity. Instead, the NADPH oxidase site of ROS generation has to be brought into close proximity of the reactive cysteine of the target protein to warrant specific signal transduction.

Crucial for its function is that NADPH oxidases are targeted to their site of action as part of a multi-protein complex. This has extensively been studied in neutrophilic granulocytes. However, recent studies report that the same is true for vascular cells. The NADPH oxidase subunit p47phox namely has been demonstrated to form an adaptor between NADPH oxidase and its targeting complexes in caveolae/lipid rafts of angiotensin II-stimulated human vascular smooth muscle cells, and in lamellipodial leading edges of vascular endothelial growth factor (VEGF)- and tumor necrosis factor alpha (TNFα)-stimulated human endothelial cells. In endothelial cells stimulated with TNFα also an increase in p47phox/p22phox complex formation were found in immunoprecipitations of whole-cell extracts. Next to endothelial cells, in hearts of spontaneously hypertensive rats as well as salt-sensitive hypertensive rats quantitative PCR showed an increased left ventricular mRNA of p47phox and p22phox, coinciding with elevated ROS levels. Furthermore, superoxide
release (determined by chemiluminescence) coinciding with increased p47phox expression in cardiomyocyte membrane extractions has also been found in human left ventricular myocardium from patients with ischemic cardiomyopathy. However, in these studies total membrane extractions were used without distinction between subcellular compartments. We now show that NOX2, p47phox and p22phox are targeted at the NPC in ischemic cardiomyocytes. This coincided with a significant increase in (peri)nuclear nitrotyrosine, indicative for ROS production at that location. Furthermore, the NADPH oxidase inhibitors apocynin and DPI both significantly counteracted this ischemia-induced (peri)nuclear ROS production, indicating that the NADPH oxidase components at the nucleus form an active complex.

The NPC is a large channel-like structure in the nuclear envelope that bridges the gap between the external and internal layers of the envelope. Composed of >500-1000 proteins that represent >30 different NUP’s, the obvious function of the pore complex is to control the passage of molecules between the nucleus and the cytoplasm. However, in recent years it has been shown to also play an important role in the regulation of nuclear processes, such as DNA replication, DNA repair, transcription and RNA processing. Currently we can only speculate regarding the exact mechanism(s) whereby NOX2-related ROS interfere with apoptotic signalings in ischemic cardiomyocytes. The specific localization we now found of an active ROS producing NOX2-containing NADPH oxidase complex at the NPC offers some interesting mechanistic possibilities regarding its function. There are a number of redox-sensitive transcription factors that are either activated or inactivated through redox modifications. Two redox-sensitive transcription factors, activator protein 1 (AP-1) and nuclear factor kappa B (NF-kB), have been implicated in the regulation of cardiomyocyte apoptosis. NOX2-related ROS production at the NPC can thus regulate gene expression via redox modification of transcription factors and in this way contribute to the induction of apoptosis in ischemic cardiomyocytes. On the other hand, ROS, via the formation of peroxynitrite, may introduce DNA damage directly and, in this way, can also contribute in the process of apoptosis. Studies in C. elegans and rat brain have shown an age-dependent deterioration (i.e. increased leakiness) of the NPC linked to oxidative stress. It is well-known that during apoptosis the permeability of the NPC increases through caspase-mediated proteolysis of specific NUP’S. ROS-mediated oxidation of NPCs may therefore contribute to the increased permeability of the NPC perhaps during early stages of apoptosis. Other mechanism(s) of NOX2-mediated ROS involved in induction of cardiomyocyte apoptosis, however, can not be excluded.

Taken together, we have shown that NOX2, p22phox and p47phox are targeted to the NPC in ischemic cardiomyocytes colocalizing with local ROS production.
Chapter 2