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NOX2, p22^{phox} and p47^{phox} are Targeted to the Nuclear Pore Complex in Ischemic Cardiomyocytes Colocalizing with Local Reactive Oxygen Species

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Key Words

Cardiomyocytes • NADPH oxidase • Reactive oxygen species • Nuclear pore complex • Apoptosis

Abstract

Background: 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. Methods: The effect of ischemia, mimicked by metabolic inhibition, on nuclear localization of NOX2 and the NADPH oxidase subunits p22^{phox} and p47^{phox}, was analyzed in rat neonatal cardiomyoblasts (H9c2 cells) using Western blot, immuno-electron microscopy and digital-imaging microscopy. Results: 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, p22^{phox}, p47^{phox} 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. Conclusion: We for the first time show that NOX2, p22^{phox} and p47^{phox} 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 [1]. 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 [2]. 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 [3].

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The multi-component NADPH oxidase has been shown to play an essential role in redox-dependent signaling [4, 5]. 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 [6]. The flavocytochrome b_{558} is the central membrane-associated component and is composed of the catalytic subunit gp91^{phox} (NOX2) non-covalently bound to p22^{phox} that provides membrane stabilization and a docking site for the cytosolic subunit/activating protein p47^{phox} [7]. As such, at the onset of the respiratory burst in intact neutrophilic granulocytes, p47^{phox} is phosphorylated and translocates to the cytoplasmic region of p22^{phox} [7]. Like p22^{phox}, NOX2 also contains binding sites for phosphorylated p47^{phox} to form the active enzyme [8].

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 NOX2 expression in cardiomyocytes [9, 10]. This (peri)nuclear NOX2 expression colocalized with local ROS generation resulting in apoptosis. Inhibition of ROS, using the flavoenzyme inhibitor diphenylene iodonium (DPI) or the NADPH oxidase inhibitor apocynin namely led to a significant decrease of apoptosis [9]. The NADPH oxidase subunits p22^{phox} and p47^{phox} have both been demonstrated to coincide with elevated ROS levels in rat hearts [11-13]. Quantitative PCR namely showed increased levels of left ventricular mRNA of p22^{phox} and p47^{phox} in spontaneously hypertensive rats [11] as well as salt-sensitive hypertensive rats [12, 13]. Next to p22^{phox} and p47^{phox}, increased levels of left ventricular mRNA of gp91^{phox} were also found [11, 13]. However, in these studies total left ventricular mRNA was used and therefore no distinction was made between cardiomyocytes and other cardiovascular cells [11, 13]. Neither protein levels, nor subcellular locations of these components were analyzed. Furthermore, in human left ventricular myocardium from patients with ischemic cardiomyopathy, superoxide release (determined by chemiluminescence) coincided with increased cardiomyocyte membrane expression of p47^{phox} [14]. However, for this total membrane extractions were used without distinction between subcellular compartments [14]. As far as we know, targeting of NOX2, p22^{phox} and p47^{phox} to (peri)nuclear regions in ischemic cardiomyocytes has not been studied before. This we have analyzed in the present study in ischemic cardiomyocytes.

Materials and Methods

Cell-culture and metabolic inhibition

Rat cardiomyoblasts cells (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 ug/ml streptomycin (Radiopharma Fisiopharme, Palomonte, Italy) and 2 mM L-glutamine (Invitrogen Corporation, Carlsbad, CA, USA). H9c2 cells were cultured at a 5% CO_2 atmosphere at 37°C. To mimick ischemia H9c2 cells were incubated for 2 hours with a metabolic inhibition buffer (0.9 mM $CaCl_2$ H_2O, 106 mM NaCl, 3.8 mM $NaHCO_3$, 4.4 mM KCL, 1 mM $MgCl_2$ H_2O, pH 6.6), including 20 mM (2-deoxy)glucose to impair glycolysis, and 5 mM NaCN to impair the mitochondrial electron transport chain [15].

Western blotting

H9c2 cells were grown to a confluency of 70-90%. After treatment the cells were lysed in buffer and nuclear and cytosol/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 ([16], 1:250) or rabbit anti-Lamin B1 (1:250, Santa Cruz Biotechnology Inc, Heidelberg, Germany) for 1 hour at RT, followed by incubation o/n at 4°C. The following day, blots were washed and incubated with rabbit anti-mouse 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 [17], was performed. Lamin B 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 0.1 M

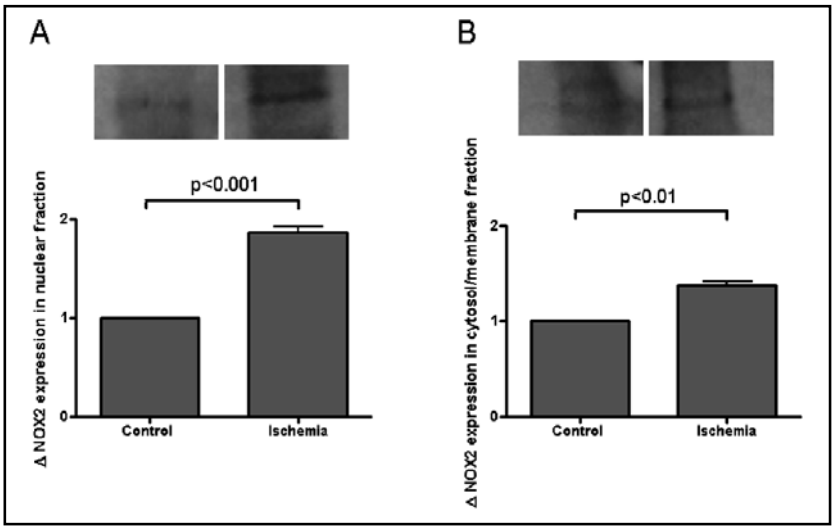

Fig. 1. Nuclear NOX2 expression is upregulated in ischemic cardiomyocytes. Western blot analysis of NOX2 expression in nuclear (A) and cytosol/membrane (B) fractions of control and ischemic H9c2 cells. 30 μg per lane of protein was added. The graphs represent the signal intensities relative to those in control cells (n=3).

PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9) and then processed for ultrathin cryosectioning as previously described. Briefly, 50-nm cryosections were cut at -120º 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. Ultrathin frozen sections were incubated at room temperature (RT) with mouse anti-NOX2 ([16], 1:30) followed by a rabbit anti-mouse bridging antibody and then incubated with 10-nm protein A-conjugated colloidal gold (EM Lab, Utrecht University, Netherlands) as described [18]. After immunolabeling, the sections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM10 electron microscope (FEI company, Eindhoven, The Netherlands).

Digital-Imaging Microscopy

Two days before metabolic inhibition H9c2 cells were passaged onto sterile Lab-Tek II 4-well chamber CC2 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 [9]. 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-$gp91^{phox}$ (NOX2: 1:50, Upstate, North Billerica, MA, USA), mouse anti-$p22^{phox}$ ([19], 1:25), goat anti-$p47^{phox}$ (1:50, Santa Cruz Biotechnology Inc, Heidelberg, Germany), rabbit anti-nitrotyrosine, as an indirect marker of ROS generation [20] (1:50, Invitrogen, Carlsbad, California, USA) and mouse anti-nucleoporins (monoclonal 414 [21], NUP62, NUP153, NUP214 and NUP358, protein complexes associated with the nuclear pore complex [21]: 1:25). 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 3I Marianas™ digital-imaging microscopy workstation (Zeiss Axiovert 200 M inverted microscope; Cark Zeiss, Sliedrecht, The Netherlands) equipped with a nanostepper motor (Z-axis increaments: 10 nm) and a thermo-electrically cooled EMCCD camera (QuantEM: 512C, 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 [9]. Now, we quantified NOX2 in nuclear fraction of non-ischemic (control) and ischemic cardiomyocytes using Western blot analysis.

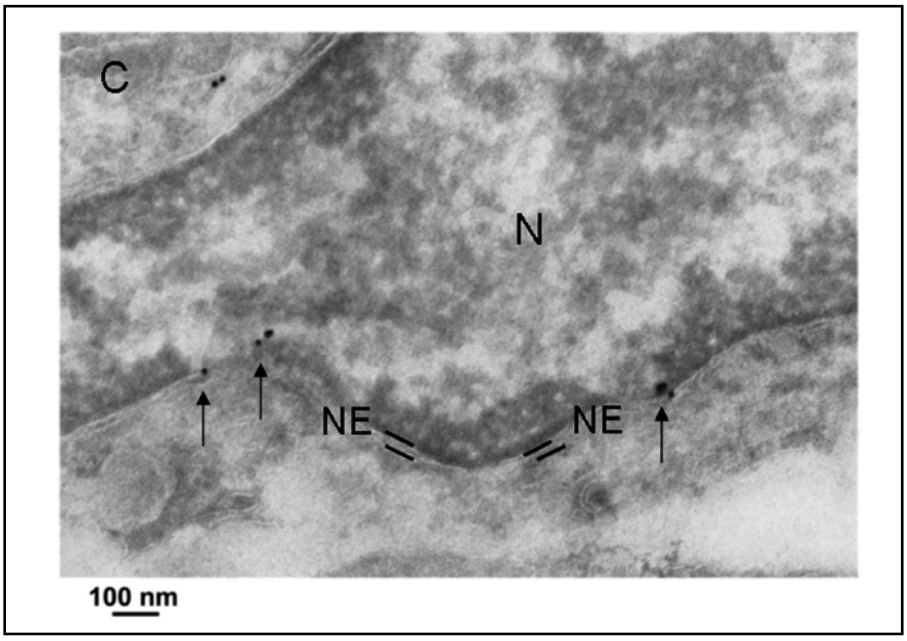

Fig. 2. Localization of NOX2 at the nuclear envelope in ischemic cardiomyocytes. Immuno-electron microscopy showing the association of NOX2 with the nuclear envelope in ischemic H9c2 cells. The nuclear envelope (NE) is separating the nucleus (N) from the cytosol (C). Gold particles labeling NOX2 (arrows) are located in close proximity of the nuclear envelope.

NOX2 protein was detected at approximately 60 kDa (Fig. 1). In the nuclear fraction of control cardiomyocytes a low basal level of NOX2 was found (Fig. 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 ($p<0.01$, Fig. 1B).

NOX2 colocalizes with the nuclear pore complex and ROS at the nuclear envelope 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 (Fig. 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 (Fig. 3). In ischemic H9c2 cells 3D stack images showed (peri)nuclear NOX2 expression (Fig. 3-IA, IIA, red signal). NOX2 was found to colocalize with NUP (Fig. 3-IB, green signal) as shown in Fig. 3-IC (yellow signal, arrows). NOX2 was found dispersed (peri)nuclear and also colocalized with local ROS (Fig. 3-IIB, green signal) as shown in Fig. 3-IIC (yellow signal, arrows). In control cells 3D stack images showed a very low basal (peri)nuclear presence of NOX2 (Fig. 3-IE), while no (peri)nuclear nitrotyrosine was found (Fig. 3-IIE).

The NADPH oxidase subunits $p47^{phox}$ and $p22^{phox}$ colocalize with NOX2 at the nuclear pore complex in ischemic cardiomyocytes

To analyze whether the NADPH oxidase subunits $p47^{phox}$ (needed for activation and targeting of NADPH oxidase) [25] and $p22^{phox}$ (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 (Fig. 4).

In ischemic H9c2 cells (peri)nuclear NOX2 (Fig. 4-IA, blue signal) colocalized with both $p47^{phox}$ (Fig. 4-IB, red signal) and $p22^{phox}$ (Fig. 4-IC, green signal) as shown in Fig. 4-ID (white signal, arrows). This colocalization of NOX2 with $p47^{phox}$ was found to be homogenous, while $p22^{phox}$ was found dispersed (Fig. 4-ID). This (peri)nuclear expression of $p47^{phox}$ (Fig. 4-IIB, red signal) and $p22^{phox}$ (Fig. 4-IIC, green signal) also colocalized with NUP (Fig. 4-IIA, blue signal) as shown in Fig. 4-IID (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 $p47^{phox}$, while $p22^{phox}$ was virtually absent (Fig. 4-IIE).

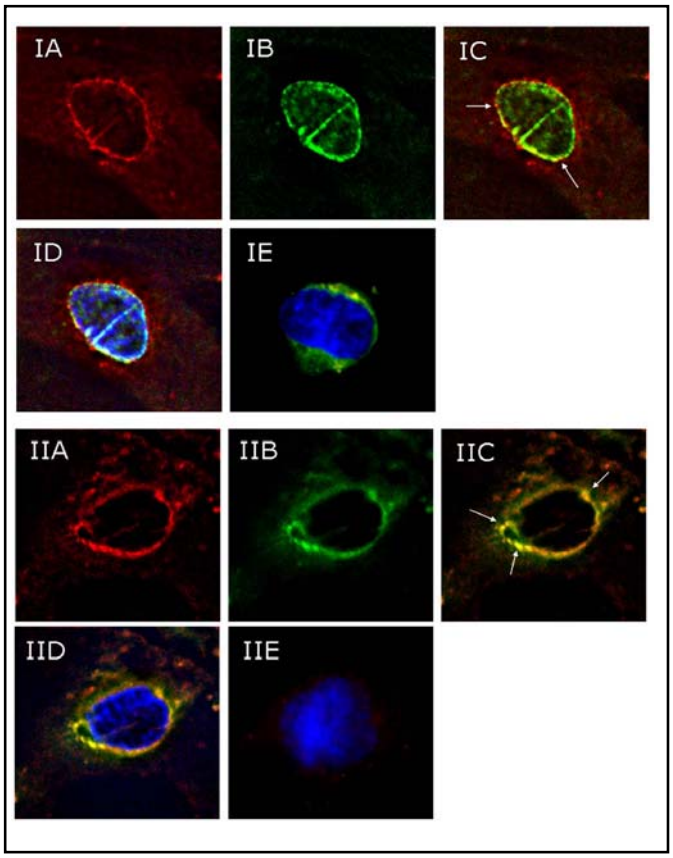

Fig. 3. NOX2 colocalizes with ROS production and the nuclear pore complex in ischemic cardiomyocytes. Nuclear localization of NOX2 in H9c2 cells as shown by digital-imaging microscopy. Cells were stained for NOX2 (red; I and II), NUP (green; I) or nitrotyrosine (green; II). DNA was stained by DAPI (blue). Pictures D demonstrate that under ischemia (peri)nuclear NOX2 focally coincides with NUP (ID) or ROS (IID), visible as yellow signal (arrows). Pictures E demonstrate that under control very low basal (peri)nuclear NOX2 is present (IE), while no (peri)nuclear nitrotyrosine was found (IIE). Original magnification 63x (n=4).

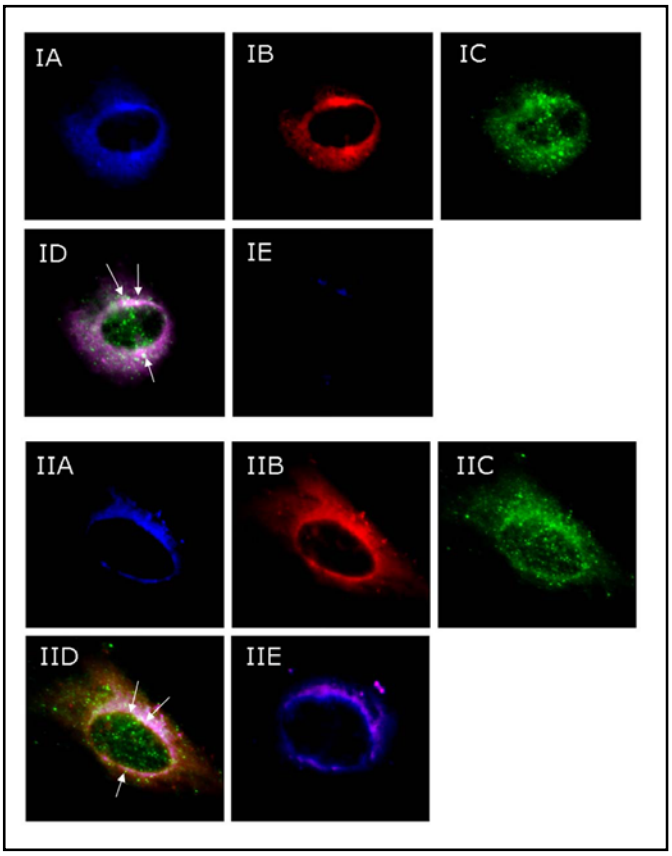

Fig. 4. The NADPH oxidase subunits $p47^{phox}$ and $p22^{phox}$ colocalize with NOX2 at the nuclear pore complex in ischemic cardiomyocytes. Nuclear localization of NOX2 and the NADPH oxidase subunits $p47^{phox}$ and $p22^{phox}$ in H9c2 cells as shown by digital-imaging microscopy. Cells were stained for $p47^{phox}$ (red; I and II), $p22^{phox}$ (green; I and II), NOX2 (blue; I) or NUP (blue; II). Picture D depicts that under ischemia (peri)nuclear $p47^{phox}$ and $p22^{phox}$ colocalize with NOX2 (ID) or NUP (IID), visible as white signal (arrows). Pictures E demonstrate that under control low basal $p47^{phox}$ is present, while $p22^{phox}$ was virtually absent (IIE). Original magnification 63x (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 (Fig. 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

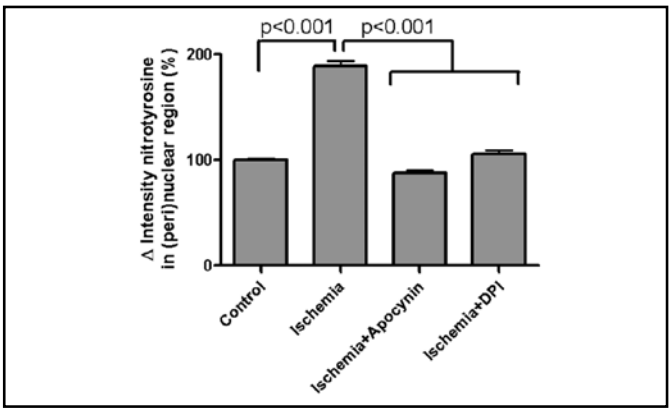

Fig. 5. Inhibition of NADPH oxidase reduces ischemia-induced (peri)nuclear ROS production. Quantification of digital-imaging microscopy analysis of (peri)nuclear nitrotyrosine expression in H9c2 cells. The changes are shown as the difference (Δ) in the staining intensity compared to control cells (n=4).

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 [9, 10]. We, to the best of our knowledge, are the first to show now that NOX2, $p22^{phox}$ and $p47^{phox}$ 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 [26]. 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 [27, 28]. Redox signaling is mediated by generation of ROS that alter the oxidation state of specific reactive-cysteine residues in target proteins [27, 29]. There are several different forms of modification possible that all lead to an altered structural and functional state of the target protein and consequently adapted signal transmission [30, 31].

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 [32]. 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 [27, 29]. In redox signaling this principle is all the more important because ROS are very short-lived and ready to react with a multitude of non-specific biomolecules [27, 29].

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 $p47^{phox}$ 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 [33], and in lamellipodial leading edges of vascular endothelial growth factor (VEGF)- [34, 35] and tumor necrosis factor alpha (TNFα)- [36] stimulated human endothelial cells. In endothelial cells stimulated with TNFα also an increase in $p47^{phox}$-$p22^{phox}$ complex formation was found in immunoprecipitations of whole-cell extracts [36]. Next to endothelial cells, in hearts of spontaneously hypertensive rats [11] as well as salt-sensitive hypertensive rats [12, 13] quantitative PCR showed an increased left ventricular mRNA of $p47^{phox}$ and $p22^{phox}$, coinciding with elevated ROS levels. Furthermore, superoxide release (determined by chemiluminescence) coinciding with increased $p47^{phox}$ expression in cardiomyocyte membrane extractions has also been found in human left ventricular myocardium from patients with ischemic cardiomyopathy [37]. However, in these studies total membrane extractions were used without distinction between subcellular compartments [14]. We now show that NOX2, $p47^{phox}$ and $p22^{phox}$ 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 [22, 23]. Composed of ~500-1000 proteins that represent ~30 different NUPs [21], the obvious function of the pore complex is to control the passage of molecules between the nucleus and the cytoplasm [38]. 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 [24, 39]. 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 [40, 41]. Two redox-sensitive transcription factors, activator protein 1 (AP-1)

and nuclear factor kappa B (NF-kappaB), have been implicated in the regulation of cardiomyocyte apoptosis [42]. 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 [43]. Studies in C.elegans and rat brain have shown an age-dependent deterioration (i.e. increased leakiness) of the NPC linked to oxidative stress [44]. It is well-known that during apoptosis the permeability of the NPC increases through caspase-mediated proteolysis of specific NUPs [45]. 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, $p22^{phox}$ and $p47^{phox}$ are targeted to the NPC in ischemic cardiomyocytes colocalizing with local ROS production.

Abbreviations

AP-1 (Ativator protein 1); DPI (Diphenylene iodonium); MI (Metabolic inhibition); NF-kappaB (Nuclear factor kappa B); NOX (NADPH oxidase); NPC (Nuclear pore complex); NUP (Nucleoporin); ROS (Reactive oxygen species); TNFα (Tumor necrosis factor alpha); VEGF (Vascular endothelial growth factor).

Acknowledgements

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