ISCHEMIA INDUCES NUCLEAR NOX2 EXPRESSION IN CARDIOMYOCYTES AND SUBSEQUENTLY ACTIVATES APOPTOSIS


Ischemia induces nuclear NOX2 expression in cardiomyocytes and subsequently activates apoptosis


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In previous work we have demonstrated increased expression of NOX2 in cardiomyocytes of infarcted human hearts. In the present manuscript we investigated the functional role of NOX2 in ischemically challenged H9c2 cells, a rat cardiomyoblast cell line, and adult rat cardiomyocytes. Expression of NOX2 in H9c2 cells was confirmed by RT-PCR. In Western-blot experiments, increased NOX2 expression was detected during ischemia, which was inhibited by transcription and translation inhibitors. Surprisingly, under ischemia, in addition to an increased cytosolic expression, NOX2 was localized mainly in the nucleus of apoptotic cardiomyocytes, where it coocccasen with nitroreosine resuces and acti-vated caspase 3. Inhibition of reactive-oxygen-species generation with the flavonolyn inhibitor diphenylalanol (DPI) and the NADPH-oxidase inhibitor apocynin led to a significantly decreased induction of apoptosis as assessed by quantification of caspase-3 activity and by TUNEL analysis. These results demonstrate that NOX2 is expressed in the nucleus of cardiomyocytes during apoptosis and that it likely participates in proapoptotic signaling. To the best of our knowledge, this is the first demonstration of nuclear NOX2 expression and its involvement in cardiomyocyte apoptosis.

Keywords: apoptosis; free radicals; ischemia; NADPH oxidase.

Introduction

Redox-based signaling has emerged in recent years as an important and often essential part of most signal transduction pathways. Disturbances of redox homeostasis, therefore, have been implicated in a multitude of disease states.

A major advance in the elucidation of redox-based signaling has been the recent discovery of a group of NADPH oxidases as a principal source of signaling-related reactive oxygen species (ROS). Described in almost all tissues since their initial discovery, these highly homologous transmembrane proteins utilize NADPH to generate superoxide. Superoxide, in turn, can give rise to more radical ROS. While these basic mechanisms are quite well understood, the regulation of the NADPH oxidases in different tissues and their downstream signaling targets remains, for a large part, unknown.

In cardiovascular pathology, ROS are well-recognized mediators of, for instance, atherosclerosis and hypertension. Consequently, several NADPH oxidases have been characterized in the vasculature. In the heart itself, NADPH oxidases have been implicated in hypertension or cardiac failure. So far, NOX2 and NOX4 have been described in human heart tissue and in non-human cardiomyocytes. In human cardiomyocytes our group has recently been able to demonstrate the presence of NOX2, and its upregulation in patients with acute myocardial infarction (AMI). However, the functional significance of this upregulation remained to be established. NOX2 has been described to participate both in the regulation of proliferation and of apoptosis in different cell types. However, the exact role of the NADPH oxidases in the context of the remaining signal-transduction pathways seems to be unique and tissue dependent and remains largely unknown. Since both ischemia- and reperfusion-induced apoptosis and compensative proliferation/hypertrophy are processes that determine the final extent of the tissue damage of an acute myocardial infarction, we sought to determine which role NOX2 plays in cardiomyocytes that have been exposed to ischemia/ reperfusion.
We now present evidence that nuclear upregulation of NOX2 in cardiomyocytes is an important step in the induction of apoptosis in these cells.

Materials and methods

Cell culture

H9c2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Cambrex Corporation, East Rutherford, NJ, USA) containing 10% (v/v) heat inactivated fetal calf serum (FCS; Cambrex Corporation), 100 IU penicillin per ml (Yamanouchi, Tokyo, Japan), 100 µg streptomycin per ml (Radiopharma-Fison, Palomonte, Italy) and 2 mM L-glutamine (In-vitrogen Corporation, Carlsbad, CA, USA), under 5% CO2 at 37°C.

Isolation of adult rat cardiomyocytes

Animals were treated according to the national guidelines and with permission of the Animal Experimental Committee of the VU University of Amsterdam.

Wistar rats were heparinized by administering 10,000 U heparin/kg body weight i.p. After deep anesthesia with halothane, the heart was excised and perfused retrogradely (Langendorff) at 37°C for 2 min with Tyrode buffer (containing 120 mM NaCl, 27 mM NaHCO3, 5.0 mM KCl, 2.0 mM NaH2PO4, 1.2 mM MgSO4, 10 mM glucose, 5 mM creatine, 5 mM taurocholate; gassed with 95% O2-5% CO2) supplemented with 1 mM Ca2+, followed by perfusion for 2 min with Tyrode buffer supplemented with 10 µM Ca2+. Next, the heart was perfused with recirculating Tyrode buffer supplemented with 10 µM Ca2+, 150 U/ml collagenase (Class II, Worthington Biochemical Corp.) and 0.5 mg/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) at a flow rate of 10 ml/min. After 20 min of perfusion, the heart was removed from the perfusion system, the atria and large vessels were removed and the ventricular tissue was minced with scissors. Tissue fragments were incubated with HEPES buffer (containing 116 mM NaCl, 20 mM HEPES, 5.3 mM KCl, 1.13 mM NaH2PO4, 1.2 mM MgSO4, 10 mM glucose, 5 mM creatine, 5 mM taurocholate, 100 U/ml penicillin/streptomycin, 0.2% (v/v) fatty-acid-free BSA and 10 µM Ca2+; pH 7.35) containing 150 U/ml collagenase and 0.5 mg/ml hyaluronidase during 8 min in a shaking water bath at 37°C. Dissociated cells were removed and the remaining tissue was incubated for another 8 min. The obtained cell suspension was mixed 1:1 with HEPES buffer supplemented with 0.4 mM Ca2+, passed through a 200-µm mesh nylon gauze and centrifuged for 1 min at 43 g. The cells were suspended in 55 ml of a HEPES-buffer/M199 mixture containing 0.4 mM Ca2+ and were allowed to sediment in a 50-ml test tube during 10 min at room temperature. This process was repeated with 1 HEPES/M199 mixtures of 0.8 and 1.2 mM Ca2+, respectively. Finally, the cells were resuspended in M199 (supplemented with 5 mM creatine, 5 mM taurocholate, 100 U/ml penicillin/streptomycin and 0.2% (v/v) fatty-acid-free BSA), seeded onto laminin-coated LabTek II chamber slides (Nalge Nunc International, Rochester, NY, USA) and allowed to adhere to the substratum for 1–2 h. Medium was then changed to M199 supplemented with 5 mM creatine, 5 mM taurocholate, 100 U/ml penicillin/streptomycin, 0.2% (v/v) fatty-acid-free BSA, 100 nM insulin, 0.1 nM L-triiodothyronine and 2 mM carnitine.

Isolation of rat leukocytes

Rat leukocytes were isolated from 10 ml of EDTA-anticoagulated blood by centrifugation at 1000 x g for 10 min, resuspension of the supernatant in 10 ml of phosphate-buffered saline and renewed centrifugation at 1000 x g for 10 min. The resulting cell pellet was confirmed by microscopic inspection to be highly enriched (>95%) in leukocytes.

Isolation of mRNA, reverse transcription, PCR and cycle sequencing

Total RNA from H9c2 cells and rat leukocytes was isolated with the RNaseasy Fibrous Tissue Kit (Qiagen) and RNA-fee (Tel-Test, Inc., Friendswood, TX, USA), respectively, and cDNA was synthesized. The coding region of CYBB was then amplified by PCR in five overlapping fragments on the Rapid Cycler (Idaho Technology, Idaho Falls, ID, USA) with 50 cycles at 95°C for 10 s, 60°C for 30 s and 72°C for 30 s and slope 0.9. The following primer combinations were used: 5'-ATGGGGACTGGCGTGGTATGAGG-3' and 5'-TGGGTGTAAGTGCCCGTGTAAGTG-3'; 5'-TTCACA CCGCCATTCACACATGGC-3' and 5'-GGCTACATGTC ATTTGCTCAGCTCC-3'; 5'-GGAGCTGAGCAGGATT GTACGTTGAC-3' and 5'-GTCCTGCTAGTACGTT GATACGTT-3'; 5'-AGTACGAGCTGATGAGTC CC-3' and 5'-AACCACCTCAAAGATCCAGGTGTC TCCC-3'; 5'-GACAGCCATGTTTGTTGTTGCG-3' and 5'- TGTGTGAAGATGAAGTGGCCACCG-3'. The reaction volume of 15 µl contained 2 U of Taq DNA polymerase (Promega, Madison, WI, USA), 2 U of TaqStart antibody (Clontech Laboratories, Palo Alto, CA, USA), 50 ng of each primer, 3 mM of each of the dNTPs (Promega) and reaction buffer (50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 10 mM Tris and 8% DMSO, pH 9 at 25°C). The PCR reaction took place in 10-µl glass capillaries (Idaho Technology).

The purified templates were cycle-sequenced with the above primers and the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer Applied Biosystems, Warrington, Cheshire, UK) and run on an ABI 373 XL.
Automated DNA Sequencer (Perkin Elmer Applied Biosystems). Sequence analysis was performed by means of the Sequence Analysis, Sequence Navigator and Auto Assembler software (all Perkin Elmer Applied Biosystems).

Metabolic inhibition

We used metabolic inhibition as an experimental *in vitro* model system for ischemia as described elsewhere. Briefly, cells were exposed to metabolic-inhibition buffer (5 mM NaCN, 20 mM 2-deoxyglucose, 0.9 mM CaCl$_2$, 106 mM NaCl, 38 mM NaHCO$_3$, 4.4 mM KCl and 1 mM MgCl$_2$, pH 6.0 for 1 h (adult rat cardiomyocytes) or 2 h (H9c2 cells) under 5% CO$_2$ at 37°C). When analysing the effects of inhibitors, the cells were preincubated for 1 h with actinomycin D and/or cycloheximide (both 10 µM final concentration), diphenylene iodonium (DPI; 10 µM final concentration) or apocynin (100 µM final concentration) prior to metabolic inhibition.

Western blotting

H9c2 cells were harvested into modified Laemmli sample buffer (0.33 M Tris-HCl (pH 6.8), 30% (v/v) glycerol, 3.5% (v/v) 2-mercaptoethanol) mixed rigorously and incubated for 30 min on ice. After determination of the protein concentration of the samples with the Bio-Rad Protein assay (Bio-Rad, Hercules, CA, USA), 0.4 volumes of 10% sodium dodecyl sulphate (SDS) with bronnophenol blue were added and the samples were mixed and heated at 95°C for 10 min. The samples were then subjected to SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted for NOX2 expression with monoclonal antibody 48 (Ref. 12: 1:250 dilution) and subsequently with horseradish peroxidase-conjugated rabbit-anti-mouse immunoglobulins (R+M-HRP: 1:1000 dilution; DakoCytomation, Glostrup, Denmark). The blots were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences AB; Uppsala; Sweden).

Immunocytochemistry

Two days before metabolic inhibition, H9c2 cells were passaged onto sterile Lab-Tek II chamber CC2 glass slides (Nalgé Nunc International, Naperville, IL, USA) to reach about 80–90% confluency at the day of the experiment.

After treatment, the cells were washed, paraformaldehyde fixed, permeabilized with ice-cold acetone/methanol (70%/30% (v/v)) and, subsequently, incubated with the primary antibody. Primary antibodies were monoclonal antibody 48 (used at a dilution of 1:50), anti-nitrotyrosine antibody (Molecular Probes, Eugene, OR, USA; used at a dilution of 1:50) and anti-active caspase-3 pAb (Promega; used at a dilution of 1:100). The corresponding isotype controls were purified mouse myeloma protein IgG1, MOPC21 (MP Biomedicals, Irvine, CA, USA) and polyclonal rabbit anti-human calcitonin (DakoCytomation), used at the same concentrations as the corresponding primary antibodies.

The cells were then incubated with Alexa-Fluor-568-labeled goat-anti-mouse-IgG$_1$ antibodies (Molecular Probes; used at a dilution of 1:75) and FITC-conjugated polyclonal swine Fabh (DAKO), anti-rabbit immunoglobulins (DakoCytomation; used at a dilution of 1:10). Finally, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting medium (H1200, Vectashield; Vector Laboratories, Burlingame, CA, USA).

Sections were qualitatively analyzed by use of a 3i Marians™ digital imaging microscopy workstation (Zeiss Axiosver 200 M inverted microscope; Carl Zeiss, Sliedrecht, The Netherlands), equipped with a nanostepper motor (Z-axis increments: 10 nm) and a cooled CCD camera (Cooke Sensicam, 1280 × 1024 pixels; Cooke Co., Tonawanda, NY, USA). The motorized filter turret allowed acquisition of one composite image of up to 4 different fluorescent wavelengths as well as a brightfield image (several options). Exposures, objective, montage, and pixel binning were automatically recorded with each image and stored in memory (Dell Dimension workstation: 1.7 GHz Xenon dual processor, 2 GB RAM). The microscope, camera and data viewing process were controlled by SlideBook™ software (version 4.0.8.1; Intelligent Imaging Innovations, Denver, CO, USA).

Caspase 3 activity assay

Caspase 3 activity was measured with a fluorometric homogeneous caspase assay from Roche (Basel, Switzerland) according to the manufacturer’s recommendations. Briefly, cells were lysed and incubated with DEVD-homocysteinyldipeptide (DEVD-homocysteinyldipeptide) substrate for one hour at 37°C. Subsequently the amount of free rhodamine was determined fluorimetrically in a microplate fluorescence reader (SPECTRAFluor, TECAN, Durham, NC, USA). The developed fluorochrome was proportional to the concentration of activated caspase and was quantified by a calibration curve of diluted free rhodamine.

Caspase activation measurements were done in triplicate. Statistics were performed with the SPSS statistics program (Windows version 9.0). To evaluate whether observed differences were significant, a one-way ANOVA analysis was performed with Bonferroni’s multiple comparison test. A *p*-value (two-sided) of less than 0.05 was considered to represent a significant difference.

TUNEL analysis

TUNEL analysis was performed with the APO-BrdU™ TUNEL Assay Kit (Invitrogen) according to the manufacturer’s instructions. Briefly, ~2 × 10⁶ cells/ml in 0.5 ml PBS were fixed in 5 ml 1% (w/v) paraformaldehyde for
15 min on ice and permeabilized with 70% ethanol for at least 24 h at −20°C. After washing of the permeabilized cells, they were resuspended in 50 μl DNA-labeling solution and incubated for 60 min at 37°C. Subsequently the samples were rinsed repeatedly, resuspended in 100 μl antibody-staining solution and incubated for 30 min at room temperature in the dark. For quantification of the viable and apoptotic cell populations 10^5 cells were measured on a FACS Calibur (BD Biosciences, San Jose, CA, USA). The analysis of the results was performed with CellQuest Pro software (BD Biosciences).

Positive and negative cells provided by the manufacturer were used as positive and negative controls, respectively.

To evaluate whether observed differences were significant, a one-way ANOVA analysis was performed with Bonferroni’s multiple comparison test. A p-value (two-sided) of less than 0.05 was considered to represent a significant difference.

Results

NOX2 is expressed in H9c2 cells at the mRNA level

Although we have previously identified NOX2 in human cardiomyocytes and although NOX2-mRNA expression in adult rat ventricular myocytes has been described, we first determined the presence of NOX2 in H9c2 cells. For this purpose we amplified by RT-PCR about eighty percent of the coding region of the NOX2 transcript from H9c2-derived mRNA (Figure 1). Rat leukocyte mRNA was taken along as positive control. The sequence of the PCR products, as shown by cycle sequencing, was identical to the NOX2 sequence for Rattus norvegicus (Genbank accession code: AF298656).

NOX2 expression in H9c2 cells is upregulated under metabolic inhibition

In a previous study we found upregulation of NOX2 in the infarcted human heart. To investigate whether this upregulation resulted from ischemia, we analysed in vitro putative NOX2 expression by Western-blot analysis of H9c2 cells incubated with metabolic-inhibition buffer. We found that NOX2 is constitutively present in H9c2 cells and is clearly upregulated under metabolic inhibition, with maxim...
Figure 3. Digital-imaging microscopy of H9c2 cells: Fixed H9c2 cells were stained with antibodies against NOX2 (red) and nitrotyrosine or active caspase 3 (green). DNA staining by DAPI is shown in blue (magnification 10 ×). (I) H9c2 cells at normoxia: (a) (weak) nuclear expression of NOX2 is shown in pink; (b) (weak) nuclear presence of nitrotyrosine residues is shown in turquoise; (c) merge of a and b: (weak) nuclear colocalization of NOX2 expression and nitrotyrosine residues is shown by a whitish staining. (II) H9c2 cells after 2 h of metabolic inhibition: (a) nuclear expression of NOX2 is shown in pink; (b) nuclear presence of nitrotyrosine residues is shown in turquoise; (c) merge of a and b: nuclear colocalization of NOX2 expression and nitrotyrosine residues is shown by a white staining. (III) H9c2 cells, exposed to the flavoprotein inhibitor DPI, after 2 h of metabolic inhibition: (a) (uninhibited) nuclear expression of NOX2 is shown in pink; (b) inhibition of nuclear generation of ROS by DPI is shown by absence of turquoise staining; (c) merge of a and b: only nuclear NOX2 expression is seen in pink. (IV) H9c2 cells after 2 h of metabolic inhibition: (a) nuclear expression of NOX2 is shown in pink; (b) nuclear activation of caspase 3 is shown in turquoise; (c) merge of a and b: nuclear colocalization of NOX2 expression and caspase-3 activation is shown by a white staining.
Figures 1-4. Analysis by TUNEL of the effect of DPI and apocynin on apoptosis of H9c2 cells induced by metabolic inhibition: The effect of DPI (10 μM final concentration) or apocynin (100 μM final concentration) was assessed under control as well as under ischemic (2 h of metabolic inhibition) conditions. Shown are the percentages of apoptotic cells as assessed by TUNEL. Data represent mean and standard error of mean. *p < 0.001, **p < 0.05, ***p < 0.001, †p < 0.01.

Figure 5. Effect of DPI on apoptosis induced by metabolic inhibition in H9c2 cells as assessed by measurement of caspase-3 activity. Using fluorogenic DEVD-rhodamine-110 as substrate for the active form of caspase 3, activation of the caspase under metabolic inhibition and the inhibitory effect of 10 μM DPI were analyzed. Data represent mean and standard error of three experiments. Shown are the differences in percent with the control, the average of the control values was taken as 100% value and set at zero. *p < 0.001, †p < 0.001, ‡p < 0.01

Nuclear NOX2 upregulation is pronounced in apoptotic H9c2 cells and colocalizes with nitrotyrosine residues

To analyze the intracellular localization of NOX2, digital-imaging microscopy was used. In control H9c2 cells, a diffuse cytoplasmic as well as some nuclear expression of NOX2 was visible (Figure 5-I). Upon metabolic inhibition both cytosolic and nuclear expression increased. The increase in nuclear NOX2 expression seemed to be especially pronounced in condensed, presumably apoptotic nuclei (Figure 5-IIa).

To analyze a possible relationship between NOX2 expression and apoptosis, we also analyzed caspase-3 activation, a marker of apoptosis, in H9c2 cells. Under metabolic inhibition a clear increase in caspase 3-positive nuclei was seen (Figure 5-IIb). This upregulation of caspase 3 in the majority of nuclei coincided with nuclear NOX2 expression (Figure 5-IIc).

To determine whether NOX2 expression was associated with functional oxidase activity, we probed the fixed H9c2 cells with an antiserum specific for nitrotyrosine residues. Nitrotyrosine is formed when tyrosine residues are modified by peroxynitrite, the reaction product of nitric oxide and superoxide. As can be seen in Figure 5-IIIc, in the majority of cells nitrotyrosine colocalized with NOX2 expression in the nucleus. Nitrotyrosine formation, indicative of reactive-oxygen-species (ROS) production, was observed in the cytosol (Figure 5-IIIb). This suggested that ROS production due to NOX2 activation was limited to nuclei.

To assess whether the observed nuclear ROS was, indeed, generated by NOX2, we tested the effect of the flavonoid inhibitor DPI on the increase in nitrotyrosine formation. As would be expected for NOX-dependent ROS generation, the addition of 10 μM DPI had no effect on NOX2 expression (Figure 5-IIIb) but led to a clear reduction of the metabolically induced increase in nuclear nitrotyrosine formation (Figure 5-IIIb).

To investigate the relationship between NOX2-generated ROS and the putative activation of apoptosis, we analyzed by TUNEL the effect of the NADPH-oxidase inhibitors DPI and apocynin on apoptosis induced in H9c2 cells by metabolic inhibition (ischemia). As shown in Figure 4, ischemia induced a significant increase in the percentage of apoptotic cells (p < 0.001). Apocynin and DPI both significantly reduced apoptosis induced by metabolic inhibition (p < 0.001 for apocynin and p < 0.01 for DPI). However, while apocynin reduced apoptosis to a level not significantly different from control cells, DPI reduced apoptosis only to a level that still was significantly higher than that in control cells (p < 0.05). Under normal cell-culture conditions no effect or apoptosis was seen with both inhibitors.

To confirm these results with a second, principally different method, we also analyzed the effect of DPI on caspase-3 activity in a fluorometric caspase assay.
Figure 6. Nuclear colocalization of NOX2 expression and nitrotyrosine residues in adult rat cardiomyocytes under ischemic conditions: Isolated adult ventricular rat cardiomyocytes were subjected to 1 h of metabolic inhibition and then fixed and stained with antibodies against NOX2 (red; a) and nitrotyrosine (green; b). DNA was stained blue by DAPI (magnification 63x). Picture a shows a vesicular or granular staining pattern of NOX2 that is especially pronounced in the nucleus. While there is a diffuse background signal for nitrotyrosine in the cytosol (b), only in the nucleus a granular staining pattern comparable to that of NOX2 is visible. The merged image (c) confirms the nuclear colocalization of NOX2 and nitrotyrosine residues, which are indicative of ROS production (white signal). N = nucleus.

Figure 5. Metabolic inhibition of H9c2 cells induced a significant increase in caspase-3 activity ($p < 0.001$) compared with control cells. DPI significantly inhibited the metabolic-inhibition-induced upregulation of caspase-3 activity ($p < 0.01$), but caspase-3 activity with DPI was again significantly higher than in control cells ($p < 0.001$). These results underscore the role of NOX2-generated ROS in the induction of apoptosis in cardiomyocytes exposed to ischemia.

Nuclear NOX2 expression and generation of reactive oxygen species are also found in adult rat cardiomyocytes.

H9c2 cells are a rat cardiomyoblast cell line. To exclude the possibility that our findings represent a cell-line- or immortalisation-specific phenomenon, we also analyzed primary adult rat cardiomyocytes for NOX2 expression and ROS generation. In these cells, at higher magnification,
a granular or vesicular cytosolic as well as a clear nuclear expression of NOX2 was seen under ischemic conditions (Figure 6a). However, while there was a diffuse background staining for nitrotyrosine visible in the cytosol, only in the nucleus a vesicular staining pattern was seen that was indicative of localized ROS production and colocalized with the nuclear staining pattern of NOX2 (Figure 6b). The white signal in the merged picture (Figure 6c) confirms the nuclear colocalization of NOX2 expression and nitrotyrosine residues. In summary, therefore, adult rat cardiomyocytes, just like H9c2 cells, under ischemic conditions display a pattern of less intense cytosolic NOX2 expression and of dense nuclear expression. Also, as in H9c2 cells, ROS production—as detected by the presence of nitrotyrosine residues—can be seen only in the nucleus, where it colocalizes with NOX2 expression.

Discussion

In previous work we have demonstrated the expression of NOX2 in human cardiomyocytes after acute myocardial infarction (AMI). In this follow-up study we investigated the functional role of NOX2 upregulation under ischemic conditions. We found that NOX2 in ischemia is upregulated mainly in the nucleus of apoptotic cells where it colocalizes with nitrotyrosine residues, which are indicative of the generation of reactive oxygen species (ROS). In line with these observations, inhibition of ROS generation with the flavonoid inhibition diphenyl ether indoxim (DPI) or with the NOX2 inhibitor apocynin led to significantly decreased apoptosis as assessed by a decrease in caspase-3 activation and by TUNEL. To the best of our knowledge, this is the first demonstration of nuclear NOX2 expression and its involvement in cardiomyocyte apoptosis induced by ischemia.

Reactive oxygen species are well-known mediators of apoptosis, and considerable evidence has accumulated in recent years concerning their role in cardiomyocyte cell death and cardiovascular pathophysiology in general. However, the exact sources of the ROS as well as their downstream targets are as yet unknown. NADPH oxidases are an important source of redox signaling-related ROS, and cardiac expression of NOX2 and NOX4 has been implicated in cardiac hypertrophy and chronic heart failure. Inhibition of the angiotensin II-initiated apoptotic cascade in rat neonatal cardiomyocytes by DPI has also suggested the involvement of an NADPH oxidase in this process. However, this latter article identified neither the exact isotype of the NADPH oxidase involved, nor its subcellular localization.

Our results, therefore, represent the first evidence for NOX2 participating in cardiomyocyte apoptosis. AMI-induced cardiomyocyte apoptosis is known to arise primarily during the reperfusion phase following ischemia, not during ischemia itself. However, while the majority of apoptotic events indeed probably takes place during the reperfusion phase, apoptosis has also been described to take place during the ischemic phase itself, a finding that is corroborated by the present study.

Induction of apoptosis in general follows one of two pathways. The first pathway, the death-receptor or extrinsic pathway, is activated by ligand binding of cell-surface-expressed death receptors, while the second pathway, the mitochondrial or intrinsic pathway, is promoted by physical or chemical stress agents. However, the response of cardiomyocytes to ischemia and reperfusion a complex, and the involved pathways have not been fully described. Since activation of caspase 3 is a late event of both apoptotic cascades, our results do not allow an assessment as to which apoptotic pathway is affected by NOX2 activation.

The new finding of NOX2 being expressed in the nucleus is remarkable but makes sense on a functional and biophysical level. There are numerous redox-sensitive transcription factors that are either activated or inactivated by redox modifications. On the other hand, ROS, via the formation of peroxynitrite, introduce DNA damage and thereby contribute to the apoptotic cascades. Given the highly reducing intracellular milieu, it is hard to imagine that these processes can be effectively controlled by cytosolic generation of ROS. Our results support a scenario in which cytosolic NOX2, upon induction of apoptotic signaling, translocates to the nucleus and is activated to generate superoxide. This signaling mechanism would eliminate the necessity of ROS diffusing from the cytosol into the nucleus.

In our previous study we have described a primarily cytosolic localization of NOX2 in cardiomyocytes, as have others. However, in the present study cytosolic expression of NOX2 did not coincide with ROS production. Furthermore, it is known that in human tissue sections only in rare instances apoptotic nuclei can be found in cardiomyocytes. This phenomenon has been explained by the fact that primarily apoptotic cardiomyocytes quickly become secondarily necrotic due to the rapidly arising lack of ATP. It is to be expected, therefore, that in human tissue sections nuclear NOX2 staining is visible only exceptionally, since it is restricted to apoptotic nuclei.

Conclusion

Taken together, our results present evidence that NOX2 is expressed in the nucleus of cardiomyocytes and is likely involved in the regulation of cardiomyocyte apoptosis. Future research needs to elucidate in more detail the participants and mechanisms of the apoptotic signaling cascade described here and has to investigate the possibility of an independent functional role of cytosolic NOX2 under non-ischemic conditions.

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References


