NADPH oxidases in the cardiovascular system

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2014

document version
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Chapter 6

NOX5 expression is increased in intramyocardial blood vessels and cardiomyocytes after acute myocardial infarction in humans

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Am J Pathol. 2012 Jun; 180(6): 2222-9

Abstract

Reactive oxygen species (ROS)-producing NADPH oxidases have been shown to play an important role under different (patho)physiological conditions. Especially NOX1, NOX2 and NOX4 have been proven as important sources of ROS in the heart. Knowledge of the calcium-dependent NOX5 in the heart is lacking.

The presence of NOX5 was studied via RT-PCR in heart tissue from end-stage heart failure patients, obtained during cardiac transplantation surgery. In addition, NOX5 positivity and cellular localization was studied via immunohistochemistry and digital-imaging microscopy in heart tissue of patients who did not suffer from heart disease, and in infarction areas of patients who died of myocardial infarction of different duration. Furthermore, NOX5 was analyzed in isolated human cardiomyocytes via Western blot analysis.

NOX5 RNA was found in the human heart of controls and ischemic cardiomyopathy patients. In control hearts NOX5 localized in the endothelium of a limited number of intramyocardial blood vessels and in a limited number of scattered cardiomyocytes. In infarcted hearts, NOX5 expression increased especially in infarctions older than twelve hours. This manifested itself as an increase in NOX5-positive intramyocardial blood vessels, wherein NOX5 was present in endothelium and smooth muscle, but also in cardiomyocytes. In cardiomyocytes NOX5 was found in the cytoplasm, plasma membrane, intercalated discs and cross striations. Western blot analysis confirmed NOX5 expression in isolated human cardiomyocytes.

This study for the first time demonstrates NOX5 expression in intramyocardial blood vessels and cardiomyocytes of the human heart, which increased significantly in the affected myocardium after AMI.

Introduction

Reactive oxygen species (ROS) have been implicated in the regulation of numerous biological processes and play an important role in the development and progression of heart failure.[1/2] Different cell types within the heart, e.g. cardiomyocytes, endothelial cells and vascular smooth muscle cells can produce ROS. Particularly under pathological conditions, ROS levels are elevated.[3] The family of NADPH oxidases (NOXes) has been shown to be especially important in ROS-related cellular signaling, the so called redox signaling, by producing ROS in specific subcellular locations in response to different stimuli.[4/5]

So far the NOX family members NOX1, NOX2, NOX4 and DUOX1/DUOX2 have been identified either in cardiomyocytes and/or cells of the intramyocardial (micro)vasculature. As such, they have been shown to be involved in a wide array of (patho) physiological processes in the heart.[6-8] For instance, NOX2 and NOX4 were shown to be involved in pro-apoptotic[9-11] as well as pro-hypertrophic[12/13] signaling in cardiomyocytes and in pro-inflammatory[14] as well as pro-angiogenetic[15] responses of endothelial cells after ischemia. DUOX1/DUOX2 even were found to be expressed in ischemic cardiomyocytes that were capable of generating thyroglobulin in response to ischemia.[16]

The latest identified member of the NOX family is NOX5. In contrast to most other NOX isoforms, NOX5 does not appear to require cytoplasmic subunits for its activation,[17] but instead is regulated through calcium, which induces a conformational change of the NOX5 N-terminus, leading to enzyme activation.[18] Although expression of NOX5 has been shown in for instance atherosclerotic diseased human epicardial coronary arteries,[19] in cultured platelet-derived growth factor (PDGF)-stimulated human aortic smooth muscle cells[20] and in angiotensin II-stimulated immortalized human myometrial smooth muscle cells,[21] knowledge of the NOX5 isoform in the heart is lacking. Therefore, in this study, we have analyzed the expression and (sub) cellular localization of NOX5 in non-diseased human hearts as well as in human hearts after acute myocardial infarction (AMI).

Materials and Methods

RNA isolation

Samples were obtained after informed consent and with approval of the local ethical committee. Tissue from the free left ventricle wall from end-stage heart failure patient, clinically characterized with familial ischemic cardiomyopathy, was obtained during cardiac transplantation surgery. Control cardiac left ventricle tissue was obtained from donor hearts for which no suitable transplant recipient was found. The donors had no history of cardiac disease, a normal ECG and normal ventricular function on echocardiography performed within 24 hours prior to heart explantation. The tissue was collected in cold cardioplegic solution, divided into pieces of approximately 2 grams wet weight and immediately frozen in liquid nitrogen.[22]

Total RNA from these left ventricle biopsies was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. RNA quality was checked by analysis on the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

Real time PCR

RNA extracts were reverse transcribed and the resulting cDNA was used as template to amplify NOX5 transcripts. RNA of human control testis was included as positive control. 0.5 µg RNA was added in a 50 µl reaction as described before,[23] containing forward primer 5'-GTGCTACATCGATGGGCCTTATG-3' and reverse primer 5'-CCCCGTGATGGAGTCTTTCTTCT-3', in supplied buffer from Promega. The 2 minutes initial denaturation at 94°C was followed by 38 cycles of PCR, each consisting of 30 seconds denaturation at 94°C, 30 seconds annealing at 65°C, and 60 seconds extension at 72°C. The PCR finished after a further 7 minutes extension.

Patients

Heart tissue was obtained from patients referred to the department of pathology for necropsy. Autopsies were performed as soon as possible, within 24 hours after death. Decolorization of lactate dehydrogenase (LDH) staining of the heart was used to determine and localize myocardial infarction. Heart tissue samples were taken from the left ventricular infarction area of AMI patients (n=17) or from the left ventricular wall of control patients (n=4) who did not suffer from heart disease (table 1). To estimate infarct duration, next to LDH staining, microscopic criteria were used, as described previously.[9] Early-phase infarction (n=7; infarct age 3-12 hours) was defined by LDH decolorization without microscopically morphological changes and without infiltration of polymorphonuclear neutrophils (PMNs). PMN-phase infarction (n=6; infarct age 12 hours-5 days) was defined by extravascular localization of PMNs within the LDH decolorized area. Chronic-phase infarction (n=4; infarct age 5-14 days) was defined by infiltration of lymphocytes and macrophages, and granulation tissue formation.

Our study was approved by the ethics committee of the VU University Medical Center, Amsterdam, and conforms to the principles of the Declaration of Helsinki. The use of tissues left over after the pathological examination for research purposes is part of the standard patient contract in our hospital.

Immunohistochemistry

Before cryosection, human heart tissue was stored at -196°C (liquid nitrogen). Frozen sections (4 µm thick) were mounted onto SuperFrost® Plus glass slides (Menzel-Gläser, Braunschweig, Germany), air-dried for 1 hour and fixed in acetone.

table 1. Clinical data of patients included in the study

	Controls (n=4)	Early phase (n=7)	PMN phase (n=6)	Chronic phase (n=4)
Age range (in years) *	55-73	47-88	62-84	61-74
Male/Female *	3/1	6/1	5/1	2/2
Cause of death	Aneurysm of artery communicans (n=1)	AMI left ventricle 3-12 hours (n=7)	AMI left ventricle 12 hours-5 days (n=6)	AMI left ventricle 5-14 days (n=4)
	Aneurysm of aorta abdominalis (n=1)			
	Metastasis of carcinoma (n=2)			
Medication at time of death				
Beta-blocker		n=4		n=1
Ang II receptor antagonist		n=3		
Phosphodiesterase inhibitors	n=1			
Cardiac glycoside		n=1		
Nitrates		n=3		
Alpha-adrenergic blocker				n=1
NSAIDs	n=1	n=2	n=1	n=1
Platelet aggregation inhibitors §	n=1	n=3		
Statins	n=1	n=2		
Diuretics		n=4		
Insulin		n=2	n=1	n=2

AMI: acute myocardial infarction, PMN: polymorphonuclear neutrophils, Ang: angiotensin, NSAIDSs: non-steroidal anti-inflammatory drugs
There was no statistical difference in age nor in male/female composition between the groups (*) (One-way Anova with Bonferroni post-test)
There was a statistical difference in medication between the groups for platelet aggregation inhibitors (§ $p<0.05$) (Chi-square test with ordinal gamma test)

Sections were pre-incubated with normal swine serum (NSS; 1:10, Dako, Glostrup, Denmark) and subsequently incubated with rabbit anti-human NOX5 antibody (1:25). Anti-human NOX5 antibody was made in rabbits immunized with synthetic peptides corresponding to the residues 491-506 and 577-588 of human NOX5α.[24] Sections were then incubated with swine anti-rabbit-HRP (1:300, Dako) for 30 minutes at RT. Staining was visualized with 3,3'-diaminobenzidine (DAB; 0.1 mg/ml, 0.02% H_2O_2, Sigma-Aldrich, Milwaukee, Wisconsin, USA). Thereafter, sections were counterstained with haematoxylin, dehydrated and covered. All dilutions were made in 1% (w/v) bovine serum albumin (BSA; Boehringer, Mannheim, Germany) in PBS.
Since NOX5 has been shown in the testis in several studies,[18/25/26] we included staining of human control testis as a positive control. To assess nonspecific binding, isotype controls with normal rabbit serum immunoglobulin were also included.
Two investigators (NEH and PAJK) each judged and scored independently all slides for infarct age and anatomical localization of NOX5. NOX5-positive intramyocardial blood vessels (positive for endothelial cells and/or smooth muscle cells) and NOX5-positive cardiomyocytes were scored by determining the number of positive cells counted in 25 high power fields (HPF; magnification 400×). The average number of NOX5 positivity per HPF was used in the calculations.

Digital-imaging microscopy

Frozen sections (4 μm thick) were mounted onto SuperFrost® Plus glass slides, air-dried for 1 hour and fixed in acetone. Sections were pre-incubated with NSS (1:10, Dako) and subsequently incubated with rabbit anti-human NOX5 antibody (1:25)[24] for 1 hour at RT, followed by incubation overnight at 4°C. The following day, sections were washed repeatedly with PBST (0.05% (v/v) Tween-20 in PBS) and incubated in the dark with the secondary antibody, Alexa Fluor 647-labeled donkey anti-rabbit-Cy5 (1:40, Invitrogen) for 30 minutes at RT. Nuclei were visualized with 4',6 diamidino-2-phenylindole (DAPI) in Vectashield® Mounting medium (Vectorlabs, Pieterborough, United Kingdom).
2D/3D optical sections were acquired and analyzed with a 3I Marianas™ digital-imaging microscopy workstation (Zeiss Axiovert 200M inverted microscope; Carl Zeiss, Sliedrecht, The Netherlands). 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).[27]

Preparation of human testis homogenates

Frozen tissue from human normal testis was used for homogenate preparation. Tissue was pulverized in a mortar with liquid nitrogen and subsequently resuspended in lysis buffer containing 250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES pH 7.0, 5 mM EDTA and 0.5 mM DTT in H_2O, supplemented with protease inhibitor cocktail (1:40, Sigma, St. Louis, MO, USA). Then the suspension was centrifuged (4000 RPM for 10 min at 4°C) and the supernatant was used for additional Western blot analysis.

Isolation of human cardiomyocytes

Specimen of control and PMN-phase infarcted left ventricle were used for isolation of cardiomyocytes. Isolation was performed as described previously.[9] Approximately 5 cm^3 of heart tissue was cut into small pieces, rinsed twice in PBS and pelleted by centrifugation (6 minutes at 100×*g* on a low brake). The tissue was then incubated at 37°C in a solution of collagenase type 2 (Worthington Biochemical Corporation, Lakewood, New Jersey, USA) at 0.8 mg/ml in calcium-free Krebs Ringer buffer (pH 7.4).[28] After separation of cardiomyocytes (containing ≈99% morphologically purified human cardiomyocytes) the suspension was filtered through a 100 µm filter and centrifuged again (6 minutes at 100×*g* on a low brake).

Western blotting

After determination of the protein concentration of the samples with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), an equal amount (20 µg) of human testis homogenate and isolated human cardiomyocytes 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 rabbit anti-human NOX5 antibody (1:100)[24] for 1 hour at RT, followed by incubation overnight at 4°C. Mouse anti-human actin antibody (1:500, Sigma-Aldrich) was used as loading control. The following day, blots were washed and incubated with goat anti-rabbit-HRP (1:500, Dako) for 30 minutes at RT. Blots were visualized by enhanced chemiluminescence (ECL; 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).

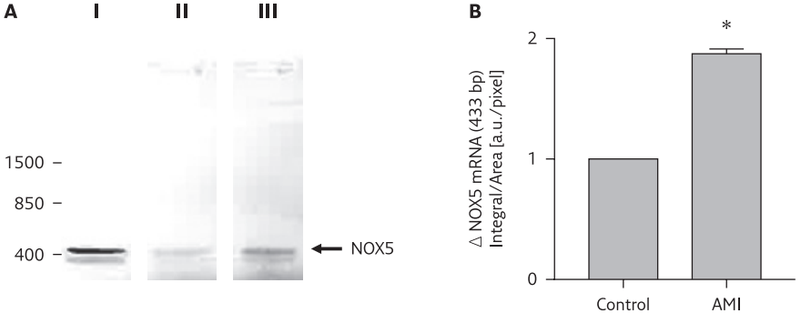
Statistics

The GraphPad Prism program (Windows version 5) was used for statistical analysis. To evaluate whether observed differences were significant, One-way ANOVA with post hoc Bonferroni tests and Chi-square with ordinal gamma 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

Presence of NOX5 in the human heart

Using RT-PCR the presence of NOX5 RNA was analyzed in control heart tissue and ischemic cardiomyopathy heart tissue derived from patients undergoing cardiac transplantation surgery (figure 1).
We then found a double RNA transcript of 390-433 bp in human control testis (figure 1A-I, arrow). We found the same transcript in control heart tissue and ischemic cardiomyopathy heart tissue (figure 1A-II/III, arrow), showing that NOX5 is expressed in human heart tissue. Quantification indeed demonstrated a significant increased NOX5 RNA in ischemic cardiomyopathy hearts compared to controls (figure 1B, $p<0.01$).



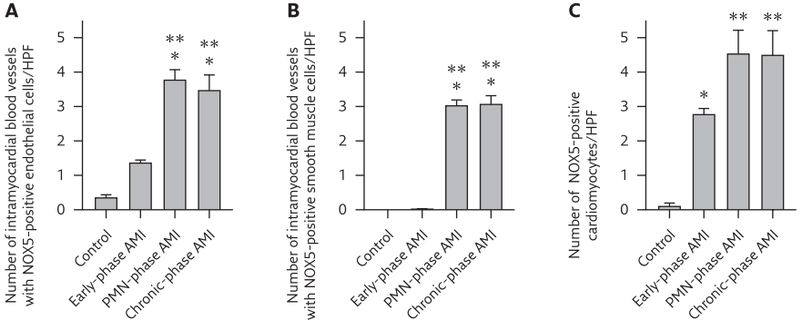
1. NOX5 RT-PCR | (A) RT-PCR of NOX5 mRNA in human (I) control testis, (II) control heart tissue and (III) ischemic cardiomyopathy heart tissue. NOX5 mRNA was detected at 390-433 bp. (B) Quantitative analysis of the NOX5 mRNA intensities relative to those in controls (n=3). *p<0.01 compared to controls.

Presence and subcellular localization of NOX5 in the human heart

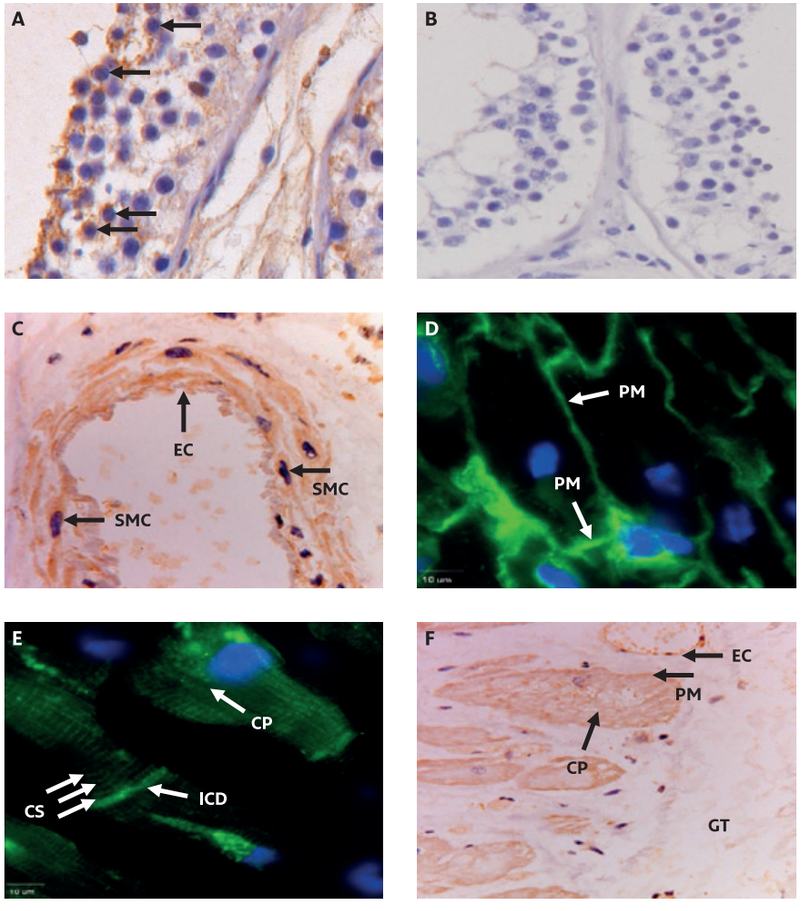
To verify the presence of NOX5 in human heart tissue, we subsequently analyzed the subcellular localization in heart tissue derived from controls and in macroscopical infarction areas of patients who died of AMI (table 1), using immunohistochemistry and digital-imaging microscopy (figure 2/3).

As expected, NOX5 positivity was found in spermatozoa of human control testis (figure 3A),[25/26] whereas the isotype control showed no staining (figure 3B).
In hearts of controls, a limited number of intramyocardial blood vessels did express NOX5. In these vessels NOX5 was only found in endothelial cells (figure 2A, 0.4±0.1 vessels/HPF) but not in smooth muscle cells (figure 2B). NOX5 was also found in a limited number of scattered cardiomyocytes (figure 2C, 0.1±0.1 cells/HPF), staining the cytoplasm and plasma membrane.
In early-phase infarctions (LDH decolorization without microscopically morphological changes), a non-significant increase of NOX5 was found in endothelial cells (figure 2A, 1.4±0.1 vessels/HPF) compared with controls, while the smooth muscle cells again were all negative (figure 2B). The number of NOX5-positive cardiomyocytes, however, was significantly increased to 3.1±0.3 cells/HPF (figure 2C, $p<0.05$) compared with controls. Also here, NOX5-positive cardiomyocytes were found scattered. These cells not only stained the cytoplasm and plasma membrane, but also intercalated discs (not shown).
In PMN-phase infarctions (LDH decolorization with infiltration of PMNs), significantly more intramyocardial blood vessels were positive for NOX5 as compared to controls and early-phase infarctions. In addition to endothelial cells (figure 2A, 3.8±0.3 vessels/HPF, $p<0.001$), NOX5 was now also found in smooth muscle cells (figure 3C) (figure 2B, 3.0±0.2 vessels/HPF, $p<0.001$). Both NOX5-positive intramyocardial blood vessels staining endothelial cells as well as smooth muscle cells were found. NOX5 staining in cardiomyocytes again was found scattered, in both morphological necrotic as non-necrotic cardiomyocytes within the macroscopical infarction area. NOX5-positive cardiomyocytes were significantly increased up to 4.7±0.6 cells/HPF (figure 2C, $p<0.01$) compared to controls. Also an increase compared with early-phase infarctions was found, however, this was not significant. As in early-phase infarctions, NOX5 staining was found in the cytoplasm, plasma membrane (figure 3D) and intercalated discs, but now also in cross striations (figure 3E). No differences were found in NOX5 staining between morphological necrotic and non-necrotic cardiomyocytes within the macroscopical infarction area.

In chronic-phase infarctions (granulation tissue with infiltration of lymphocytes and macrophages), expression of NOX5 was found in intramyocardial blood vessels comparable to PMN-phase infarctions. Namely, a significant increase in endothelial NOX5 expression of 3.5±0.5 vessels/HPF (figure 2A, $p<0.001$) and in smooth muscle NOX5 expression of 3.1±0.5 vessels/HPF (figure 2B, $p<0.001$) as compared to controls or early-phase infarctions. NOX5 expression in cardiomyocytes was also comparable to PMN-phase infarctions, both in number (figure 2C, 4.7±0.3 cells/HPF, $p<0.01$ compared to controls) and subcellular localization. Interestingly, NOX5 staining was also found in blood vessels of the granulation tissue as well as in cardiomyocytes adjacent to granulation tissue (figure 3F). In contrast with cardiomyocytes distant to granulation tissue, in these borderzone cardiomyocytes NOX5 stained only the cytoplasm and plasma membrane. In none of the examined hearts NOX5 was found in the extracellular matrix (not shown).
Although a relatively low number of subjects was included in this study, no significant differences between the age range nor the male/female composition were found (table 1). There was a significant difference between the patient groups related to platelet aggregation inhibitors ($*p<0.05$). However, since patients included in this study with PMN-phase infarction and chronic-phase infarction did not receive platelet aggregation inhibitors, and nothing is known about the effects of these inhibitors on NOX5 expression, it is less likely that this would significantly influence the NOX5 expression we now found.



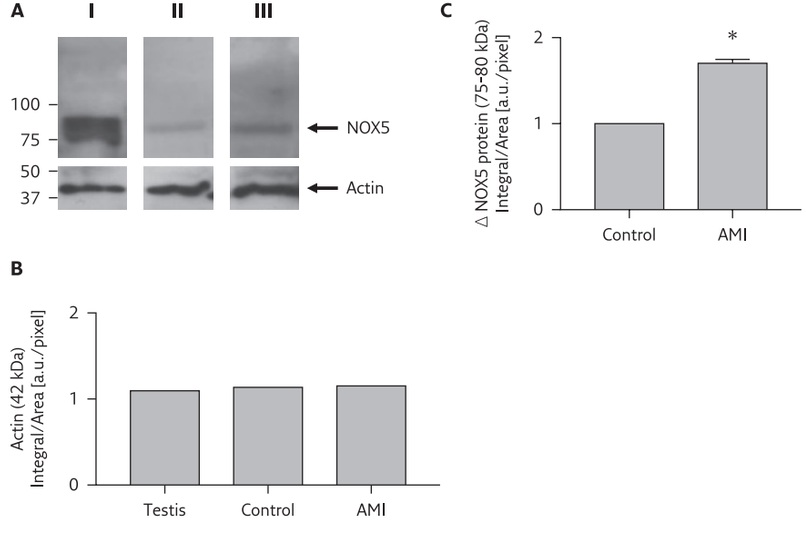
2. Immunoscoring of NOX5 in human hearts | Immunoscoring of the number of (A) intramyocardial blood vessels with NOX5-positive endothelial cells, (B) intramyocardial blood vessels with NOX5-positive smooth muscle cells and (C) NOX5-positive cardiomyocytes per high powered field (HPF) in control, early-phase, PMN-phase and chronic-phase infarcted human heart tissues. For each patient the average number of NOX5-positive vessels/cardiomyocytes was determined from 25 HPF's scored. (A/B) $*p<0.001$ compared to control, $**p<0.001$ compared to early-phase AMI. (C) $*p<0.05$ and $**p<0.01$ compared to control.



3. (Sub)cellular localization of NOX5 in human hearts | The (sub)cellular localization of NOX5 in human testis and human infarcted heart tissue using immunohistochemistry (A-C/F, brown signal) and immunofluoresence (D/E, green signal). Nuclei were stained blue (hematoxylin and DAPI, respectively). Arrows indicate localization of NOX5 in (A) spermatozoa, (C) endothelial cells (EC) and smooth muscle cells (SMC), (D) plasma membrane (PM) of cardiomyocytes, (E)intercalated discs (ICD), cross striations (CS) and cytoplasm (CP) of cardiomyocytes and (F) cardiomyocytes adjacent to granulation tissue (GT). (B) Testis stained with isotype control to assess nonspecific binding. Original magnifications 400x (A-C/F).

NOX5 in isolated human cardiomyocytes

We wanted to verify the presence of NOX5 in human cardiomyocytes using Western blot analysis (figure 4). As NOX5 was also found in intramyocardial endothelial cells and smooth muscle cells we have isolated cardiomyocytes from human heart tissue derived from the left ventricular area of a PMN-phase infarction patient, rather than using homogenates. Furthermore, a homogenate of human control testis was used as a positive control. Immunoblotting indeed revealed a single band that migrated to approximately 75 kDa, typical for NOX5[24] (figure 4A-I, arrow). Both in isolated cardiomyocytes from control and PMN-phase infarctions a single band was detected at approximately 75 kDa, albeit that in cardiomyocytes isolated from PMN-phase infarctions this band was more intense (figure 4A-II/III, arrow). Quantification of the loading control actin (at 42 kDa) demonstrated no significant difference (figure 4B), whereas the presence of NOX5 was indeed significantly increased in PMN-phase cardiomyocytes compared to control cardiomyocytes (figure 4C, $p<0.01$).



4. NOX5 expression in isolated human cardiomyocytes | (A) Western blot analysis of NOX5 and actin expression in (I) human control testis homogenate, (II) isolated human control cardiomyocytes and (III) isolated human cardiomyocytes from left ventricular PMN-phase infarcted heart tissue. NOX5 protein was detected at 75-80 kDa (arrow). (B) Quantitative analysis of the actin signal intensities. (C) Quantitative analysis of the NOX5 signal intensities relative to those in control cardiomyocytes (n=3). *$p<0.01$ compared to control.

Discussion

NADPH oxidases have been shown to play important roles in the physiology and pathophysiology of the heart. So far the NOX isoforms NOX1, NOX2, NOX4 and DUOX1/DUOX2 have been identified either in cardiomyocytes and/or cells of the intramyocardial (micro)vasculature.[6-8] However, knowledge of the NOX5 isoform in the heart is lacking. We now show the presence of NOX5 RNA and protein in the human heart. In control hearts NOX5 was present both in a limited number of endothelial cells of intramyocardial blood vessels and in a limited number of scattered cardiomyocytes. In infarcted hearts, within the affected myocardium, NOX5 expression increased, especially in infarctions older than twelve hours. This manifested itself as increased NOX5-positive intramyocardial blood vessels, wherein NOX5 was present in endothelial cells and/or smooth muscle cells, and increased NOX5-positive cardiomyocytes, wherein NOX5 was found in the cytoplasm, plasma membrane, intercalated discs and cross striations.

This increase in NOX5 expression in intramyocardial blood vessels and cardiomyocytes, together with the different subcellular localizations, suggest that NOX5 is involved in different processes in the infarcted heart. However, so far we can only speculate as to what these roles may be.

Recently, NOX5 expression was shown in the endothelium and smooth muscle cells of epicardial coronary arteries obtained from explanted hearts of patients with coronary artery disease and AMI.[19] In these arteries increased NOX5 expression was found in atherosclerotic lesions and correlated with the stage of atherosclerosis, suggesting a role for NOX5 in atherosclerosis development.[19] However, atherosclerotic lesions, as occurring in epicardial coronary arteries, are usually not found in the intramyocardial vasculature,[29] making it unlikely that the NOX5 we found in these blood vessels, is involved in atherosclerosis development. On the other hand, structural and functional aberrations have been shown to occur in intramyocardial blood vessels in the affected myocardium subsequent to and/or preceding AMI, also independent of aberrations of the epicardial coronary arteries. For instance, we have found thickening of the basement membrane, accumulation of advanced glycation end products (AGEs) and expression of the adhesion molecule E-selectin in intramyocardial blood vessels of AMI patients,[30/31] suggestive of a pro-inflammatory status of these blood vessels in AMI. Recently, NOX5 expression was shown to be increased by angiotensin II, playing a role in the upregulation of vascular cell adhesion molecule-1 (VCAM-1) in human dermal microvascular endothelial cells.[32] Also in human myometrial smooth muscle cells NOX5 was shown to be increased by angiotensin II suggesting a role for myometrial hypertrophy in pregnant women.[21] Although angiotensin II levels are increased after AMI, it remains to be established

whether angiotensin II indeed is related to the NOX5-dependent changes occurring in the microvasculature of the infarcted heart.

Revascularization of ischemic myocardial tissue occurs in the remodeling heart after AMI[33] and we found NOX5 in blood vessels of granulation tissue in chronic phase infarctions. Interestingly, overexpression of NOX5 was found to induce proliferation and the formation of capillary-like structures in human dermal microvascular endothelial cells,[34] suggesting a role in the process of angiogenesis. A role for NOX5 in new blood vessel formation in the infarcted heart therefore appears possible.

In addition to intramyocardial blood vessels, NOX5 was also found in cardiomyocytes. Because in infarcted hearts increased NOX5 expression was detected in necrotic cardiomyocytes, a cell-damaging role for NOX5 cannot be excluded. On the other hand, non-necrotic cardiomyocytes also strongly expressed NOX5, which argues with such a role. Furthermore, we also found NOX5-positive cardiomyocytes adjacent to granulation tissue in chronic-phase infarctions. In chronic-phase infarctions hypertrophic remodeling of cardiomyocytes is known to occur.[35] Recently, NOX5 expression was related to angiotensin II-induced hypertrophy in human myometrial smooth muscle cells.[21] As such a role for NOX5 in post-AMI cardiomyocyte hypertrophy can also be postulated.

In addition to an increase in expression, with increasing infarction age, NOX5 was detected in distinct subcellular locations within cardiomyocytes. In non-diseased control hearts NOX5 was detected in the cytoplasm and plasma membrane, whereas in infarcted hearts, with increasing infarction age, NOX5 was detected in the cytoplasm, plasma membrane, cross striations and intercalated discs. It is known that NOX-derived ROS can mediate cell signaling through the modulation of the structure and/or activity of signaling proteins.[36] To do this NOXes must produce ROS in close proximity to the target protein and consequently NOXes can be found in specific subcellular locations as was shown for instance for NOX2 and NOX4.[12/37/38] The subcellular location of NOX5 at the intercalated disc therefore may relate to cell adhesion or cell-cell junction remodeling in cardiomyocytes after AMI. It is known that infarction can induce (temporal) remodeling of intercalated discs,[39/40] such as weakened cardiomyocyte adhesion and activation of matrix metalloproteinases which are associated with infarct rupture.[41/42] Furthermore, cell-cell junction can propagate hypercontracture and cell death after AMI.[43/44]

In conclusion, we for the first time demonstrate NOX5 in the human heart and its presence is increased significantly after AMI. The multiple different cellular and subcellular locations of NOX5 suggest multiple roles for NOX5 in the infarcted heart.

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