Short Communication

NOX5 Expression Is Increased in Intramyocardial Blood Vessels and Cardiomyocytes after Acute Myocardial Infarction in Humans

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Reactive oxygen species producing NADPH oxidases play important roles under different (patho)physiological conditions. NOX1, NOX2, and NOX4 are important sources of reactive oxygen species in the heart, but knowledge of the calcium-dependent NOX5 in the heart is lacking. The presence of NOX5 was studied via RT-PCR in heart tissue from patients with end-stage heart failure; the tissue was obtained during cardiac transplantation surgery. NOX5 positivity and cellular localization were studied via IHC and digital-imaging microscopy in heart tissues of patients who did not have heart disease and in infarction areas of patients who died of myocardial infarctions of different durations. Furthermore, NOX5 expression was analyzed *in vitro* by using Western blot analysis. NOX5 RNA was found in the hearts of controls and patients with ischemic cardiomyopathy. In controls, NOX5 localized to the endothelium of a limited number of intramyocardial blood vessels and to a limited number of scattered cardiomyocytes. In infarcted hearts, NOX5 expression increased, especially in infarctions >12 hours, which manifested as an increase in NOX5-positive intramyocardial blood vessels, as well as in endothelium, smooth muscle, and cardiomyocytes. NOX5 was found in cardiomyocyte cytoplasm, plasma membrane, intercalated disks, and cross striations. Western blot analysis confirmed NOX5 expression in isolated human cardiomyocytes.

For the first time to our knowledge, we demonstrate NOX5 expression in human intramyocardial blood vessels and cardiomyocytes, with significant increases in the affected myocardium after acute myocardial infarction. (Am J Pathol 2012, 180:2222–2229; <http://dx.doi.org/10.1016/j.ajpath.2012.02.018>)

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 (eg, 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 especially important in ROS-related cellular signaling, the so-called reduction-oxidation signaling, by producing ROS in specific subcellular locations in response to different stimuli.^{4,5}

Thus far, the NOX family members NOX1, NOX2, NOX4, and dual oxidase (DUOX)1/DUOX2 have been identified in cardiomyocytes and/or cells of the intramyocardial (micro)vasculature. As such, they have been involved in a wide array of (patho)physiological processes in the heart.^{6–8} For instance, NOX2 and NOX4 were involved in pro-apoptotic^{9–11} and prohypertrophic^{12,13} signaling in cardiomyocytes and in pro-inflammatory¹⁴ and pro-angiogenic¹⁵ responses of endothelial cells after ischemia. DUOX1/DUOX2 even were expressed in ischemic cardiomyocytes that were capable of generating thyroglobulin in response to ischemia.¹⁶

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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 activation¹⁷; instead, it 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 cultured platelet-derived growth factor-stimulated human aortic smooth muscle cells,²⁰ and 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 nondiseased human hearts and 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 patients with end-stage heart failure, 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 before heart explantation. The tissue was collected in cold cardioplegic solution, divided into pieces of approximately 2 g wet weight, and immediately frozen in liquid nitrogen.²² Total RNA from these left ventricle biopsy specimens was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), ac-

cording to the manufacturer's 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 a template to amplify NOX5 transcripts. RNA of human control testis was included as a positive control. RNA, 0.5 μ g, was added in a 50- μ L reaction, as previously described,²³ containing forward primer (5'-GTGCTACATCGATGGGCCTTATG-3') and reverse primer (5'-CCCCGTGATGGAGTCTTTCTTCT-3'), in supplied buffer from Promega (Leiden, The Netherlands). The 2-minute initial denaturation at 94°C was followed by 38 cycles of PCR, each consisting of a 30-second denaturation at 94°C, a 30-second annealing at 65°C, and a 60-second extension at 72°C. The PCR finished after a further 7-minute extension.

Patients

Heart tissue was obtained from patients referred to the Department of Pathology, VU University Medical Center, Amsterdam, the Netherlands, 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 patients with AMI ($n = 17$) or from the left ventricular wall of control patients ($n = 4$) who did not have heart disease (Table 1). To estimate infarct duration, next to LDH staining, microscopic criteria were used, as previously described.⁹ Early-phase infarction ($n = 7$; infarct age, 3 to 12 hours) was defined by LDH decolorization without microscopically

Table 1. Clinical Data of Patients Included in the Study

Variable	Controls ($n = 4$)	Early phase ($n = 7$)	PMN phase ($n = 6$)	Chronic phase ($n = 4$)
Age range (years)*	55–73	47–88	62–84	61–74
Male/female ratio*	3:1	6:1	5:1	2:2
Cause of death	Aneurysm of artery communicans ($n = 1$) Aneurysm of aorta abdominalis ($n = 1$) Metastasis of carcinoma ($n = 2$)	AMI left ventricle; 3–12 hours	AMI left ventricle; 12 hours–5 days	AMI left ventricle; 5–14 days
Medication at death				
β -Blocker	0	4	0	1
Ang II receptor antagonist	0	3	0	0
Phosphodiesterase inhibitors	1	0	0	0
Cardiac glycoside	0	1	0	0
Nitrates	0	3	0	0
α -Adrenergic blocker	0	0	0	1
NSAIDs	1	2	1	1
Platelet aggregation inhibitors†	1	3	0	0
Statins	1	2	0	0
Diuretics	0	4	0	0
Insulin	0	2	1	2

Data are given as number of each group, unless otherwise indicated.

*There was no statistical difference in age or in male/female composition between the groups (one-way analysis of variance with Bonferroni post test).

† $P < 0.05$, χ^2 test with ordinal γ test. There was a statistical difference in medication between the groups for platelet aggregation inhibitors.

Ang, angiotensin; NSAID, nonsteroidal anti-inflammatory drug.

morphological changes and without infiltration of polymorphonuclear neutrophils (PMNs). PMN-phase infarction ($n = 6$; infarct age, 12 hours to 5 days) was defined by extravascular localization of PMNs within the LDH decolorized area. Chronic-phase infarction ($n = 4$; infarct age, 5 to 14 days) was defined by infiltration of lymphocytes and macrophages and by granulation tissue formation.

Our study was approved by the ethics committee of the VU University Medical Center, 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.

IHC Data

Before cryosection, human heart tissue was stored at -196°C (liquid nitrogen). Frozen sections ($4\text{ }\mu\text{m}$ thick) were mounted onto SuperFrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany), air dried for 1 hour, and fixed in acetone. Sections were pre-incubated with normal swine serum (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 to 506 and 577 to 588 of human NOX5 α .²⁴ Sections were then incubated with swine anti-rabbit horseradish peroxidase (1:300, Dako) for 30 minutes at room temperature. Staining was visualized with 3,3'-diaminobenzidine (0.1 mg/mL 0.02% H_2O_2 ; Sigma-Aldrich, Milwaukee, WI). Thereafter, sections were counterstained with hematoxylin, dehydrated, and covered. All dilutions were made in 1% (w/v) bovine serum albumin (Boehringer, Mannheim, Germany) in PBS.

Because 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 Ig were also included.

Two investigators (N.E.H. and P.A.J.K.) independently judged and scored all slides for infarct age and anatomical localization of NOX5. The 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 (HPFs; original magnification, $\times 400$). The average number of NOX5-positive specimens per HPF was used in the calculations.

Digital-Imaging Microscopy

Frozen sections ($4\text{ }\mu\text{m}$ thick) were mounted onto SuperFrost Plus glass slides, air dried for 1 hour, and fixed in acetone. Sections were pre-incubated with normal swine serum (1:10; Dako) and subsequently incubated with rabbit anti-human NOX5 antibody (1:25)²⁴ for 1 hour at room temperature, followed by incubation overnight at 4°C . The following day, sections were washed repeatedly with 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 room temperature. Nuclei were visualized with DAPI in Vectashield Mounting medium (Vectorlabs, Pieterborough, UK).

The two- and three-dimensional optical sections were acquired and analyzed with a 3I Marianas digital-imaging microscopy workstation (Zeiss Axiovert 200 M inverted microscope; Carl Zeiss, Sliedrecht, the Netherlands). The microscope, camera, and all other aspects of data acquisition, and data processing were controlled by Slidebook software, version 4.2 (Intelligent Imaging Innovations, Denver, CO).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 mmol/L NaCl, 0.1% Nonidet P-40, 50 mmol/L HEPES (pH 7.0), 5 mmol/L EDTA, and 0.5 mmol/L dithiothreitol in H_2O , supplemented with protease inhibitor cocktail (1:40; Sigma, St Louis, MO). Then, the suspension was centrifuged ($1200 \times g$ for 10 minutes at 4°C), and the supernatant was used for additional Western blot analysis.

Isolation of Human Cardiomyocytes

Specimens of control and PMN-phase infarcted left ventricle were used for isolation of cardiomyocytes. Isolation was performed as previously described.⁹ Approximately 5 cm^3 of heart tissue was cut into small pieces, rinsed twice in PBS, and divided into pellets by centrifugation (for 6 minutes at $100 \times g$ on a low brake). The tissue was then incubated at 37°C in a solution of collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ) at 0.8 mg/mL in calcium-free Krebs Ringer buffer (pH 7.4).²⁸ After separation of cardiomyocytes (containing approximately 99% morphologically purified human cardiomyocytes), the suspension was filtered through a $100\text{-}\mu\text{m}$ filter and centrifuged again (for 6 minutes at $100 \times g$ on a low brake).

Western Blot Analyses

After determination of the protein concentration of the samples with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), equal amounts ($20\text{ }\mu\text{g}$) of human testis homogenate and isolated human cardiomyocytes were dissolved in Laemmli SDS sample buffer, stirred, and heated at 95°C for 10 minutes. The samples were subjected to 10% SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with rabbit anti-human NOX5 antibody (1:100)²⁴ for 1 hour at room temperature, followed by incubation overnight at 4°C . Mouse anti-human actin antibody (1:500; Sigma-Aldrich) was used as a loading control. The following day, blots were washed and incubated with goat anti-rabbit horseradish peroxidase (1:500; Dako) for 30 minutes at room temperature. Blots were visualized by enhanced chemiluminescence (1:40; Amersham Biosciences AB, Uppsala, Sweden) and quantified with a charge-coupled device camera

Figure 1. NOX5 RT-PCR. **A:** RT-PCR of NOX5 mRNA in human control testis (1), control heart tissue (2), and ischemic cardiomyopathy heart tissue (3). NOX5 mRNA was detected at 390 to 433 bp. **B:** Quantitative analysis of the NOX5 mRNA intensities relative to those in controls ($n = 3$). * $P < 0.01$ versus controls.

(Fuji Science Imaging Systems, Düsseldorf, Germany) in combination with AIDA Image Analyzer software version 3.52 (Isotopenmessgeräte, Staubenhardt, Germany).

Statistics

The GraphPad Prism program version 5 (GraphPad Software, Inc., San Diego, CA) was used for statistical analysis. To evaluate whether observed differences were significant, one-way analysis of variance with post hoc Bonferroni tests and χ^2 with ordinal γ tests were used. All values are expressed as mean $\pm$ SEM. $P \leq 0.05$ (two sided) was considered significant.

Results

Presence of NOX5 in the Human Heart

By 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 to 433 bp in human control testis (Figure 1A). We found the same transcript in control heart tissue and ischemic cardiomyopathy heart tissue (Figure 1A), showing that NOX5 is expressed in human heart tissue. Quantification demonstrated significant increased NOX5 RNA in ischemic cardiomyopathy hearts compared with controls ($P < 0.01$, Figure 1B).

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 (IHC) and digital-imaging microscopy (Figures 2 and 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 (0.4 ± 0.1 vessels per HPF; Figure 2A), not in smooth muscle cells (Figure 2B). NOX5 was also found in a limited number of scattered cardiomyocytes (0.1 ± 0.1 cells per HPF; Figure 2C), staining the cytoplasm and plasma membrane.

In early-phase infarctions (LDH decolorization without microscopically morphological changes), a nonsignificant increase of NOX5 was found in endothelial cells (1.4 ± 0.1 vessels per HPF; Figure 2A), compared with controls, whereas the smooth muscle cells were all negative (Figure 2B). The number of NOX5-positive cardiomyocytes, however, was significantly increased to 3.1 ± 0.3 cells per HPF ($P < 0.05$, Figure 2C), compared with controls. Also, NOX5-positive cardiomyocytes were scattered. These cells stained the cytoplasm, plasma membrane, and intercalated disks (data not shown).

In PMN-phase infarctions (LDH decolorization with infiltration of PMNs), significantly more intramyocardial blood vessels were positive for NOX5, compared with controls and early-phase infarctions. In addition to endothelial cells (3.8 ± 0.3 vessels per HPF, $P < 0.001$; Figure 2A), NOX5 was also found in smooth muscle cells (3.0 ± 0.2 vessels per HPF, $P < 0.001$; Figure 2B) (Figure 3C). Both NOX5-positive intramyocardial blood vessels staining endothelial cells and smooth muscle cells were found. NOX5 staining in cardiomyocytes was scattered, in both morphological necrotic and nonnecrotic cardiomyocytes within the macroscopical infarction area. NOX5-positive cardiomyocytes were significantly increased, up to 4.7 ± 0.6 cells per HPF ($P < 0.01$, Figure 2C), compared with 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 disks; in addition, it was also in cross striations (Figure 3E). No differences were found in NOX5 staining between morphological necrotic and nonnecrotic 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 was found in endothelial NOX5 expression, of 3.5 ± 0.5 vessels per HPF ($P < 0.001$, Figure 2A), and in smooth muscle NOX5 expression, of 3.1 ± 0.5 vessels per HPF ($P < 0.001$, Figure 2B), compared with

Figure 2. Immunoscoring of NOX5 in human hearts. Immunoscoring of the number of intramyocardial blood vessels with NOX5-positive endothelial cells (A), intramyocardial blood vessels with NOX5-positive smooth muscle cells (B), and NOX5-positive cardiomyocytes (C) per HPF in control, early-phase, PMN-phase, and chronic-phase infarcted human heart tissues. For each patient, the average number of NOX5-positive vessels per cardiomyocyte was determined from 25 HPFs scored. * $P < 0.001$ versus control, ** $P < 0.001$ versus early-phase AMI (A and B); * $P < 0.05$, ** $P < 0.01$ versus control (C).

controls or early-phase infarctions. NOX5 expression in cardiomyocytes was also comparable to PMN-phase infarctions, both in number (4.7 ± 0.3 cells per HPF; $P < 0.01$ compared with controls; Figure 2C) and subcellular

localization. Interestingly, NOX5 staining was also found in blood vessels of the granulation tissue and in cardiomyocytes adjacent to granulation tissue (Figure 3F). In contrast to cardiomyocytes distant to granulation tissue, in these border zone cardiomyocytes, NOX5 stained only the cytoplasm and plasma membrane. NOX5 was not found in the extracellular matrix in any of the examined hearts (data not shown).

Although relatively few subjects were included in this study, no significant differences between the age range and 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, because patients included in this study with PMN-phase and chronic-phase infarctions 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 found.

NOX5 in Isolated Human Cardiomyocytes

We wanted to verify the presence of NOX5 in human cardiomyocytes using Western blot analysis (Figure 4). Because NOX5 was also found in intramyocardial endo-

Figure 3. (Sub)cellular localization of NOX5 in human hearts. The (sub)cellular localization of NOX5 in human testis and human infarcted heart tissue using IHC (brown signal; A–C and F) and immunofluorescence (green signal; D and E) with nuclei were stained blue (hematoxylin and DAPI, respectively). **Arrows**, localization of NOX5 in spermatozoa (A); endothelial cells (ECs) and smooth muscle cells (SMCs) (C); plasma membrane (PM) of cardiomyocytes (D); intercalated disks (ICDs), cross striations (CSs), and cytoplasm (CP) of cardiomyocytes (E); and cardiomyocytes adjacent to granulation tissue (GT; F). **B**: Testis stained with isotype control to assess non-specific binding. Original magnification, $\times 400$ (A–C and F).

Figure 4. NOX5 expression in isolated human cardiomyocytes. **A:** Western blot analysis of NOX5 and actin expression in human control testis homogenate (1), isolated human control cardiomyocytes (2), and isolated human cardiomyocytes (3) from left ventricular PMN-phase infarcted heart tissue. NOX5 protein was detected at 75 to 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 ($n = 3$). * $P < 0.01$ versus control.

thelial and smooth muscle cells, we isolated cardiomyocytes from human heart tissue derived from the left ventricular area of a patient with PMN-phase infarction, rather than using homogenates. Furthermore, a homogenate of human control testis was used as a positive control. Immunoblotting revealed a single band that migrated to approximately 75 kDa, typical for NOX5²⁴ (Figure 4A). In isolated cardiomyocytes from both control and PMN-phase infarctions, a single band was detected at approximately 75 kDa; however, in cardiomyocytes isolated from PMN-phase infarctions, this band was more intense (Figure 4A). Quantification of the loading control actin (at 42 kDa) demonstrated no significant difference (Figure 4B), whereas the presence of NOX5 was significantly increased in PMN-phase cardiomyocytes compared with control cardiomyocytes ($P < 0.01$, Figure 4C).

Discussion

The NOXes have played important roles in the physiological and pathophysiological characteristics of the heart. Thus far, the NOX isoforms NOX1, NOX2, NOX4, and

DUOX1/DUOX2 have been identified in cardiomyocytes and/or cells of the intramyocardial (micro)vasculature.^{6–8} However, knowledge of the NOX5 isoform in the heart is lacking. We showed the presence of NOX5 RNA and protein in the human heart. In control hearts, NOX5 was present both in a few endothelial cells of intramyocardial blood vessels and in a few scattered cardiomyocytes. In infarcted hearts, within the affected myocardium, NOX5 expression increased, especially in infarctions >12 hours. This manifested itself as increased NOX5-positive intramyocardial blood vessels, in which NOX5 was present in endothelial and/or smooth muscle cells, and increased NOX5-positive cardiomyocytes, in which NOX5 was found in the cytoplasm, plasma membrane, intercalated disks, and cross striations.

This increase in NOX5 expression in intramyocardial blood vessels and cardiomyocytes, together with the different subcellular localizations, suggests that NOX5 is involved in different processes in the infarcted heart. However, thus 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 occur 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 occurred 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, and expression of the adhesion molecule E-selectin in intramyocardial blood vessels of patients with AMI,^{30,31} suggestive of a pro-inflammatory status of these blood vessels in AMI. Recently, NOX5 expression was increased by angiotensin II, playing a role in the up-regulation of vascular cell adhesion molecule-1 in human dermal microvascular endothelial cells.³² Also, in human myometrial smooth muscle cells, NOX5 was 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 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 induced proliferation and the formation of capillary-like structures in human dermal microvascular endothelial cells,³⁴ suggesting a role in the process of angiogenesis.

Therefore, a role for NOX5 in new blood vessel formation in the infarcted heart appears possible.

In addition to intramyocardial blood vessels, NOX5 was also found in cardiomyocytes. In infarcted hearts, because increased NOX5 expression was detected in necrotic cardiomyocytes, a cell-damaging role for NOX5 cannot be excluded. On the other hand, nonnecrotic 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 nondiseased 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 disks. 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 shown (eg, for NOX2 and NOX4).^{12,37,38} Therefore, the subcellular location of NOX5 at the intercalated disk may relate to cell adhesion or cell-cell junction remodeling in cardiomyocytes after AMI. It is known that infarctions can induce (temporal) remodeling of intercalated disks,^{39,40} such as weakened cardiomyocyte adhesion and activation of matrix metalloproteinases, which are associated with infarct rupture.^{41,42} Furthermore, cell-cell junctions can propagate hypercontracture and cell death after AMI.^{43,44}

In conclusion, we, for the first time to our knowledge, 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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