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Original article

Role of NADPH oxidase isoforms NOX1, NOX2 and NOX4 in myocardial ischemia/reperfusion injury

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ABSTRACT

Myocardial reperfusion injury is mediated by several processes including increase of reactive oxygen species (ROS). The aim of the study is to identify potential sources of ROS contributing to myocardial ischemia–reperfusion injury. For this purpose, we investigated myocardial ischemia/reperfusion pathology in mice deficient in various NADPH oxidase isoforms (Nox1, Nox2, Nox4, as well as Nox1/2 double knockout). Following 30 min of ischemia and 24 h of reperfusion, a significant decrease in the size of myocardial infarct was observed in Nox1-, Nox2- and Nox1/Nox2-, but not in Nox4-deficient mice. However, no protection was observed in a model of chronic ischemia, suggesting that NOX1 and NOX2-mediated oxidative damage occurs during reperfusion. Cardioprotective effect of Nox1 and Nox2 deficiencies was associated with decrease of neutrophil invasion, but, on the other hand an improved reperfusion injury was also observed in isolated perfused hearts (Langendorff model) suggesting that inflammatory cells were not the major source of oxidative damage. A decrease in global post-reperfusion oxidative stress was clearly detected in Nox2-, but not in Nox1-deficient hearts. Analysis of key signaling pathways during reperfusion suggests distinct cardioprotective patterns: increased phosphorylation was seen for Akt and Erk in Nox1-deficient mice and for Stat3 and Erk in Nox2-deficient mice. Consequently, NOX1 and NOX2 represent interesting drug targets for controlling reperfusion damage associated with revascularization in coronary disease.

1. Introduction

The prolonged interruption of the coronary blood supply results in myocardial tissue death and jeopardized ventricular function. This physiopathological event is defined as acute myocardial infarction and represents one of the leading causes of morbidity worldwide [1]. The recommended initial treatment is directed toward the prompt mechanical restoration of myocardial perfusion by coronary angioplasty. This approach is essential for the cardiomyocyte salvation, resulting in the improvement of myocardial damage and cardiac dysfunction [2]. However, the reperfusion itself is harmful for the cardiac tissue. Indeed, the restoration of blood flow and the supply of nutrients and oxygen increases post-ischemic detrimental inflammatory and oxidative processes [1]. At the onset of reperfusion, several studies showed that a fast and marked increase of ROS generation occurs in post-ischemic tissues [3–5]. Although multiple sources of ROS have been identified, evidence supports Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidases (NOXs) as major contributors to oxidant generation during hypoxia-reoxygenation in different organs [6].

The NOX family is composed of seven members (Nox1–Nox5, Duox1, and Duox2) that transfer electrons across the biological membranes to generate ROS [7]. Nox isoforms have different patterns of expression within the cardiovascular tissues. In particular, Nox1 is expressed in endothelial cells, smooth muscle cells, and cardiomyocytes, while Nox2 is also expressed in adventitial and cardiac fibroblasts and leukocytes. Importantly, Nox4 is expressed in cardiomyocytes, but not in circulating leukocytes, suggesting that different isoforms might selectively contribute to ROS generation within cardiac and recruited inflammatory cells [8–10]. Several regulatory subunits are essential for the activity of Nox1 (NOXO1, NOXA1, Rac) and Nox2 ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, Rac), whereas NOX4 is constitutively active [11]. While Nox1 and Nox2 generate large amounts of superoxide anion as primary product, NOX4 appears

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to generate hydrogen peroxide enzymes without apparent detection of superoxide [12].

Due to this complex background, controversial results have been published on the potential role of Nox isoforms in ischemia–reperfusion injury in different organs [6]. In acute myocardial infarction, previous studies have shown no difference in infarct size between knock-out mice for *Ncf1*, which is the gene coding for the p47*phox* subunit of Nox2, and heterozygous controls following 24 h of reperfusion [13], neither in Nox2-deficient mice following short reperfusion (2 h) [14], nor an ex vivo model of MIR [15]. In the present study, we compared Nox1, Nox2, Nox4 and Nox1/Nox2 double deficient mice with control mice in in vivo and ex vivo models of reperfusion injury and chronic myocardial ischemia for infarct size, inflammation and oxidative modifications. We show that deficiency in Nox1 and Nox2, but not in Nox4, is strongly protective for myocardial reperfusion injury through activation of different molecular pathways.

2. Materials and methods

2.1. Animals

Adult males (aged 8–12 weeks) from the C57Bl/6 background were used. Nox-deficient and wt male mice (aged 10–12 weeks) were inbred in specific pathogen-free conditions. They were healthy without signs of disease during the study and all experiments were approved by local authorities. Nox1Y/− and Nox4 −/− mice were generated and genotyped as described [16,17], while Nox2Y/− mating couples were purchased from Jackson laboratories. Breeding was performed using Nox1Y/− males bred with Nox1 +/− females, Nox2 Y/− bred with Nox2 +/− females and Nox4 +/− males bred with Nox4 +/− females, thereby generating littermate controls. *Ncf1* mutant mice were in the B10.Q background as well as the controls used for the subset of this study as described [18,19]. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and has been approved by the local and ethics authorities (Geneva Veterinary Office and the Ethic Commission of Animal Experimentation of the University of Geneva).

2.2. In vivo ischemia–reperfusion protocol

2.2.1. Surgery

Mice were anesthetized with 4% isoflurane and intubated. Mechanical ventilation was performed (150 μl, 120 breaths/min) using a rodent respirator (model 683; Harvard Apparatus). Anesthesia was maintained with 2% isoflurane delivered in 100% O_2 through the ventilator. During surgery adequacy of anesthesia was monitored by careful visual and tactile control of mouse consciousness (changes in breathing rate and volume, heart rate, sweating and tearing). Body temperature was maintained at 37 °C using a rectal thermometer coupled with a heating pad (TCAT-2 Temperature Controller, Physitemps Instruments Inc). Before surgical procedure, buprenorphine HCl (0.05 mg/kg in 100 μl) was subcutaneously administered. Then, a thoracotomy was performed and the pericardial sac was removed. An 8–0 prolene suture was passed under the left anterior descending (LAD) coronary artery at the inferior edge of the left atrium and tied with a slipknot to produce occlusion. A small piece of polyethylene tubing was used to secure the ligature without damaging the artery. Ischemia was confirmed by the visualization of blanching myocardium, downstream of the ligation. After 30 min of ischemia, LAD coronary artery occlusion was released by removing the polyethylene tube and reperfusion occurred. The suture thread was left in place. Reperfusion was confirmed by visible restoration of red color to the ischemic tissue. The chest was closed and pneumothorax was evacuated from the chest cavity. The ventilator was removed and normal respiration was restored. At different reperfusion time points, animals were anesthetized with 4% isoflurane and sacrificed (by intraperitoneal injection of ketamine/xylazine [4 mg/0.2%]) for infarct size determination and immunohistochemical analysis.

2.2.2. Evaluation of the area at risk and infarct size

At mouse sacrifice (after 24 h of reperfusion) the LAD coronary artery was re-occluded. The prolene suture thread was previously left in place in order to re-occlude the left descending coronary artery, exactly at the same ligation site. Evan's blue dye 2% (Sigma) was injected in the left ventricle to delineate in vivo area at risk (AAR). Hearts were rapidly excised, rinsed in NaCl 0.9%, frozen and sectioned into 2-mm transverse sections from apex to base (5–6 slices/heart). To distinguish viable (AAR) from necrotic tissue (infarction [I]), heart sections were incubated at 37 °C with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 15 min, fixed in 10% formaldehyde solution and photographed with a digital camera (Nikon Coolpix). The viable myocardium was visualized in red, whereas necrotic tissue appeared in white. The different zones were determined using a computerized planimetric technique (MetaMorph v6.0, Universal Imaging Corporation).

2.3. Ex vivo ischemia/reperfusion protocol

We used the technique of Langendorff isolated buffer-perfused mouse heart preparation that has been previously described [20]. Briefly, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg). The heart was rapidly excised and placed in ice-cold Krebs–Henseleit bicarbonate buffer consisting of (in mmol/l): 118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, and glucose 5 and equilibrated with 95% O_2/5% CO_2 (pH 7.4). Following the removal of extraneous tissues (pericardium, lung, trachea, etc.), the aorta was cannulated with an 18-G plastic cannula (1.5 cm length; 0.95 mm, inner diameter) for a Langendorff retrograde perfusion. After stabilization, local ischemia was induced by LAD coronary artery occlusion. This procedure was performed as described in the in vivo ischemia–reperfusion protocol. After 30 min of ischemia, reperfusion was allowed for 2 h by removing the polyethylene tube. After the reperfusion period, the suture was re-occluded and Evan's blue dye 2% (Sigma) was injected within the heart through the cannulated aorta to delineate in vivo AAR. The TTC staining was performed as described before in the in vivo ischemia–reperfusion protocol section.

2.4. Immunostaining

Hearts isolated from animals were perfused with NaCl 0.9% to remove blood, cleaned, and frozen in OCT compound (Tissue-Tek). They were then cut serially from the occlusion locus to the apex in 7 μm serial sections.

Immunostainings for neutrophils (anti-mouse Ly-6B.2 antibody, ABD Serotec) and macrophages (anti-mouse CD68, ABD Serotec) were performed on 5 midventricular cardiac sections per animal, as previously described [21]. Quantifications were performed with MetaMorph v6.0 software (Universal Imaging Corporation). Results were expressed as number of infiltrating cells on mm^2 of total heart surface area.

Before 4-hydrox-2-nonenal (4-HNE) and 3,5-dibromotyrosine (DiBrY) stainings, we used, according to the manufacturer's recommendation, the Vector M.O.M. immunodetection kit (VECTOR Laboratories) to block detection with anti-mouse immunoglobulin secondary antibody of endogenous mouse immunoglobulins in the tissue. To detect 4-HNE that is a highly toxic aldehyde product of lipid peroxidation, mouse antibody against 4-HNE (Percipio Biosciences) has been used. Activation of neutrophil, monocyte and eosinophil is known to catalyze the formation of hypochlorous acid that reacts with proteins and induces tyrosine halogenation such as 3,5-dibromotyrosine [22]. Therefore, to assess leukocyte-derived oxidative stress, we also performed immunostaining for 3,5-dibromotyrosine using a mouse anti-dibromotyrosine monoclonal antibody (NNS-MBY-020P-EX; Cosmo Bio Co., LTD). Quantification of stained area was performed by computer

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analysis using MetaMorph v6.0 (Universal Imaging Corporation). The results of oxidative modification were expressed as percentages of stained area on total heart surface area.

2.5. Measurement of oxidative stress by hydroethidine fluorescence

Oxidative stress was assessed in myocardium 24 h after reperfusion. We used the ROS-sensitive dye hydroethidine (HE, Molecular Probes). ROS rapidly oxidized HE to fluorescent ethidium, which is then intercalated into nucleic acids. Fluorescent ethidium is therefore a presumptive marker of oxidative stress. Hearts of control or treated mice were washed in cold saline and embedded in OCT for cryosectioning. Frozen sections (7 μm) of the myocardium were stained with 10 μM HE at 37 °C for 30 min in a light-protected and humidified chamber. Stained sections were mounted with the mounting medium for fluorescence with DAPI (Vectashield). In situ fluorescence was assessed using fluorescence microscopy. The quantification of fluorescence was performed using MetaMorph v6.0 (Universal Imaging Corporation) on 3 fields per section and 5 sections per animal.

2.6. Western blot analysis

After 10 min of reperfusion, proteins from hearts were harvested in ice-cold radioimmunoprecipitation (RIPA) lysis buffer. Total protein concentrations were determined using the bicinchoninic acid (BCA) quantification assay (Pierce). Blots were blocked in 5% dry milk–phosphate-buffered saline (PBS)–0.1% Tween and incubated for 1.5 h at room temperature with the following primary antibodies: anti-ERK1/2 (R&D Systems, AF1576) and anti-phospho-ERK1/2 (T202/Y204) (R&D Systems, AF1018). The first antibody incubation was followed by 1 hour incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch). Membranes were developed using an enhanced chemiluminescence system (Millipore) to obtain autoradiograms. After scanning, the blots were analyzed for optical density (ImageJ 1.43u). Relative intensities were calculated by the ratio between phosphorylated and total protein amounts after stripping on the same gel membrane.

2.7. Serum cytokine measurement

Twenty four hours after reperfusion, sera from wt, Nox1 −/− and Nox2 −/− mice were collected. Flow cytometry was performed to assess systemic concentration of IL-6, IL-10, IL-12p70, CCL2, TNF-α and IFN-γ, using BD^{tm} Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences) as recommended by the supplier.

2.8. Statistical analysis

Statistical analysis was performed with Sigmastat software. Data are expressed as mean ± SEM. Paired groups were compared using either t-test. Multiple groups were compared using one way ANOVA followed by post-hoc Bonferroni t-tests. p values below 0.05 were considered significant.

3. Results

3.1. Genetic deficiencies in Nox1, Nox2, and double Nox1/Nox2 but not in Nox4 are associated with decreased myocardial reperfusion injury

To investigate the involvement of the ROS generating Nox enzymes in myocardial ischemia–reperfusion physiopathology, we submitted wild-type (wt), Nox1, Nox2, Nox1/Nox2 (double KO), and Nox4 deficient C57Bl/6 mice to 30 min of ischemia followed by 24 h of reperfusion. Severity of the ischemic insult was similar in the different NOX knockout mice as well as controls, as shown by the comparable ratio between the area at risk (AAR) and total ventricle area (V) (Fig. 1A). In Nox1 −/−, Nox2 −/−, and double KO animals, we observed a significant decrease in myocardial infarct size (I/AAR) compared to control (wt) mice. However, no significant difference was observed between Nox4 deficient mice versus wt (Fig. 1B). Fig. 1C illustrates representative examples of heart slices from wt, Nox1 −/−, Nox2 −/− (single and double knockout), and Nox4 −/− mice that have been used for quantification of the histological infarct size parameters. Considering previous results from Hoffmeyer and co-workers showing that deletion of *Ncf1* gene coding for the p47phox subunit did not influence myocardial reperfusion injury in vivo in mice [13], we repeated the ischemia/

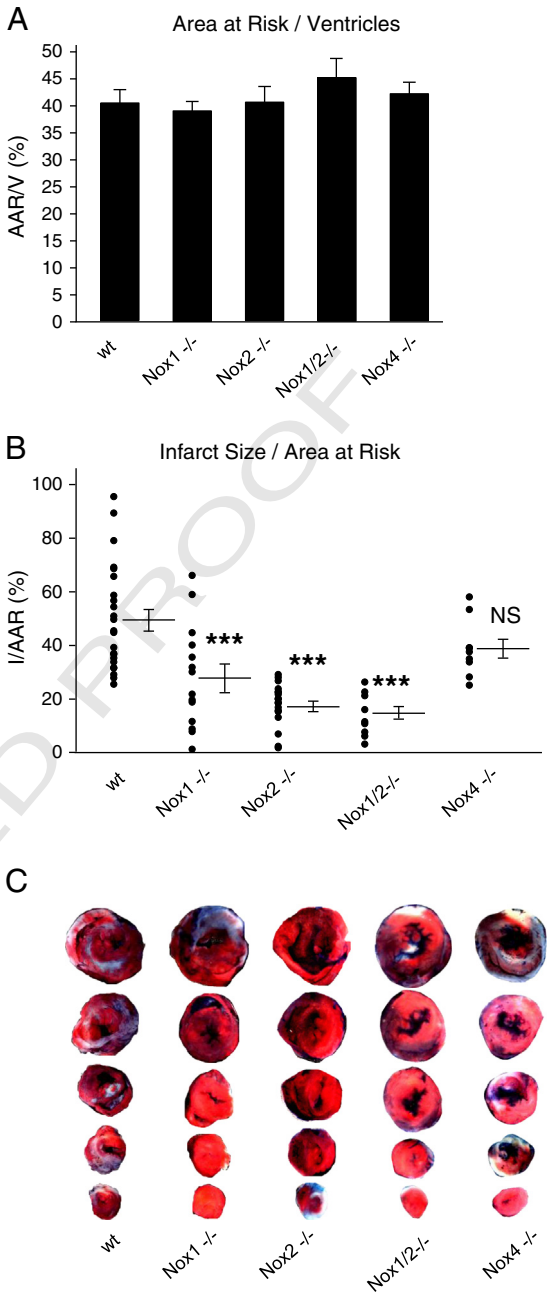

Fig. 1. Nox1, Nox2 and Nox1/Nox2, but not Nox4 deficient mice are protected from myocardial ischemia/reperfusion injury in vivo. Data are expressed as mean ± SEM. A. Quantification of area at risk (AAR) per ventricle surface (V) in wild type (wt) (n = 23), Nox1 −/− (n = 14), Nox2 −/− (n = 18), Nox1/2 double knockout (double KO) (n = 9), and Nox4 −/− (n = 9) animals. B. Quantification of infarct size (I) per AAR: ***p < 0.001 vs. wt; N.S.: not significant vs. wt. C. Representative images of TTC stained middle heart sections used for infarct size quantifications.

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reperfusion protocol using *Ncf1* mutant mice (B10.Q background). No difference in infarct size was observed in *Ncf1* mutant compared to wild-type mice, thereby confirming previous observations in our experimental model (Supplementary Fig. 1).

3.2. The myocardial damage in chronic myocardial ischemia is not affected by Nox1 or Nox2 deficiencies

In order to investigate whether the NOX1/NOX2-mediated protection occurred in the 30 min of ischemia or later during reperfusion, we submitted the mice to 24 h of permanent occlusion of the left coronary artery. The area at risk (AAR) was similar in the mouse groups, indicating that ligatures were reproducibly performed at the same level of the left anterior coronary arteries (Fig. 2A). Infarct size was comparable between wild type control and Nox1 and Nox2 (single and double knockout) deficient mice (Figs. 2B and C). As evidence of the permanent coronary ligature and chronic ischemia, the heart slices showed more atrophic ventricle walls as compared to the previous ischemia–reperfusion model (Figs. 1C and 2C). Taken together, these results suggest that ROS generated by Nox1 and Nox2 during the reperfusion are critical determinants of cardiac infarct injury. Note that additional control animals were not included (in particular NOX4 KO animals) in order to conform to the 3R (Reduce, Refine, Replace) criteria.

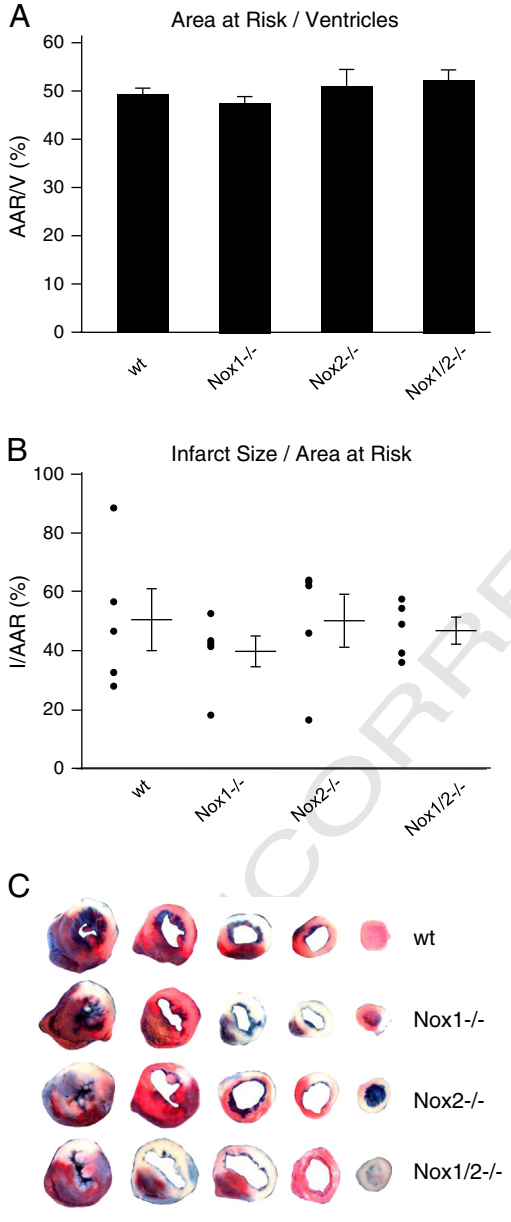

Fig. 2. Nox1, Nox2 and Nox1/Nox2 deficient mice are not protected from ischemic at 24 h of chronic ischemia. Data are expressed as mean ± SEM, n = 5 per group. A. Quantification of area at risk (AAR) per ventricle surface (V) in wild type (wt), Nox1 −/−, Nox2 −/−, and Nox1/2 double knockout (double KO) animals. B. Quantification of infarct size (I) per AAR. C. Representative sections of hearts submitted to 24 h of permanent occlusion stained with TTC and used for quantification. Differences between all knockouts vs. wt were not significant.

3.3. The post-ischemic infiltration of neutrophils, but not macrophages is decreased in Nox2 deficient mice

In order to investigate whether the deficiencies for Nox1 and Nox2 genes influence neutrophil and macrophage infiltration within the ischemic myocardium, we performed immunohistochemical stainings of heart cryosections at 24 h of reperfusion with specific markers for mouse neutrophils (Ly-6B.2 + cells) and macrophages (CD68 + cells). Nox2 −/− mice presented a significant decrease of neutrophil recruitment within the post-ischemic myocardium when compared to controls (Fig. 3A). On the other hand, although the count of infiltrating neutrophils appeared decreased between Nox1 −/− and wild type animals (Fig. 3A), it did not reach statistical significance. In consecutive cryosections, no difference in macrophage recruitment was observed in Nox1 −/− nor in Nox2 −/− as compared to wt controls (Fig. 3B). To evaluate the systemic inflammatory state of the different strains, the serum levels of both anti- and pro-inflammatory cytokines and chemokines were assessed at 24 h of reperfusion (Table 1). No significant difference between the groups for all the molecules tested (IL-6, -10, -12, CCL2, TNF-α, and IFN-γ) was shown.

3.4. Nox1 −/− and Nox2 −/− hearts are protected from ischemia/reperfusion injury ex vivo (Langendorff model)

Finally, the effect of Nox1 and Nox2 genetic deficiency was evaluated in an ex vivo model of ischemia/reperfusion injury. Since this protocol is performed using excised buffer-perfused hearts in a system free of blood, we were capable of evaluating the selective response of the cardiac tissue in the absence of circulating inflammatory cells and molecules. Mouse hearts were submitted to 30 min of ischemia by LAD coronary occlusion followed by 2 h of reperfusion. AAR was similar for all groups, proving similar ischemic insult in different groups (Fig. 4A). Confirming in vivo results, both Nox1 and Nox2 single knockout mice showed smaller infarct size than wt controls (Figs. 4B and C), indicating that at least part of the protective effect mediated by Nox1 and Nox2 deficiencies during reperfusion was not due to post-ischemic inflammation, but it is rather intrinsic to the ischemic myocardium.

3.5. Myocardial ROS are differently influenced by Nox2 and Nox1 at 24 h of reperfusion

In myocardial injury during reperfusion, ROS have been shown as crucial mediators. We used different methods to compare relative oxidative stress levels between Nox deficient mice and controls. Hydroethidine (HE) is a hydrophobic compound able to cross cell membranes. Inside cells, HE reacts with superoxide, hydrogen peroxide and activity of heme-containing enzymes to form different products with overlapping red fluorescence [23]. HE fluorescence is therefore not specific for a single type of ROS, but represents an indicator of general oxidation state of a cell or tissue. 4-hydroxynonenal (4-HNE) is a marker of lipid peroxidation and 3,5-dibromotyrosine (DiBrY) is formed by halogen radicals generated by neutrophil activity. We first investigated the basal release of ROS in myocardium of wt and Nox deficient mice and did not observe any significant differences between the groups (Supplementary Fig. 2). After 24 h of reperfusion, we also measured these oxidative stress markers within sections of infarcted hearts from

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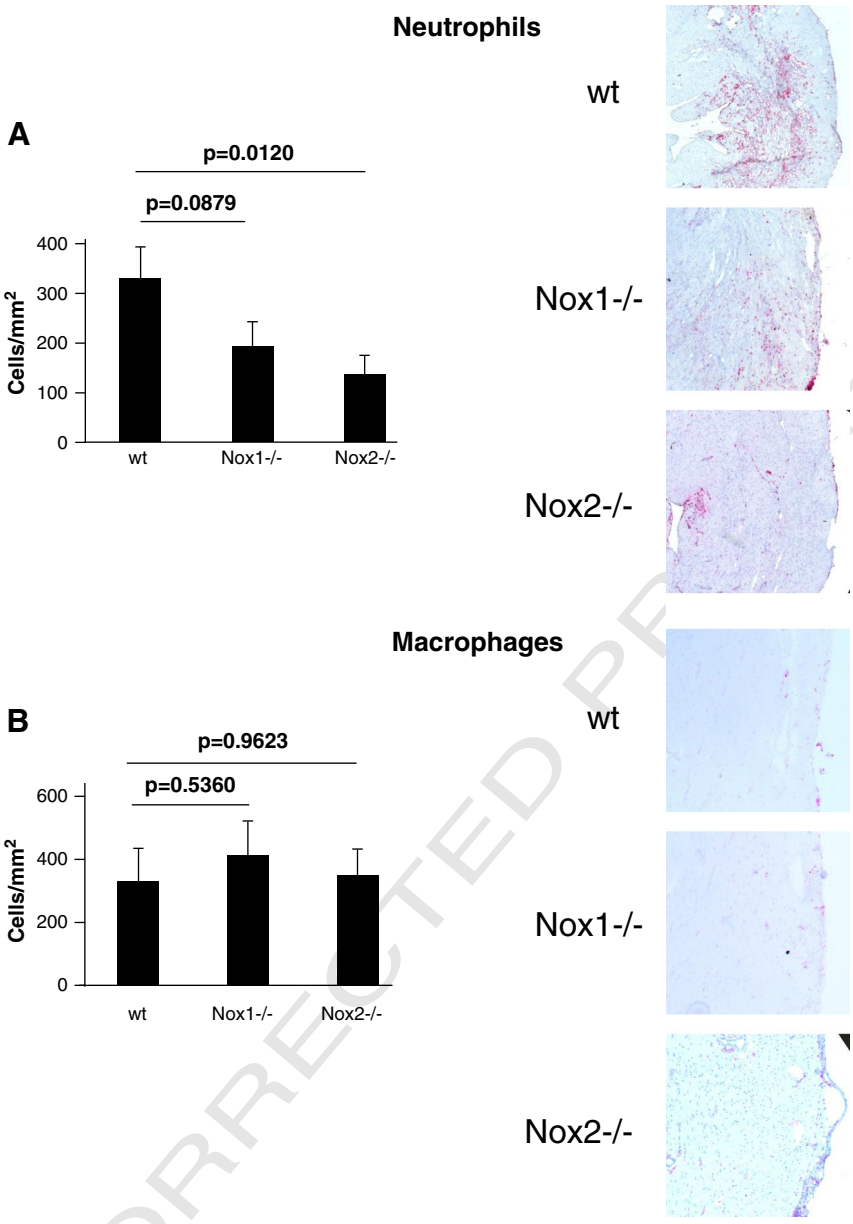

Fig. 3. Nox2, but not Nox1 deficiency is associated with reduced neutrophil myocardial infiltration after 24 h of reperfusion. Quantification and representative images of the immunostaining of heart frozen sections using specific antibodies directed against neutrophils (A) and macrophages (B). Positive cells (purple) were counted using morphometric computer analysis. Data are expressed as mean ± SEM, n = 7 for controls and n = 10 for Nox1 −/− and Nox2 −/− animals. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Nox1 −/−, Nox2 −/− and wt mice. HE fluorescence was significantly reduced in Nox2 −/−, but not in Nox1 −/− hearts as compared to wt controls (Fig. 5A). At the same reperfusion time point, no significant difference in 4-HNE myocardial production was observed between the mouse groups (Fig. 5B). Both Nox1 and Nox2 single knockout animals had a significant reduction in DiBrY myocardial content when compared to wt controls (Fig. 5C).

Table 1
Cytokine and chemokine serum levels after 30 min of ischemia and 24 h of reperfusion.

Cytokine	Wt (pg/ml) n = 4	Nox1 −/− (pg/ml) n = 9	Nox2 −/− (pg/ml) n = 5	ANOVA test
IL-6	63.84 ± 26.57	59.54 ± 9.43	76.99 ± 26.59	NS
IL-10	165.84 ± 59.75	192.6 ± 30.99	108.80 ± 36.59	NS
IL-12p70	39.19 ± 14.17	42.02 ± 7.84	28.23 ± 5.40	NS
CCL2	206.82 ± 39.17	169.32 ± 22.72	166.11 ± 5.04	NS
TNF-α	19.94 ± 1.19	20.98 ± 1.98	28.35 ± 9.63	NS
IFN-γ	39.19 ± 14.17	42.02 ± 7.84	28.23 ± 5.4	NS

3.6. Nox1 and Nox2 deficiencies are associated with the activation of different cardioprotective intracellular phosphorylation pathways

To identify the molecular mechanisms downstream of Nox1 and Nox2 at an early stage (10 min) during reperfusion, we investigated the potential concomitant activation of intracellular signaling pathways, previously shown to salvage cardiomyocytes from ischemic injury [24]. To this end, the activation of Reperfusion Injury Salvage Kinase (RISK) and Survivor Activating Factor Enhancement (SAFE) pathways was explored. At 10 min of reperfusion, Nox1 and Nox2 deficiencies were associated with an increase in extracellular signal-regulated kinase (ERK) phosphorylation when compared with wt controls, suggesting that ROS are inhibiting ERK phosphorylation at the early stage of reperfusion

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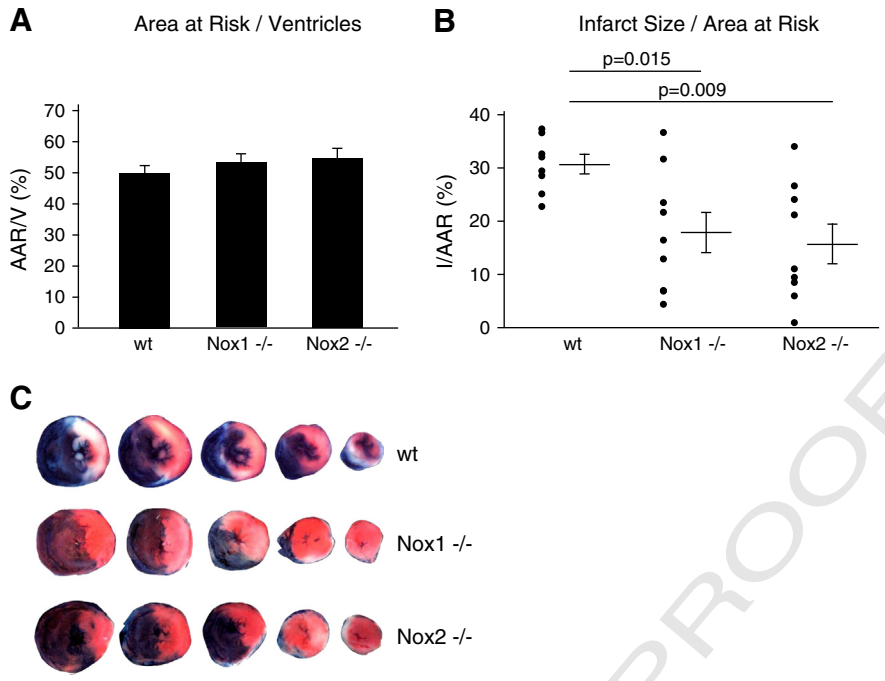

Fig. 4. Nox1 and Nox2 hearts are protected from myocardial ischemia/reperfusion injury ex vivo (Langendorff model). Data are expressed as mean ± SEM. A. Quantification of area at risk (AAR) per ventricle surface (V) in wild type (wt) ($n = 8$), Nox1 −/− ($n = 8$), and Nox2 −/− ($n = 9$) mice submitted to 30 min of ischemia followed by 2 h of reperfusion. No significant differences were seen between all groups. B. Quantification of infarct size (I) per AAR after 2 h of reperfusion. C. Representative sections of infarcted hearts stained with TTC and used for quantification.

(Figs. 6A and B). Phosphorylation of Akt is increased in Nox1 −/− hearts (Fig. 6C) while, conversely, absence of Nox2 was associated with a significant phosphorylation of Signal Transducers and Activators of Transcription (STAT) 3 (Tyr-705) as compared to controls (Fig. 6D). However, no differences of basal phosphorylation of either ERK1/2, AKT or STAT3 were observed (Supplementary Fig. 3).

4. Discussion

Oxidative stress is a hallmark of myocardial ischemia/reperfusion physiopathology as increased ROS generation occurs when oxygen supply is restored following an ischemic event. Although ROS are almost invariably detected during reperfusion, their exact role is unclear. Low levels of oxidants can be cardioprotective in pre- and post-conditioning therapies, while high levels of ROS are deleterious and lead to cardiomyocyte death [3]. In addition, different types, localization and potential sources of ROS may impact cardiac recovery during reperfusion. Among potential sources of ROS, three Nox isoforms (Nox1, Nox2 and Nox4) are expressed within the cardiac tissue [7]. Nox enzymes are known to be involved in ischemia–reperfusion injury, but studies aiming at understanding the exact role of each isoform have often provided contradictory results [6,25]. In this study, we addressed the role of each Nox isoform in myocardial ischemia by applying identical protocols for in vivo and ex vivo experiments using genetically-deficient mice for Nox1 and Nox2 as well as Nox1/Nox2 double knockouts. In vivo infarct size was also measured in Nox4 knockout and Ncf1 mutant mice.

The main result of this study is that mice deficient in Nox1, Nox2, and Nox1/Nox2 presented a significant reduction in myocardial post-infarction necrosis during reperfusion when compared to control wt mice. No difference was measured in the reperfusion injury for Nox4 deficient mice. The deleterious effect of Nox1 and Nox2 derived ROS occurs during the reperfusion phase rather than during ischemic phase, because no in vivo protective effect was observed following 24 h of ischemia (i.e. without reperfusion). This finding is consistent with the concept that oxygen supply during reperfusion provides substrate for Nox-mediated ROS generation.

Previous studies suggested a role of Nox2 in myocardial ischemia–reperfusion, but, until recently there was only one study using genetically deficient mice in in vivo myocardial ischemia–reperfusion injury. This study by Hoffmeyer and co-workers showed that Ncf1 deficiency (the gene coding for p47phox, a subunit of Nox2) did not change infarct size, nor left ventricular function compared to heterozygous controls [13]. This finding suggested that Nox2 is not involved in myocardial ischemia–reperfusion because the p47phox subunit is crucial for the normal function of the NADPH oxidase. Mutations affecting both Ncf1 and Nox2 similarly result in chronic granulomatous disease (CGD), which is characterized by defective Nox2-dependent ROS generation in phagocytes [26]. We confirmed Hoffmeyer's findings in another mouse genetic background using the Ncf1 mutant mice. Such disparity between Nox2 and Ncf1 deficient or Ncf1 mutant mice has been previously observed in other disease models, such as experimental inflammatory bowel disease and murine *Listeria monocytogenes* infection [27,28]. This difference might be due to the fact that residual ROS are generated in Ncf1 knock-out and mutant Ncf1 mice. Indeed CGD patients affected by mutations in the NCF1 gene generally present a less severe phenotype than the X-linked form of the disease affecting CYBB, the gene coding for Nox2 [29], although an independent role of Ncf1 should not be excluded.

While writing this manuscript, Matsushima et al. published a study showing a decrease in myocardial damage following ischemia–reperfusion in both Nox2- and Nox4-deficient mice [30]. The Nox2-deficient mice were in the same background, thereby corroborating our findings. For Nox4-deficient mice, the difference between Matsushima's findings and our study might come to the fact that we used global Nox4 knock-out mice, compared to cardiac specific Nox4 knock-out mice. Interestingly, in contrast to the Nox1/Nox2 knock-outs which are strongly protected from the ischemic insult, myocardial injury was exacerbated in the Nox2/Nox4 double knock-out [30]. Unlike Nox1 and Nox2, which require activation for activity, Nox4 constitutively generates ROS [12]. The role of Nox4-derived ROS must be quite subtle as it can be protective in the vasculature during ischemic or inflammatory stress or deleterious in other

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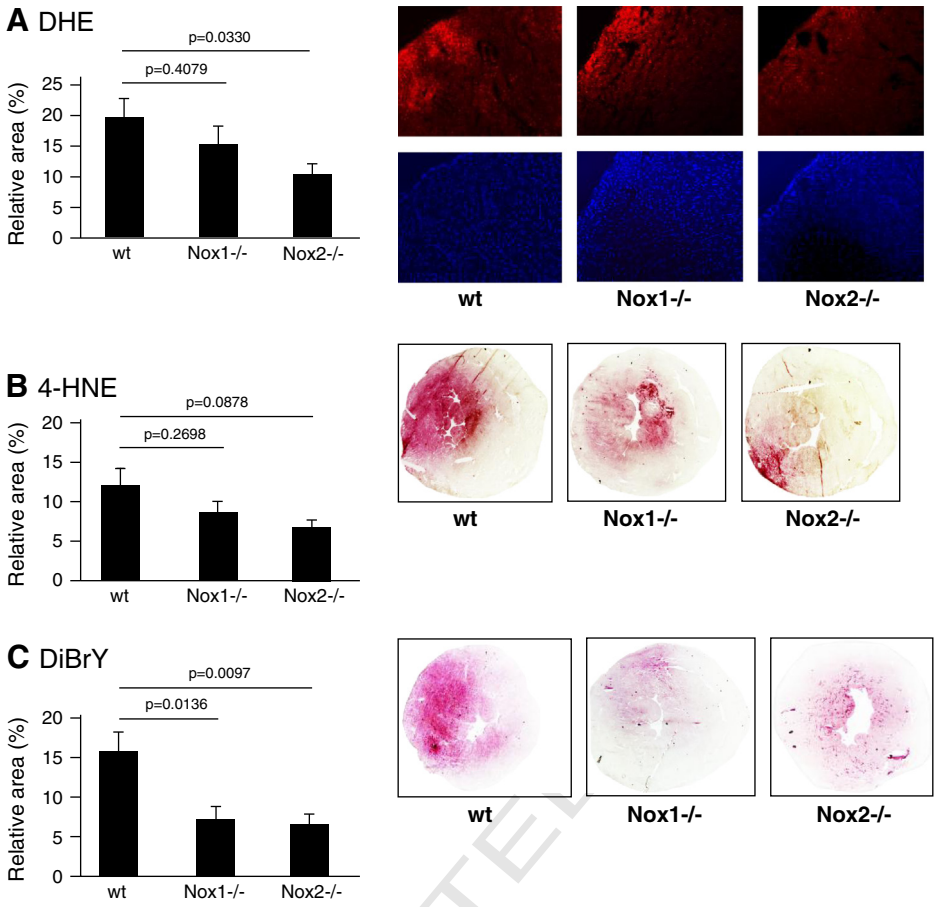

Fig. 5. ROS production is partially reduced in Nox1 and Nox2 deficient mice. A. Quantification of oxidation level (DHE fluorescence) content in frozen sections of infarcted hearts at 24 h of reperfusion. On the right, representative images of DHE stained middle heart sections of wt Nox1 −/− or Nox2 −/− mice and corresponding images of the same middle heart sections stained with 4′,6-diamidino-2-phenylindole (DAPI, visualizing nuclear DNA) are shown. B. Quantifications of 4-HNE immunostaining in frozen sections of infarcted hearts at 24 h of reperfusion. C. Dibromo-tyrosine (DiBrY) immunostaining in frozen sections of infarcted hearts at 24 h of reperfusion. Data are expressed as mean ± SEM, n = 7 for controls and n = 10 for Nox1 −/− and Nox2 −/− animals.

experimental settings, such as brain ischemia–reperfusion model [31,32]. This potential cooperative activity between different sources of ROS highlights the complex role of Nox-derived ROS in the regulation of beneficial or deleterious pathways in cardiac pathophysiology.

In terms of mechanism, we have shown that a large part of the beneficial effects of Nox1 and Nox2 deletions were intrinsic to myocardium because Nox1 and Nox2 deficient hearts were similarly protected in the ex vivo Langendorff model, where hearts are perfused with Krebs solution (i.e. in the absence of circulating cells and molecules). Decreased neutrophil infiltration and neutrophil-mediated tyrosine halogenation (DibrY, a marker of neutrophil-mediated oxidative burst) in Nox1 and Nox2 knockout mice may partially contribute to this protective effect. However, difference in inflammation in the Nox1 and Nox2-deficient mice is unlikely to account for the protective effect as there were no differences in serum cytokine levels and infiltrated macrophage count in the ischemic hearts. Recently, CRE-LOX NOX-deficient mice have been generated for different Nox isoforms [33,34]. In vivo MIR cardiac-specific deletion of different NOX isoforms is therefore possible. These novel tools will help determine to which level post-ischemic inflammation-mediated Nox-derived ROS contributes to MIR injury.

As of today technical tools allowing direct in vivo measurement of Nox activity are still missing, therefore we used several indirect markers of oxidation of post-ischemic myocardium to quantify oxidant production. Decreased DiBrY staining was observed in both Nox1 and Nox2 deficient mice. Since DiBrY is a marker of neutrophil oxidative activity, this was consistent with the decrease in neutrophil infiltration in Nox1 −/− and Nox2 −/− hearts. However, only Nox2 −/− animals showed a trend for decrease of 4-HNE staining ($p = 0.0878$) and a significant reduction in HE fluorescence ($p = 0.033$), which were both used as a measure of global oxidation. This suggests that Nox2 generates larger levels of ROS leading to direct myocardial damage, while ROS generated by Nox1 may be either lower or more localized and regulate subtle intracellular pathways. Unfortunately the above markers did not allow us to evaluate the kinetics of ROS formation by Nox1 and Nox2. Novel probed for in vivo ROS measurements are being developed [35], but the use of available probes is so far limited by several factors: (i) lack of knowledge of their pharmacokinetic/pharmacodynamic (PK/PD) characteristics, (ii) oxidative modifications outside the tissue of interest, i.e. in the circulation, liver or kidney, and (iii) lack of knowledge of their oxidation kinetics and ROS specificity.

The difference between ROS generated by Nox1 and Nox2 leads to activation of different intracellular signaling pathways. Recently, Lecour identified two different cardioprotective phosphorylation pathways critical for the cardiomyocyte salvage from ischemia–reperfusion injury [24]. The SAFE signaling pathway involves the phosphorylation of STAT3 whereas the phosphorylation of ERK1/2 and Akt enhances the RISK signaling pathway. ROS generated by Nox1 and Nox2 suppressed phosphorylation of ERK1/2, but phosphorylation of Akt and STAT3 was strikingly different. The SAFE pathway (phosphorylation of STAT3) was only induced by Nox2 deletion, while Akt phosphorylation was enhanced only in Nox1 deficient mice. Further studies using specific Akt and ERK inhibitors as well as studies on downstream targets of Akt and ERK will be required to address the link between Nox and the survival pathway.

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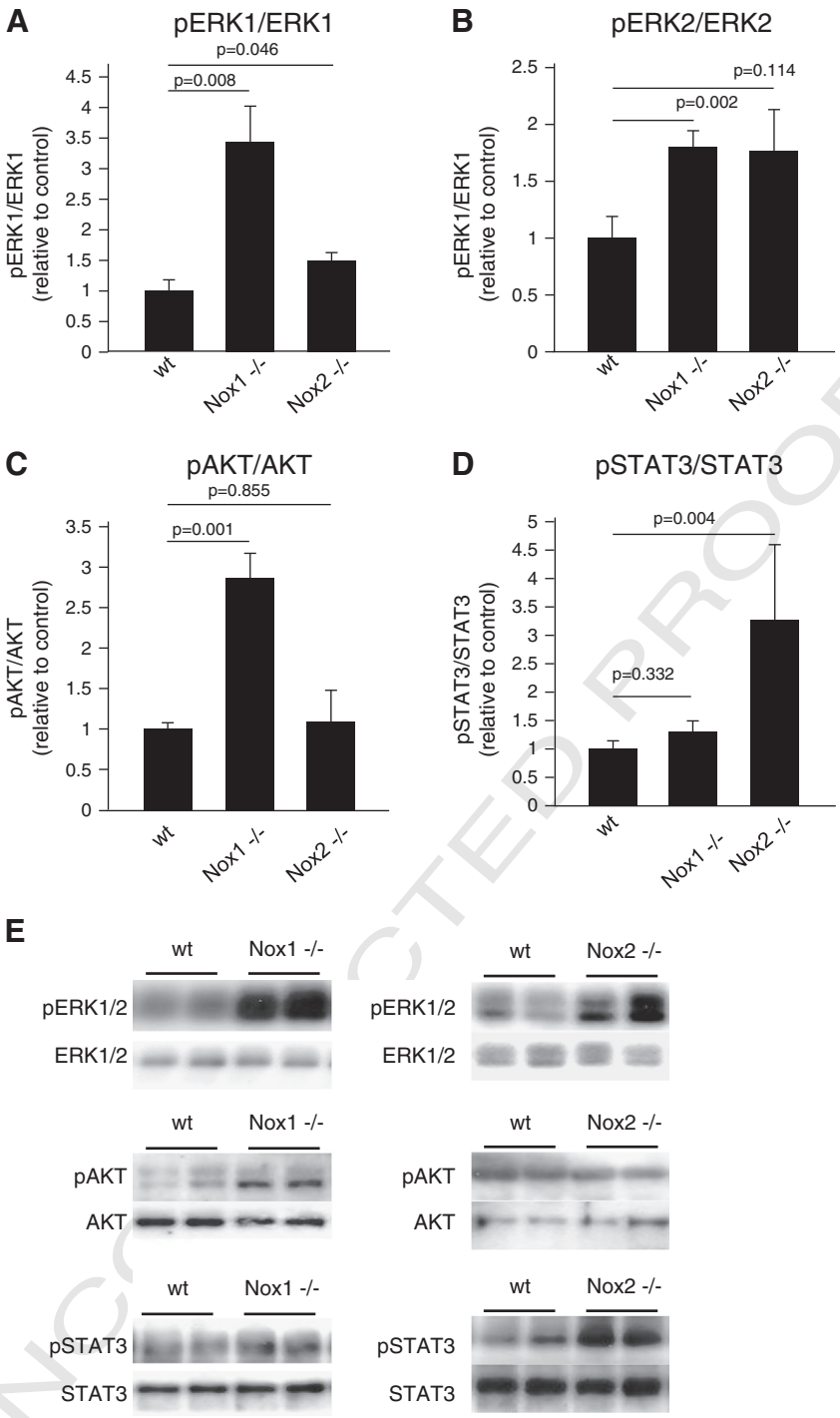

Fig. 6. Nox1 and Nox2 deficiencies are associated with the activation of different protective pathways within the infarcted myocardium early during reperfusion. Quantifications of western blots (n = 5 for sham and wt animals, n = 7 for Nox1−/− mice and n = 6 for Nox2−/− mice) for phosphorylated or total ERK1 (A), ERK2 (B), AKT (C), and STAT3 (D). Relative intensities were calculated by the ratio between phosphorylated and total protein amounts. E. Representative images of ERK, AKT and STAT3 blots.

Thus, ROS-mediated cardiotoxicity is abolished by different cardioprotective pathways in Nox1- and Nox2-deficient mice during reperfusion.

5. Conclusion

In conclusion, our findings suggest that treatments targeting Nox1 and Nox2 inhibition during myocardial reperfusion procedures (such as angioplasty) may improve cardiomyocyte survival to oxidative injury. Evidence of the benefit of pharmacological Nox inhibition during ischemia–reperfusion injury is still sparse, however, in a search for novel Nox inhibitors, ebselen, a selenium compound previously known to protect from oxidative damage in myocardial ischemia [36,37], was shown to be a potent inhibitor of Nox1 and Nox2 [38]. As small molecule Nox inhibitory drugs are emerging [39,40], one awaits this innovative approach to be evaluated in myocardial reperfusion injury in the near future. Q5

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Disclosure statement

KHK and VJ are founding members of the startup company GenKyoTex (http://www.genkyotex.com), which develops Nox inhibitors. The other authors declare no potential conflicts of interest relevant to the subject matter of this work.

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