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Cytochrome P450 enzymes but not NADPH oxidases are the source of the NADPH-dependent lucigenin chemiluminescence in membrane assays

Flávia Rezende[a], Kim-Kristin Prior[a], Oliver Löwe[a], Ilka Wittig[b], Valentina Strecker[b], Franziska Moll[a], Valeska Helfinger[a], Frank Schnütgen[c], Nina Kurrle[c], Frank Wempe[c], Maria Walter[a], Sven Zukunft[d], Bert Luck[d], Ingrid Fleming[d], Norbert Weissmann[e], Ralf P. Brandes[a,*], Katrin Schröder[a,*]

[a] Institute for Cardiovascular Physiology, Goethe-University, Frankfurt, Germany
[b] Functional Proteomics, SFB 815 Core Unit, Goethe-Universität, Frankfurt, Germany
[c] Institute for Molecular Hematology, Goethe-University, Frankfurt, Germany
[d] Institute for Vascular Signaling, Goethe-University, Frankfurt, Germany
[e] University of Giessen, Lung Center, Giessen, Germany

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ABSTRACT

Measuring NADPH oxidase (Nox)-derived reactive oxygen species (ROS) in living tissues and cells is a constant challenge. All probes available display limitations regarding sensitivity, specificity or demand highly specialized detection techniques. In search for a presumably easy, versatile, sensitive and specific technique, numerous studies have used NADPH-stimulated assays in membrane fractions which have been suggested to reflect Nox activity. However, we previously found an unaltered activity with these assays in triple Nox knockout mouse (Nox1-Nox2-Nox4$^{-/-}$) tissue and cells compared to wild type. Moreover, the high ROS production of intact cells overexpressing Nox enzymes could not be recapitulated in NADPH-stimulated membrane assays. Thus, the signal obtained in these assays has to derive from a source other than NADPH oxidases. Using a combination of native protein electrophoresis, NADPH-stimulated assays and mass spectrometry, mitochondrial proteins and cytochrome P450 were identified as possible source of the assay signal. Cells lacking functional mitochondrial complexes, however, displayed a normal activity in NADPH-stimulated membrane assays suggesting that mitochondrial oxidoreductases are unlikely sources of the signal. Microsomes overexpressing P450 reductase, cytochromes b5 and P450 generated a NADPH-dependent signal in assays utilizing lucigenin, L-012 and dihydroethidium (DHE). Knockout of the cytochrome P450 reductase by CRISPR/Cas9 technology (POR$^{-/-}$) in HEK293 cells overexpressing Nox4 or Nox5 did not interfere with ROS production in intact cells. However, POR$^{-/-}$ abolished the signal in NADPH-stimulated assays using membrane fractions from the very same cells. Moreover, membranes of rat smooth muscle cells treated with angiotensin II showed an increased NADPH-dependent signal with lucigenin which was abolished by the knockout of POR but not by knockout of p22phox. In conclusion: the cytochrome P450 system accounts for the majority of the signal of Nox activity chemiluminescence based assays.

1. Introduction

NADPH oxidases of the Nox family are important sources of reactive oxygen species (ROS). This assumption is based on several lines of evidence. The genetic deletion of Nox homologues in mice results in defined functional deficits which are accompanied by a decrease in ROS formation in the target tissue. Moreover, an increase in ROS production is detectable in cells or organs after overexpression of Nox enzymes [1–4].

The concept of Nox enzymes as sources of ROS emerged in part from inhibitor studies: It was initially observed that ROS formation of intact tissue and cells was highly sensitive to the potent Nox inhibitors diphenylene iodonium (DPI) and apocynin [5]. Later studies, however, revealed that DPI inhibits many flavoenzymes [6] whereas apocynin was found to interfere with redox-mechanisms in general. It depletes glutathione, acts as antioxidant and changes the expression of ROS generation enzymes such as cyclooxygenases [7,8].

Another “traditional” line of evidence for an important role of Nox

* Correspondence to: Institut für Kardiovaskuläre Physiologie, Fachbereich Medizin der Goethe-Universität, Theodor-Stern Kai 7, 60590 Frankfurt am Main, Germany.
E-mail addresses: brandes@vrc.uni-franfurt.de (R.P. Brandes), schroeder@vrc.uni-frankfurt.de (K. Schröder).

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enzymes for ROS production came from chemiluminescence assays that made use of the enhancer lucigenin. In addition to ROS measurements in intact cells, chemiluminescence probes are frequently used to obtain an NADPH-dependent signal in cellular homogenates or membrane preparations. As these assays depend on NADPH and as Nox family NADPH oxidases utilize NADPH to generate ROS, the resulting signal is considered a reflection of the enzymatic activity of Nox enzymes [9]. Initially, measurements were performed with a high concentration (250 µmol/L) of the enhancer, which resulted in redox-cycling. To overcome this problem, the concentration of lucigenin was reduced to 5–10 µmol/L. However, with the new low concentration some of the previous observations could no longer be confirmed and the signal basically became undetectable [10]. In addition to redox-cycling, a direct reduction of lucigenin by flavoenzymes like eNOS (nitric oxide synthase) [11] and cytochrome P450 (CYP) monooxygenases has already been proposed [12]. In a previous study we reported the formation of lucigenin chemiluminescence by eNOS, via its diaphoresis activity [10]. However, a potential role of P450 monooxygenases has not yet been studied sufficiently. This enzyme class is part of a system, where P450 reductase transfers one electron from NADPH either to Cytochrome b_5 and then to CYP or directly to CYP to hydroxylate substrates during drug detoxification and hormone synthesis. In fact, it is well known that superoxide production occurs when CYP enzymes are in a one electron reduced state [13,14]. Thus, biochemically, it is conceivable that other enzymes than those of the Nox family generate a chemiluminescence signal in the presence of enhancers and NADPH. Importantly, the "NADPH oxidase assays" have never been truly validated until recently.

In our previous work we found little evidences to support the idea that chemiluminescence-dependent "NADPH oxidase assays" detect in vitro Nox activity. The assay activity remained unchanged after the triple knockout of the main enzymatically active Nox homologues (Nox1, Nox2 and Nox4). Moreover, overexpression of Nox enzymes dramatically increased the ROS formation of intact cells but did not alter the activity in the "NADPH oxidase assay" performed using isolated membrane fractions [10]. On such basis we hypothesized that "NADPH oxidase assays" in isolated membranes detect the activity of proteins other than Nox and set out to identify those. To do so, we used a combination of native gel electrophoresis, nitroblue tetrazolium reduction, mass spectrometry as well as gain and loss of function systems to identify the enzymatic source of the signal.

2. Material and methods

2.1. Membrane fraction preparation from tissue and cells

Tissue and cells were homogenized by pottering in Hepes Tyrode buffer (HT, containing in mmol/L: 137 NaCl, 2.7 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5 glucose, 0.36 NaH_2PO_4, 10 HEPES) supplemented with a protease inhibitor mix (antipain, aprotinin, chymostatin, leupeptin, pepstatin, trypsin-inhibitor; AppliChem), okaidaic acid, calyculin A and EGTA. Homogenates were cleared by centrifugation (3000*g*, 10 min, 4 °C) and membrane fractions were obtained by centrifugation at 100,000*g* (1 h, 4 °C). The membrane pellet was resuspended in HT buffer and the protein concentration was estimated by Bradford assay. Twenty micrograms were used for measurements.

2.2. Chemiluminescence assays

Chemiluminescence in response to lucigenin (5 µmol/L), L-012 (200 µmol/L) or luminol (100 µmol/L)/horseradish peroxidase (HRP at 1 U/mL) was measured in a Berthold TriStar^2 microplate reader (LB942, Berthold, Wildbad, Germany). For the structure of these compounds see Fig. 1. Measurements with membrane fractions were initiated by addition of NADPH (100 µmol/L for lucigenin; 10 µmol/L for L-012). PEGylated superoxide dismutase (PEG-SOD, 50 U/mL) and DPI (10 µmol/L) were used as indicated. Chemiluminescence was expressed as arbitrary units.

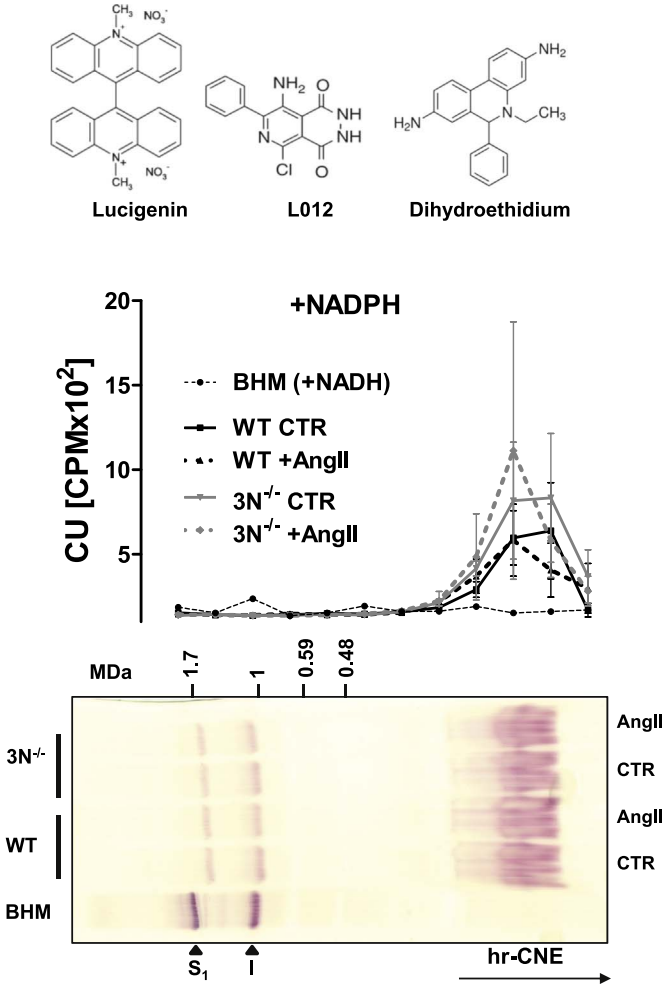

Fig. 1. Structures of compounds used (top) and NADPH reduction by membrane proteins in an in gel assay (middle and botton). WT and 3N-/- mice where treated with or without AngII. Membrane proteins from kidneys were separated by clear native electrophoresis. After separation of protein complexes under native condition, reduction of NADPH was visualized by the deposition of formazan crystals in presence of NBT (0.02%) (lower panel). A second identical set of samples was sliced and tested for lucigenin (5 µmol/L; 100 µmol/L NADPH). BHM (bovine heart mitochondria) was used as positive control in presence of NADH (100 µmol/L) as well as native molecular ladder. hr-CNE indicates high resolution clear native electrophoresis, in a range of 205–60 kDa as estimated by the molecular weight of BHM complexes. n≥3.

2.3. Dihydroethidium (DHE) assays

The oxidation products of dihydroethidium (DHE, 20 µmol/L), 2-dihydroxyethidium (2EOH) and ethidium (E), were separated by HPLC and analyzed either by absorbance (350–400 nm for DHE) and fluorescence (510 nm/595 nm exitation/emission for 2EOH and E) in intact cells and supersomes or by LC-MS/MS in membranes of HEK293 cells overexpressing Nox4 or Nox5 and knockout for cytochrome P450 reductase (POR-/-).

Briefly, intact and adherent HEK293 cells overexpressing Nox4, Nox5 and Nox5+PMA (phorbol myristate acetate, 100 nmol/L, 15 min) as well as supersomes (with or without NAPDH 100 µmol/L) (microsomes overexpressing different combinations of components of the human cytochrome P450 system) were incubated with DHE for 15 min at 37 °C in Hanks buffer containing 100 µmol/L DTPA (Diethylenetriamine-pentaacetic acid pentasodium salt).

Cellular and supersome membranes were solubilized with 1% triton

and deproteinated by precipitation with perchloric acid (0.2 mol/L) prior to HPLC injection.

For fluorescence detection, DHE and its oxidation products were separated by HPLC (Hitachi, Elite Lachrom system L3130 pump) using a C18 column (EC, Nucleosil, 100-5, 250/4.6 Macherey Nagel) and a mobile phase A of H_2O: acetonitrile: TFA (9:1:0.1) and phase B of acetonitrile +0.1% TFA. A gradient from 0% to 40% of B was achieved within 10 min and to 100% B in 20 min with a 0.5 mL/min flow.

To estimate 2EOH concentration, potassium superoxide (14 mmol/L) was incubated with DHE (20 µmol/L) and separated using the above chromatographic condition. Concentration of 2EOH was estimated as described [15].

The LC-MS/MS analysis was performed on a 1290 Infinity UHPLC system (Agilent, Waldbronn, Germany) coupled to an 5500 QTrap triple quadrupole mass spectrometer with an TurboV electro spray ionisation source (AB Sciex Deutschland GmbH, Germany). DHE and its oxidation products were separated on a C18 Phenomenex Kinetex column (150×2.1 mm), protected by a Phenomenex C18 guard cartridge, using an acetonitrile/water gradient with 0.1% formic acid. The ion source parameters were set as follows: x-axis and y-axis of the source were set to 5.0 mm, TEM=300 °C, IS=3500 V, GS1=50 p.s.i., GS2=40 p.s.i., CUR=30 p.s.i., CAD=medium. Detection of DHE oxidation products was achieved by multiple reaction monitoring (MRM) in positive ion mode. System control and analytical data analysis were processed by Analyst software 1.6.2.

2.4. Electrophoretic separation of membrane proteins followed by NADPH activity assay and mass spectrometry analysis

Heart and kidney membranes were prepared from wild type (WT) and triple Nox (Nox1-Nox2-Nox4, $3N^{-/-}$) knockout animals treated or not with AngII (0.7 mg/kg/day) as described previously [10]. Two hundred micrograms of membrane pellets were resuspended in 20 µl buffer A (50 mmol/L Imidazole pH 7, 50 mmol/L NaCl, 2 mmol/L aminocaproic acid, 1 mmol/L EDTA), solubilized by addition of 6 µl 20% (w/v) digitonin. Samples were loaded in duplicates to native gradient gels (3–16%) and proteins/ protein complexes were resolved by high resolution clear-native electrophoresis (hrCNE) as described [16]. One set of samples was used for NADPH-dependent (100 µmol/L) reduction by *in gel* deposition of formazan crystals from nitro blue tetrazolium (NBT, 0.02%) [17,18]. The second set was used for lucigenin measurements in homogenized gel slices in the presence of NADPH. Isolated bovine heart mitochondria (BHM, 200 µg) were used as positive control in presence of NADH (100 µmol/L) and as molecular weight indicator [16].

2.5. Mass spectrometry analysis

Gel slices yielding an NADPH-dependent chemiluminescent signal were excised from a corresponding sample set stained with Coomassie blue. For mass spectrometry analysis, cysteines were reduced and alkylated with DTT (10 mmol/L) and iodoacetamide (30 mmol/L), and digested with trypsin (sequencing grade, Promega). Liquid chromatography/mass spectrometry (LC/MS) was performed on Thermo Scientific™ Q Exactive coupled to an ultra-high performance liquid chromatography unit (Thermo Scientific Dionex Ultimate 3000) via a Nanospray Flex Ion-Source (Thermo Scientific). Peptides were loaded on a C18 reversed-phase precolumn (Zorbax 300SB-C18, Agilent Technologies) followed by separation on in-house packed 2.4 µm Reprosil C18 resin (Dr. Maisch GmbH) picotip emitter tip (diameter 100 µm, 15 cm long, New Objectives) using a gradient from mobile phase A (4% acetonitrile, 0.1% formic acid) to 50% mobile phase B (80% acetonitrile, 0.1% formic acid) for 30 min with a flow rate of 400 nl/min. Mass spectrometry (MS) data were recorded by data dependent Top10 acquisition (selecting the ten most abundant precursor ions in positive mode for high energy collision dissociation fragmentation (HCD)). The Full MS scan range was 300–2000 *m/z* with resolution of 70,000 at *m/z* 200, and an automatic gain control (AGC) value of $3*10^6$ total ion counts with a maximal ion injection time of 160 ms. Only higher charged ions (2+) were selected for MS/MS scans with a resolution of 17,500, an isolation window of 2 *m/z* and an automatic gain control value set to $5*10^4$ ions with a maximal ion injection time of 150 ms. Following fragmentation event all selected ions were excluded in a time frame of 30 s. Xcalibur raw files were analyzed by MaxQuant 1.5.3.30 [19]. The enzyme specificity was set to Trypsin and missed cleavages were limited to 2. Oxidation of methionine and acetylation of N-terminus were selected as variable modifications and Carbaminomethylation as fixed modification. The mouse reviewed reference proteome set (download from uniprot, April 2016, 16,764 entries) was used to identify peptides and proteins. False discovery rate (FDR) was set to 1%. Identified proteins were filtered by keyword NAD and NADPH. The sum of Intensity-based absolute quantification (IBAQ) values for each tissue was used for ranking.

2.6. Respiratory chain deficient (ρ^0) cells

The osteosarcoma cell line 143B, TK- (ρ^+) and its correspondent ρ^0 which completely lacks mitochondrial DNA and thereby is deficient of functional mitochondrial complexes I, III, IV and V was used as reported previously [20].

2.7. Recombinant cytochrome P450 overexpression system

Microsomes (Supersomes™) produced in insect cells overexpressing different combinations of components of the human cytochrome P450 system were purchased from BD Biosciences. The following combinations were used: I. control with empty baculovirus (CTR); II. P450 reductase+Cytochrome b5 (POR+Cytb5); III. P450 reductase +Cytochrome P450 (POR+CYP); IV. P450 reductase+Cytochrome b_5+Cytochrome P450 (POR+Cytb_5+CYP). Measurements were performed as described above using 20 µg microsomes in presence of NADPH.

2.8. Overexpression system in HEK293 cells

HEK293 cells were either transiently transfected with 1 µg plasmid coding for NQO1 (NAD(P)H dehydrogenase (quinone 1); Origene #sc119599) or stably transfected with plasmids coding for human Nox4 or Nox5. To activate superoxide $O_2^{\bullet-}$ production by Nox5, cells were treated with 100 nmol/L phorbol myristate acetate (PMA) for 15 min.

2.9. CRISPR/Cas9 for Cytochrome P450 reductase (POR) and p22phox

Guide RNA (gRNA) targeting human or rat POR and rat p22phox were designed with a CRISPR design web-interface (https://benchling.com). The human POR knock out vector was obtained by cloning the annealed target-specific ligonucleotides CRISPR POR sense (CACCGgtgttctacggctcccaga) and CRISPR POR antisense (AACtctgggagccgtagaacacC) [Hv1] into the BsmBI site of the pLentiCRISPRv2 plasmid (Addgene, Cat No. 52961) using the Golden Gate protocol [Hv1] By Frank Schnütgen and later Frank Wempe [21].

After cloning, plasmids were purified and verified by sequencing. HEK293T cells were used to produce viruses [22] which were also applied to control cells. Empty vector without gRNA was used as control. Positive clones were selected with puromycin (1 µg/mL) for 7 days.

Knockout efficiency for POR was accessed by western blot (Santa Cruz, F-10) and for p22phox sequencing of genomic DNA (Seqlab (Göttingen) using the following primer:

AGAGAGGACTTGCGGAGTGG) and qPCR were employed (sense: CCATGTGGGCCAACGAAC and antisense: CAGAACTAGCCCATCCTGCT).

2.10. Statistics

Unless otherwise indicated, data are given as means ± standard error of mean (SEM). Calculations were performed with Prism 5.0. In case of multiple testing, Bonferroni correction was applied. For multiple group comparisons ANOVA followed by post hoc testing was performed. Individual statistics of unpaired samples was performed by *t*-test and if not normal distributed by Mann-Whitney test. p-values of <0.05 were considered as significant. Unless otherwise indicated, n indicates the number of individual experiments.

3. Results

3.1. Mitochondrial proteins and cytochrome P450 generate an NADH/NADPH-dependent signal

Most of the previous studies utilizing cell-free assays employed crude membrane preparations. To visualize enzymatically active components and to identify proteins which could possibly contribute to NADPH-dependent assay signal, kidney and heart membrane proteins complexes were separated by native electrophoresis. After protein separation, reduction capacity in the presence of NADPH was visualized by *in gel* conversion of yellow nitro blue tetrazolium salt (NBT) to the insoluble blue formazan (Fig. 1, lower panel). In keeping with our previous observations in chemiluminescence assay, this technique failed to reveal qualitative difference between the signal obtained from samples of control WT and $3N^{-/-}$ as well as between angiotensin II-treated animals. Bovine heart mitochondria (with NADH) were used as positive control which produced a strong staining from respiratory super-complexes and complex I. In a second set of identical samples, the gel was cut into slices, which were subsequently homogenized and assayed for NADPH-dependent lucigenin chemiluminescence activity. In kidney samples this approach revealed that slices harboring NBT reduction activity also elicit an NADPH-dependent lucigenin chemiluminescent signal (Fig. 1, middle panel). The lucigenin signal in cardiac membranes in total was very low but the NBT staining pattern was similar to that of kidney samples (data not shown). To identify NADPH-binding proteins in such complexes, proteomics analyses were performed from gel slices. Among the proteins with NADPH-binding sites, P450 reductase ranked first in kidney and third in cardiac samples (Table 1). When filtered for NADH binding, mitochondrial dehydrogenases and reductases were identified. Interestingly, no Nox-family NADPH oxidases were identified using this approach.

3.2. Mitochondria do not generate the NADPH oxidase assays signal

To study a potential contribution of mitochondrial proteins to the NADPH-dependent assay signal, ρ^0 cells were used which lack mitochondrial DNA and thus a functional respiratory chain. As demonstrated by blue native electrophoresis (BNE) (Fig. 2A, left panel) only the mitochondrial intact control ρ^+ cells but not the ρ^0 cells exhibited NADH/NBT activity in the molecular weight region of the completely assembled complex I and supercomplexes S1 that contain complex I, III and IV. ρ^0 cells, without functional respiratory chain, did not show NBT reduction signals at typical positions of supercomplexes (Fig. 2A, right panel). Irrespectively of the presence or absence of functional mitochondria, both cell lineages yielded an NADPH-dependent staining in the lower part of the NBT-gel, which was similar to that observed in renal and cardiac extracts (Fig. 2A; arrow, central panel). Similarly, presence or absence of an intact respiratory chain had no impact on the NADPH-dependent lucigenin signal of the cells membrane fractions (Fig. 2B). Thus, mitochondria are unlikely to be the source of the NADPH-dependent chemiluminescence signal.

Table 1
Identification of NADPH interacting proteins by mass spectrometry. Protein complexes from heart and kidney positive for in gel lucigenin signal were identified by mass spectrometry. Proteins were filtered by keyword NADPH (Perseus software). The sum of Intensity-Based Absolute Quantification (IBAQ, x108) values for each tissue was used for ranking. Bold numbers indicate the three most abundant proteins identified in each tissue.

Protein names	IBAQ heart	IBAQ kidney
Aldose reductase	0.10	0.04
Dimethylaniline monooxygenase [N-oxide-forming] 1	0.01	**3.31**
Dimethylaniline monooxygenase [N-oxide-forming] 2	0.01	0.72
Ecto-ADP-ribosyltransferase 4	0.02	0.02
Epimerase family protein SDR39U1	**1.24**	0.16
Estradiol 17-beta-dehydrogenase 11	0.06	**6.92**
Glutamate dehydrogenase 1, mitochondrial	0.06	0.19
Isocitrate dehydrogenase [NADP], mitochondrial	**0.66**	0.15
NADPH–cytochrome P450 reductase	**0.22**	**9.76**
Oxidoreductase HTATIP2	0.02	0.14
Retinol dehydrogenase 12	0.02	0.02
Retinol dehydrogenase 13	0.01	0.03
Retinol dehydrogenase 14	0.05	0.08
Sepiapterin reductase	0.04	0.19
Sterol 26-hydroxylase, mitochondrial	0.00	0.01
Sulfide: quinone oxidoreductase, mitochondrial	0.04	0.32
Very-long-chain enoyl-CoA reductase	0.03	0.26

Another protein which uses NADPH for reduction and which has been linked to ROS production is NQO1 (NAD(P)H ubiquinone oxidoreductase) [23]. By LC-MS/MS the mitochondrial form of the enzyme was identified in the present study but an isoform of NQO1, which is tethered to the plasma membrane, is also expressed in the cytosol (Fig. 2C). Overexpressing of NQO1 in HEK293 cells, however, did not increase the NADPH-dependent lucigenin signal in cell homogenates (Fig. 2D). Thus, although NQO1 accepts electrons from NADPH at its ubiquinone group, this protein does not contribute to the signal in the chemiluminescence assays.

3.3. Cytochrome P450 is a source of the lucigenin NADPH oxidase assays signal

We previously reported that overexpression of CYP2C8 (Cytochrome P450 2C8) results in an increase in the NADPH oxidase assay signal [10]. The CYP enzymes consist of a cytochrome P450 reductase (POR), which accepts electrons from NADPH and a cytochrome P450 oxidase (CYP oxidase). To further characterize the nature of the lucigenin signal generated by these enzymes, Supersomes™ overexpressing combinations of POR, CYP oxidase and cytochrome b5 (Cyt_{b5}) were studied. Supersomes are enzymatic preparations from baculovirus-transduced insect cells. The negative control, i.e. supersomes without transduction produced only a negligible lucigenin assay signal in the presence of NADPH. Supersomes expressing either CYP oxidase or POR or Cyt_{b5} or all of them, readily produce a NADPH oxidase assay signal albeit with distinct kinetics (Fig. 3). Whereas POR +Cyt_{b5} displayed a short lasting peak, the combination of POR and CYP oxidase resulted in a sustained, but low activity signal. When all three enzymes were present, an intermediate kinetic was observed. Importantly, whereas PEG-SOD only inhibited these signals by 20–30% and PEG-catalase only inhibits POR+Cytb5+CYP by 20%, DPI almost abolished those of the triple combination and of the combination POR+CYP450 oxidase (Table 2). Interestingly, the DPI effect was lost in the assay when POR+Cyt_{b5} in the absence of the CYP oxidase was studied. This effect is most likely consequence of the fact that DPI has to be reduced by the CYP oxidase to elicit its inhibitory effect on FMN [24,25].

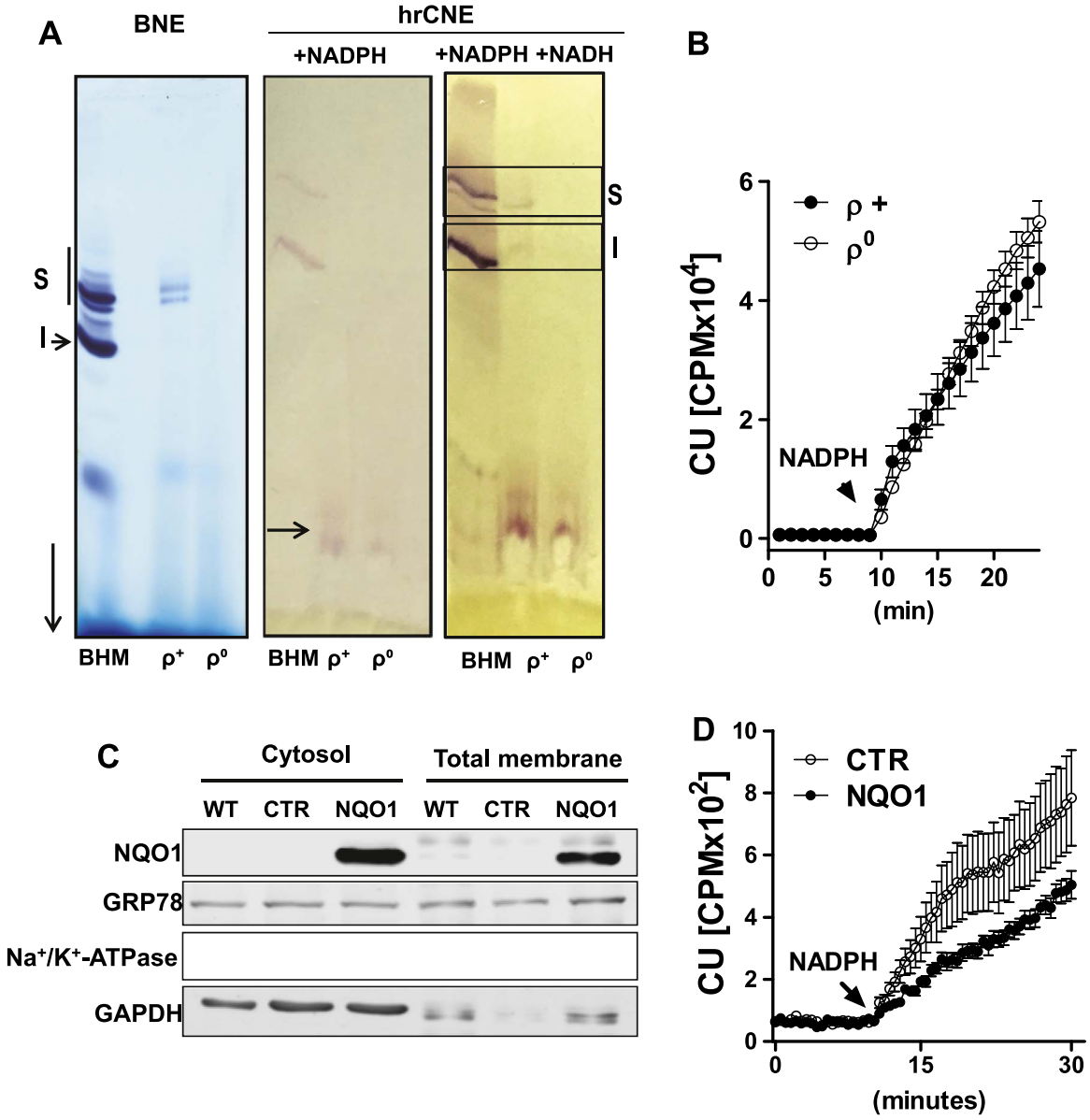

Fig. 2. Mitochondrial proteins and NAD(P)H oxidoreducase as candidates for the NADPH-dependent chemilumincescent signal with lucigenin. A: Characterization of ρ+ and ρ⁰ cells by BNE (blue native electrophoresis; left panel) and hrCNE (high resolutuion clear native electrophoresis) with NADPH and NADH reduction in presence of NBT (right panel). An NADPH-dependent signal is visualized in both cell lines (middle panel). ρ⁰ cells completely lack mitochondrial supercomplexes (S) and complex I (I), therefore no reduction of NADH is observed at their high molecular position (box, right panel). B: Lucigenin assay with membrane fraction (20 μg) of Rho and ρ⁰ cells. C: Characterization of HEK293 cells overexpressing NQO1 by western blot. NQO1 is present in cytosol and membrane fraction. D: Lucigenin assay with total cell lysate (20 μg) of HEK293 cells overexpressing NQO1. n≥3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

3.4. Cytochrome P450 also generates signals in the presence of L-012 or dihydroethium

In order to study whether the CYP effect in the Nox assay was specific for the enhancer lucigenin, L-012 and dihydroethium were utilized as detector systems. The luminol/HRP system was omitted, as the interaction of NADPH with HRP is known to generate a massive unspecific signal in this assay [26]. L-012 or DHE both failed to generate a signal if incubated with NADPH and control supersomes or supersomes containing only POR and Cyt_{b5}. Once CYP oxidase was contained in the supersomes, a strong signal was observed (Fig. 4). Importantly, different to the signal in the lucigenin assay, this signal was abolished by PEG-SOD and thus reflects the well-known $O_2^{\bullet-}$ generation of the CYP P450 oxidase-reductase system [13,14]. Thus, the NADPH-dependent assay signal is not exclusive for lucigenin, but in other assays it is mediated by the enzymatic production of $O_2^{\bullet-}$, whereas the lucigenin signal in large part is $O_2^{\bullet-}$ independent.

3.5. Knockout of POR block the NADPH oxidase assay signal

The first step in the reaction cycle of the CYP oxidoreductase system is the reduction of POR by NADPH. As this is well known [14] and supersomes lacking POR are not being produced commercially it was mandatory to genetically knockout POR in mammalian cells. If the NADPH oxidase assay signal in these cells was mediated by CYP oxidoreductases, $POR^{-/-}$ cells should not generate an assay signal. Thus, CRISPR/Cas9 technology was employed to generate $POR^{-/-}$ HEK293 cells. To study the interaction of POR with Nox enzymes in the lucigenin assay, this approach was carried out in normal HEK293 cells and cells stably transduced with either Nox4 or Nox5. Knockout efficiency of POR as determined by Western blot was >80% in all three cell lines (Fig. 5A). Knockout of POR did not affect the Nox-dependent

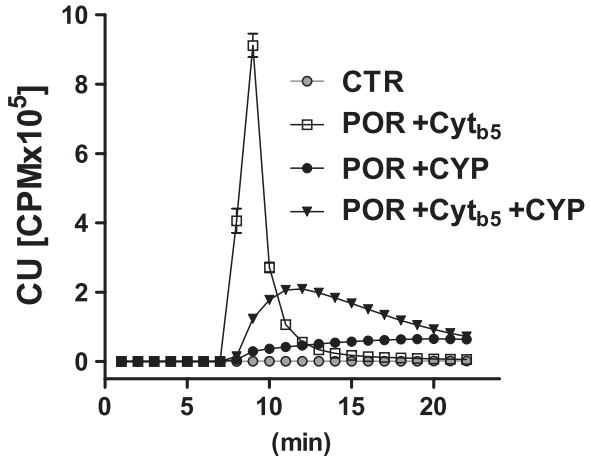

Fig. 3. Microsomes overexpressing combinations of cytochrome P450 components show an NADPH-dependent signal with lucigenin (5 µmol/L). n=4.

Table 2
Relative signal intensity of the NADPH-dependent lucigenin signal in microsomes under control conditions or in the present of DPI and PEG-SOD (± SD).

	CTR	POR+Cytb5	POR+CYP	POR+Cytb5+CYP
CTR	1 ± 0.16	1 ± 0.06	1 ± 0.13	1 ± 0.05
+DPI	0.26* ± 0.02	1.02 ± 0.04	0.08* ± 0.009	0.15* ± 0.02
+PEG-SOD	0.68* ± 0.05	0.72* ± 0.08	0.79* ± 0.06	0.72* ± 0.03
+PEG-CAT	0.97 ± 0.02	0.95 ± 0.02	0.96 ± 0.17	0.80* ± 0.05

* $p < 0.05$ for every individual combination of CYP components compared to its control.

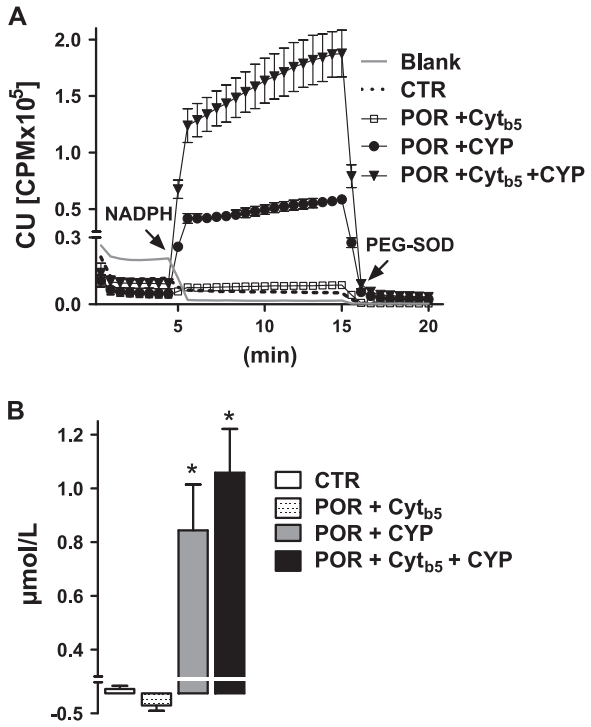

Fig. 4. NADPH-dependent signal is also detectable in microsomes overexpressing combinations of cytochrome P450 components using L-012 and DHE. A: L012 (200 µmol/L) assay with microsomes. Samples are supplemented with NADPH (10 µmol/L) and PEG-SOD (as indicated). n=3. B: 2EOH oxidation product of DHE separated by HPLC and detected by fluorescence. *p <0.05 compared to CTR. n≥4.

ROS production in intact cells as assayed by luminol/HRP, L-012 or lucigenin (Fig. 5B–D). In contrast, when the classic lucigenin assay with NADPH-incubated membranes was performed, POR$^{-/-}$ cells basically generated no signal, whereas the presence or absence of Nox enzymes did not impact on the assay (Fig. 5). Thus, the NADPH oxidase lucigenin assay signal in HEK293 cells should be attributed to the CYP system.

3.6. Deletion of POR does not influence NADPH-stimulation 2EOH production in membranes

As the experiment with supersomes suggested that CYP-derived ROS are detected by the DHE assay, we investigated whether genetic deletion of POR in HEK293 cells may reduce 2EOH formation. In intact HEK293 cells, overexpression of Nox5 but not of Nox4 increased 2EOH formation. As expected Nox5-dependent 2EOH was further increased by PMA (100 nmol/L, 15 min) (Fig. 6A). Genetic deletion of POR had no effect on 2EOH formation. In membranes supplemented with NADPH harvested from control cells or cells overexpressing Nox4 and Nox5 2EOH formation was similar. Genetic deletion of POR also did not affect 2EOH formation (Fig. 6B).

3.7. The AngII-stimulated NADPH-dependent signal is mediated by POR but not by Nox

Smooth muscle cells are among the best characterized sources of Nox-dependent ROS formation in particular when the NADPH oxidases are induced with angiotensin II. We therefore determined whether the NADPH-dependent lucigenin signal in this “physiological” model is also mediated by POR. Cells of the A7r5 rat smooth muscle cell line were subjected to CRISPR/Cas9-mediated deletion of either the essential NADPH oxidase-subunit p22phox or of POR (Fig. 7A and B). Cells were subsequently treated with or without AngII (100 nmol/L, 4 h) and NADPH-dependent chemiluminescence in membrane fractions was determined.

In keeping with previous observations [2] intact SMCs do not generate a signal in the presence of lucigenin (5 µmol/L or 250 µmol/L, 900.000 cells/condition) but in the absence of NADPH, even if pretreated with angiotensin. Nevertheless, when the intact cells are exposed to NADPH a massive increase in the signal occurs (data not shown). Similarly, membranes prepared from the A7r5 cell line, generated a strong lucigenin signal upon exposure to NADPH (Fig. 7C). Similar as the observations in membranes of HEK293 cells (Fig. 5), deletion of POR basically ablated the lucigenin signal. Knockout of p22phox, in contrast, had no effect. Thus, the basal NADPH-driven lucigenin signal in the A7r5 smooth muscle cells line is POR but not Nox-mediated.

As reported numerous times previously by others [9,27,28] AngII pretreatment results in an increase in the NADPH-induced lucigenin signalin membrane preparations, which was also observed in the present study (Fig. 7C). Unexpectedly, genetic deletion of the essential NADPH oxidase subunit p22phox did, however, not prevent this increase and rather further enhanced the lucigenin signal. In contrast, deletion of POR also blocked the lucigenin signal after AngII. Thus, the AngII-induced NADPH-dependent lucigenin signal in the A7r5 smooth muscle cell line is mediated by POR but not by NADPH oxidases.

4. Discussion

In this study we set out to identify the enzymatic source of the NADPH-dependent signal in the lucigenin membrane assay. By a proteomics approach we demonstrate that the CYP system contributes for the majority of the signal in mammalian tissue extracts. Moreover, insect cell microsomes overexpressing the CYP system also generated the assay signal. Importantly, knockout of the first enzyme of the CYP cascade, the cytochrome P450 reductase, blocked the assay signal in mammalian cells without interfering with the Nox-dependent ROS production of intact cells. In membranes of SMCs, AngII lead to an increase in NADPH-dependent signal which was abolished by knockout

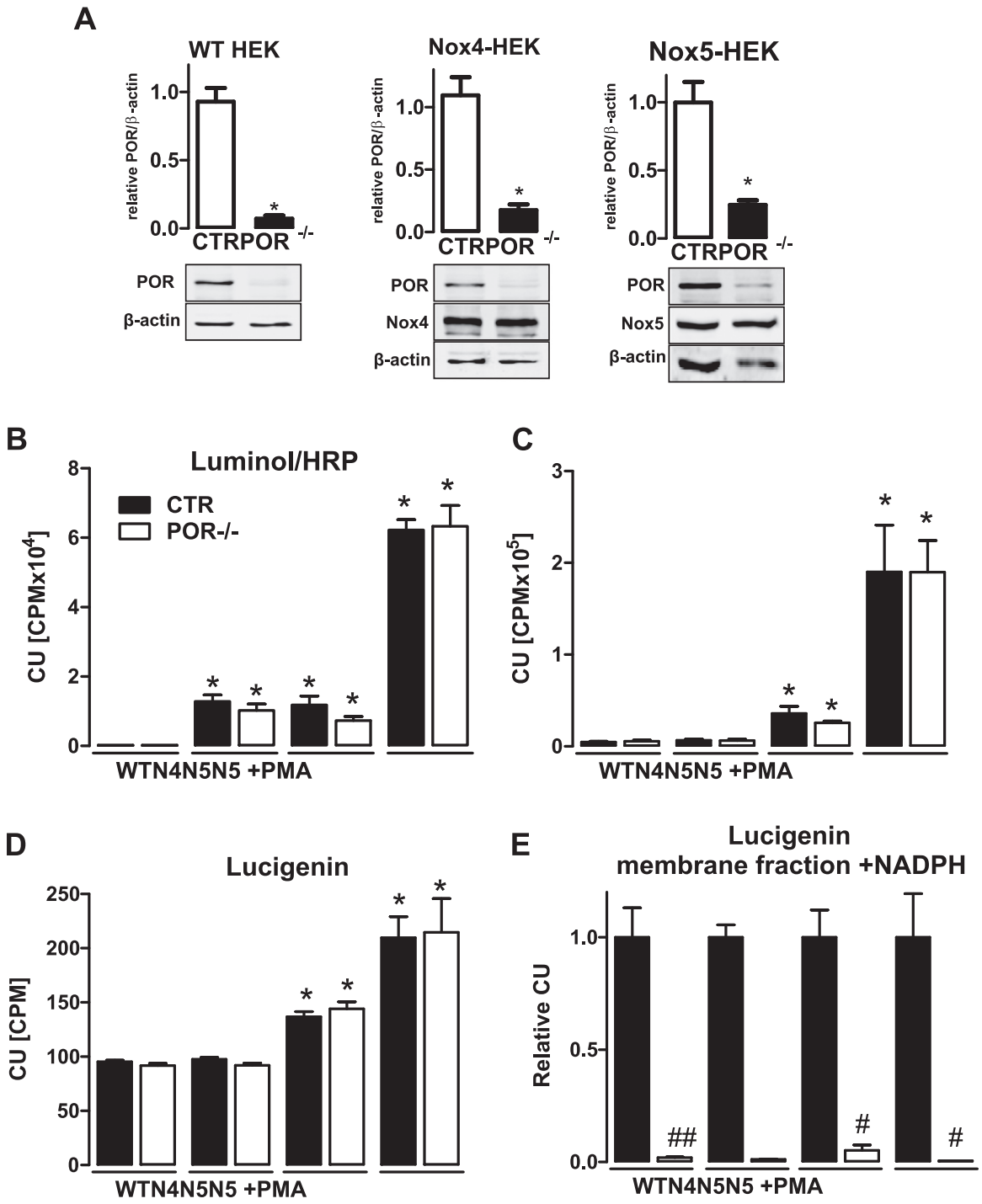

Fig. 5. Knockout of P450 reductase in HEK293 cells overexpressing Nox4 and Nox5 by CRISPR/Cas9 technology. A: Knockout efficiency evaluated by western blot. A knockout of 93%, 83% and 76% was obtained for WT, Nox4 and Nox5 HEK293 overexpressing cells, respectively. B–D: POR knockout does not affect ROS production in intact cells as measured by luminol/HRP (100 μmol/L/1 U/mL), LO12 (200 μmol/L) and lucigenin (5 μmol/L). E: NADPH-dependent signal with lucigenin is abolished by POR knockout in membrane fraction. *p <0.05WT vs. Nox, #p <0.05 with vs. without POR knockout. *p <0.05 POR-/- vs. CTR n≥4.

of POR but not of the essential NADPH oxidase subunit p22phox. We therefore conclude that the majority of the signal in the Nox NADPH chemiluminescence assays is generated by the CYP system.

The CYP enzyme family comprises numerous oxidase isoforms which are involved in xenobiotic metabolism, bioconversion and hormone synthesis [29]. The system, however, is driven by a single P450 reductase (POR), which transfers reduction equivalents from NADPH to the heme iron of the CYP oxidase. Thus, without POR the CYP reaction cycle no longer occurs and the enzyme system is inactive. The CYP system is well known as a source of $O_2^{\bullet-}$ [13,14] and gives rise to $O_2^{\bullet-}$ production even outside of the liver or the kidney, as shown for endothelial cells [30]. Thus, it is not surprising and also obvious from the reaction cycle that the CYP system generates a signal in the lucigenin assay. Interestingly, this signal is not only consequence of $O_2^{\bullet-}$ formation but also of direct electron transfer. This latter aspect, which is demonstrated by the failure of SOD to inhibit the signal, might be specific for lucigenin, as it was not observed with DHE or L-012. In fact, it is well known but also well ignored that the signal in the lucigenin Nox assay is largely SOD insensitive.

Quantitatively, the CYP system in most cells is expressed to a much

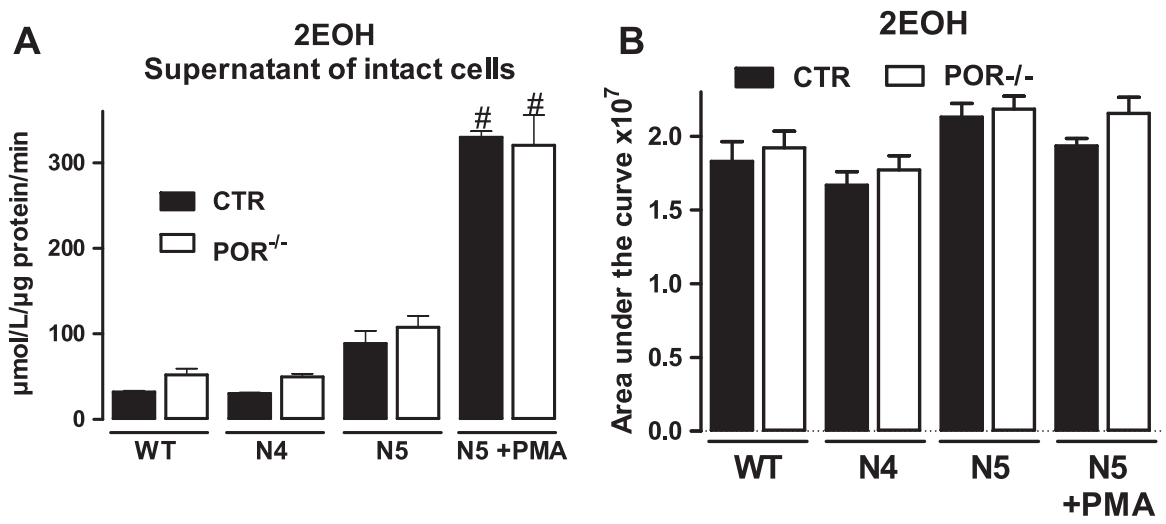

Fig. 6. Effect of POR knockout on the formation of DHE oxidation products. A: 2EOH product detected by fluorescence in supernatant of intact HEK293 cells overexpressing N4 or N5 (treated or not with PMA at 100 nmol/L) and knockout for POR. B: Detection of the oxidation product of DHE 2EOH by LC-MS/MS in membrane fractions (20 µg protein) supplemented with NADPH (100 µmol/L). #p <0.05 with vs. without PMA.

higher level than the Nox system. Although the liver and the kidney certainly exhibit a particularly high expression, also endothelial cells [31] and smooth muscle cells [32,33] contain physiologically important amounts of CYP. In the present study, we easily detected POR expression in HEK293 and SMC cells, whereas on the protein level Nox enzymes are undetectable with the exception of p22phox in HEK293 cells (Rezende et al., [10], unpublished observation). The results with SMCs knockout for POR and p22phox treated with AngII, clearly show that our findings are not restricted to overexpression systems, but can be applied to native cells as well.

This obviously does not mean that in general it is impossible to assay Nox activity by enzymatic assays. The best and first example is the membrane preparation of polymorphonuclear monocytes (PMNs) [34]. The assay, however, is considerably different. PMNs basically express no CYP enzymes but the p22phox-Nox2 complex at such a high level that even spectroscopy of the heme is possible [35]. In contrast, outside of the PMN system, heme spectra for the Nox system have basically not been reported. In our hands, the CYP heme signal overrides the spectroscopic cytochrome *b* signal of the Nox complexes in HEK293 cells, smooth muscle cells and endothelial cells (Brandes, unpublished observation). Other aspects that differ in the leukocyte Nox assay are that the enzyme system is usually activated by arachidonic acid and/or sodium dodecyl sulfate [36], that FAD and even the cytosolic phox subunits are added [34,37] and the membranes are produced by cavitation or sonification. Finally, in leukocyte membranes, usually oxygen consumption or cytochrome *c* reduction was measured, which better reflects the conversion of oxygen to $O_2^{\bullet-}$ but also requires excessive production of the radical. Thus, the peculiarities of the leukocyte system and the extensive assay development invested in measuring Nox activity in these cells by highly specialized labs resulted in the development of a functional, validated assay.

In the present study, we did not employ all peculiarities of the leukocyte assay system to set out assays. We did not test the effect of detergents nor did we substitute cytosolic co-factors of the Nox enzymes. Nevertheless, we know from unpublished work, that NADPH-dependent lucigenin chemiluminescence between crude cellular homogenates and membrane fractions is similar and thus that conditions which retain cytosolic subunits yield a similar outcome (unpublished observation).

For the non-PMN system, the situation is totally different as the “Nox assays” were accepted bona fide to reflect Nox activity. This aberration is potentially a reflection of the fact that assay development was not a focus on the Nox field but rather linking Nox enzymes to physiological and pathophysiological functions. Moreover, appropriate tools to validate the assays were not available for a long time. Finally, many publications reported an association of Nox expression with Nox activity in the membrane assay. The obvious question is why is that so? The studies in biological systems are complex and subject to interactions and coincidental changes in protein expression. Inflammatory cytokines, hormones and important stimuli of the Nox system like angiotensin II, also co-induce the CYP system [38]. Moreover, Nox enzymes are involved in signal transduction and redox-mediated gene expression. Knockdown for Nox4, for example, attenuates eNOS expression [4], which is another important source of the assay signal in the lucigenin system [10,39,40]. Interestingly, also the induction of CYP enzymes is redox-dependent for some of them [41]. These aspects, however, still do not explain observations reporting acute changes in the activity of the Nox assay. We can only speculate about the mechanistic basis of these observations but they may very well reflect changes in the cytoskeletal organization and the membrane topology of the cell. For example, translocation of enzymes into lipid rafts will change their accessibility to substrate and their activity [42] and Nox stimuli like angiotensin II are well known to change the cytoskeletal organization and membrane topology.

Probably due to the fairly low signal to noise ration of the assay and the numerous pitfalls associated with the use of lucigenin, the compound has been less frequently been used in recent years. Nevertheless, several screens for Nox inhibitors are still based on this probe and a large body of literature attributing Nox enzymes as a source of ROS in various disease conditions is based on the assay. Thus, understanding the enzymatic basis of the NADPH-stimulated lucigenin signal is not only interesting from the biochemical point of view. In the light of the present work it has to be considered an almost lucky coincident that the Nox-dependent NADPH oxidases have emerged as such important mediators of redox-biology.

In conclusion, in the present study we demonstrate that the CYP system is the major source of the signal of the chemiluminescence based Nox assay in membrane preparations of HEK293 cells. Even high Nox-dependent ROS production does not result in a specific signal in those Nox assays. Due to the capacity of the CYP system to generate $O_2^{\bullet-}$ if stimulated with NADPH, a Nox-specific assay might be possible in cells devoid of the CYP system.

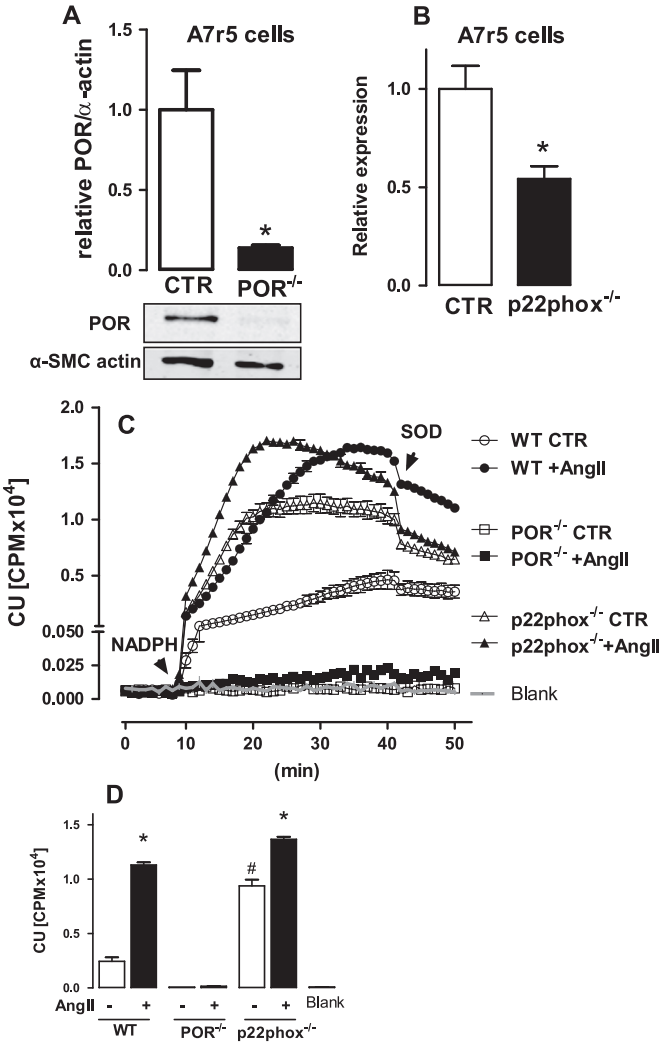

Fig. 7. Knockout of POR and p22phox in rSMC (A7r5) by CRISPR/Cas9. A: A Knockout of 90% was obtained for POR as tested by western blot. B: A knockout of 50% was achieved for p22phox as analyzed by qPCR. C: Membrane assay (20 μg protein) using lucigenin (5 μmol/L) of control (WT) and knockout cells treated or not with angiotensin II. NADPH (100 μmol/L) and SOD (100 U/mL) were given as indicated. D: Quantification of NADPH-dependent signal with lucigenin in A7r5 cells treated or not with AngII and knockout for POR and p22phox. *p <0.05 CTR vs. AngII. # p <0.05WT CTR vs. p22phox CTR.

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Disclosures

The authors declare that they have no relevant financial, personal or professional relationships to disclose which could be perceived as a conflict of interest or as potentially influencing or biasing the authors' work.

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Appendix A. Supplementary material

`Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2016.11.019.

References

[1] K. Bedard, K.H. Krause, The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology, Physiol. Rev. 87 (1) (2007) 245–313.
[2] A. Gorlach, R.P. Brandes, K. Nguyen, M. Amidi, F. Dehghani, R. Busse, A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall, Circ. Res. 87 (1) (2000) 26–32.
[3] K. Matsuno, H. Yamada, K. Iwata, D. Jin, M. Katsuyama, M. Matsuki, et al., Nox1 is involved in angiotensin II-mediated hypertension: a study in Nox1-deficient mice, Circulation 112 (17) (2005) 2677–2685.
[4] K. Schroder, M. Zhang, S. Benkhoff, A. Mieth, R. Pliquett, J. Kosowski, et al., Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase, Circ. Res. 110 (9) (2012) 1217–1225.
[5] C.A. Hamilton, M.J. Brosnan, S. Al-Benna, G. Berg, A.F. Dominiczak, NAD(P)H oxidase inhibition improves endothelial function in rat and human blood vessels, Hypertension 40 (5) (2002) 755–762.
[6] A.J. Lambert, J.A. Buckingham, H.M. Boysen, M.D. Brand, Diphenyleneiodonium acutely inhibits reactive oxygen species production by mitochondrial complex I during reverse, but not forward electron transport, Biochim. Biophys. Acta 1777 (5) (2008) 397–403.
[7] L.R.G. Castor, K.A. Locatelli, V.F. Ximenes, Pro-oxidant activity of apocynin radical, Free Radic. Biol. Med. 48 (12) (2010) 1636–1643.
[8] S. Heumuller, S. Wind, E. Barbosa-Sicard, Schmidt, H.H.W. Harald, R. Busse, K. Schroder, et al., Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant, Hypertension 51 (2) (2008) 211–217.
[9] K.K. Griendling, C.A. Minieri, J.D. Ollerenshaw, R.W. Alexander, Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells, Circ. Res. 74 (6) (1994) 1141–1148.
[10] F. Rezende, O. Lowe, V. Helfinger, K.-K. Prior, M. Walter, S. Zukunft, et al., Unchanged NADPH oxidase activity in Nox1-Nox2-Nox4 triple knockout mice: what do NADPH-stimulated chemiluminescence assays really detect?, Antioxid. Redox Signal. 24 (7) (2016) 392–399.
[11] B.T. Hope, G.J. Michael, K.M. Knigge, S.R. Vincent, Neuronal NADPH diaphorase is a nitric oxide synthase, Proc. Natl. Acad. Sci. USA 88 (7) (1991) 2811–2814.
[12] M.A. Baker, A. Krutskikh, B.J. Curry, E.A. McLaughlin, R.J. Aitken, Identification of cytochrome P450-reductase as the enzyme responsible for NADPH-dependent lucigenin and tetrazolium salt reduction in rat epididymal sperm preparations, Biol. Reprod. 71 (1) (2004) 307–318.
[13] S.C. Bondy, S. Naderi, Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species, Biochem. Pharmacol. 48 (1) (1994) 155–159.
[14] S. Puntarulo, A.I. Cederbaum, Production of reactive oxygen species by microsomes enriched in specific human cytochrome P450 enzymes, Free Radic. Biol. Med. 24 (7–8) (1998) 1324–1330.
[15] J. Zielonka, J. Vasquez-Vivar, B. Kalyanaraman, Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine, Nat. Protoc. 3 (1) (2008) 8–21.
[16] I. Wittig, M. Karas, H. Schagger, High resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes, Mol. Cell. Proteom. 6 (7) (2007) 1215–1225.
[17] R.P. Brandes, M. Barton, K.M. Philippens, G. Schweitzer, A. Mugge, Endothelial-derived superoxide anions in pig coronary arteries: evidence from lucigenin chemiluminescence and histochemical techniques, J. Physiol. 500 (Pt 2) (1997) 331–342.
[18] E. Zerbetto, L. Vergani, F. Dabbeni-Sala, Quantification of muscle mitochondrial oxidative phosphorylation enzymes via histochemical staining of blue native polyacrylamide gels, Electrophoresis 18 (11) (1997) 2059–2064.
[19] J. Cox, M. Mann, MaxQuant enables high peptide identification rates, individua-lized p.p.b.-range mass accuracies and proteome-wide protein quantification, Nat. Biotechnol. 26 (12) (2008) 1367–1372.
[20] I. Wittig, B. Meyer, H. Heide, M. Steger, L. Bleier, Z. Wumaier, et al., Assembly and oligomerization of human ATP synthase lacking mitochondrial subunits a and A6L, Biochim. Biophys. Acta 1797 (6–7) (2010) 1004–1011.
[21] C. Engler, R. Kandzia, S. Marillonnet, A one pot, one step, precision cloning method with high throughput capability, PLoS One 3 (11) (2008) e3647.
[22] G. Tiscornia, O. Singer, I.M. Verma, Production and purification of lentiviral vectors, Nat. Protoc. 1 (1) (2006) 241–245.
[23] D. Siegel, D.L. Gustafson, D.L. Dehn, J.Y. Han, P. Boonchoong, L.J. Berliner, et al., NAD(P)H:quinone oxidoreductase 1: role as a superoxide scavenger, Mol. Pharmacol. 65 (5) (2004) 1238–1247.
[24] J.T. Szilagyi, V. Mishin, D.E. Heck, Y.-H. Jan, L.M. Aleksunes, J.R. Richardson, et al., Selective targeting of heme protein in cytochrome P450 and nitric oxide synthase by diphenyleneiodonium, Toxicol. Sci. 151 (1) (2016) 150–159.
[25] V.B. O'Donnell, G.C. Smith, O.T. Jones, Involvement of phenyl radicals in iodonium inhibition of flavoenzymes, Mol. Pharmacol. 46 (4) (1994) 778–785.
[26] V. de Sandro, C. Dupuy, J. Kaniewski, R. Ohayon, D. Deme, A. Virion, et al., Mechanism of NADPH oxidation catalyzed by horse-radish peroxidase and 2,4-diacetyl-2Hheme-substituted horse-radish peroxidase, Eur. J. Biochem. 201 (2)

(1991) 507–513.
[27] R.M. Touyz, G. Yao, M.T. Quinn, P.J. Pagano, E.L. Schiffrin, p47phox associates with the cytoskeleton through cortactin in human vascular smooth muscle cells: role in NAD(P)H oxidase regulation by angiotensin II, Arterioscler. Thromb. Vasc. Biol. 25 (3) (2005) 512–518.
[28] I. Al Ghouleh, D.N. Meijles, S. Mutchler, Q. Zhang, S. Sahoo, A. Gorelova, et al., Binding of EBP50 to Nox organizing subunit p47phox is pivotal to cellular reactive species generation and altered vascular phenotype, Proc. Natl. Acad. Sci. USA 113 (36) (2016) E5308–E5317.
[29] P.A. Williams, J. Cosme, V. Sridhar, E.F. Johnson, D.E. McRee, Mammalian microsomal cytochrome P450 monooxygenase, Mol. Cell 5 (1) (2000) 121–131.
[30] I. Fleming, U.R. Michaelis, D. Bredenkotter, B. Fisslthaler, F. Dehghani, R.P. Brandes, et al., Endothelium-derived hyperpolarizing factor synthase (cytochrome P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries, Circ. Res. 88 (1) (2001) 44–51.
[31] B. Fisslthaler, R. Popp, L. Kiss, M. Potente, D.R. Harder, I. Fleming, et al., Cytochrome P450 2C is an EDHF synthase in coronary arteries, Nature 401 (6752) (1999) 493–497.
[32] S. Thomson, M.L. Edin, F.B. Lih, M. Davies, M.M. Yaqoob, B.D. Hammock, et al., Intimal smooth muscle cells are a source but not a sensor of anti-inflammatory CYP450 derived oxylipins, Biochem. Biophys. Res. Commun. 463 (4) (2015) 774–780.
[33] J.D. Imig, Epoxides and soluble epoxide hydrolase in cardiovascular physiology, Physiol. Rev. 92 (1) (2012) 101–130.
[34] E. Pick, Cell-free NADPH oxidase activation assays: "in vitro veritas", Methods Mol. Biol. 1124 (2014) 339–403.
[35] A.R. Cross, J.F. Parkinson, O.T.G. Jones, The superoxide-generating oxidase of leucocytes. NADPH-dependent reduction of flavin and cytochrome b in solubilized preparations, Biochem. J. 223 (2) (1984) 337–344.
[36] J.T. Curnutte, R. Kuver, B.M. Babior, Activation of the respiratory burst oxidase in a fully soluble system from human neutrophils, J. Biol. Chem. 262 (14) (1987) 6450–6452.
[37] D.J. Ranayhossaini, A.I. Rodriguez, S. Sahoo, B.B. Chen, R.K. Mallampalli, E.E. Kelley, et al., Selective recapitulation of conserved and nonconserved regions of putative NOXA1 protein activation domain confers isoform-specific inhibition of Nox1 oxidase and attenuation of endothelial cell migration, J. Biol. Chem. 288 (51) (2013) 36437–36450.
[38] B.L. Jennings, S. Sahan-Firat, A.M. Estes, K. Das, N. Farjana, X.R. Fang, et al., Cytochrome P450 1B1 contributes to angiotensin II-induced hypertension and associated pathophysiology, Hypertension 56 (4) (2010) 667–674.
[39] T. Takaya, K. Hirata, T. Yamashita, M. Shinohara, N. Sasaki, N. Inoue, et al., A specific role for eNOS-derived reactive oxygen species in atherosclerosis progression, Arterioscler. Thromb. Vasc. Biol. 27 (7) (2007) 1632–1637.
[40] J. Vásquez-Vivar, N. Hogg, K.A. Pritchard, P. Martasek, B. Kalyanaraman, Superoxide anion formation from lucigenin: an electron spin resonance spin-trapping study, FEBS Lett. 403 (2) (1997) 127–130.
[41] V. Lamsa, A.-L. Levonen, H. Leinonen, S. Yla-Herttuala, M. Yamamoto, J. Hakkola, The superoxide-generating oxidase of leucocytes. NADPH-dependent reduction of flavin and cytochrome b in solubilized preparations, Chem. Res. Toxicol. 23 (5) (2010) 977–985.
[42] H.H. Patel, P.A. Insel, Lipid rafts and caveolae and their role in compartmentation of redox signaling, Antioxid. Redox Signal. 11 (6) (2009) 1357–1372.