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

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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

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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.

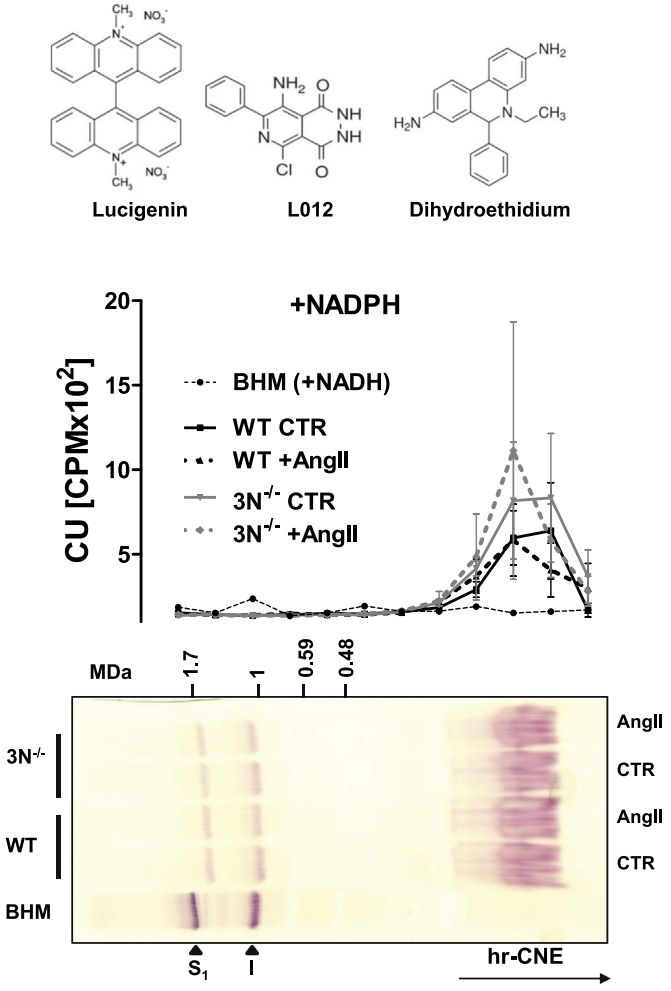

Fig. 1. Structures of compounds used (top) and NADPH reduction by membrane proteins in an in gel assay (middle and botton). WT and 3N-/- mice where treated with or without AngII. Membrane proteins from kidneys were separated by clear native electrophoresis. After separation of protein complexes under 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