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Research Paper

Cholesterol: A modulator of the phagocyte NADPH oxidase activity - A cell-free study

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ABSTRACT

The NADPH oxidase Nox2, a multi-subunit enzyme complex comprising membrane and cytosolic proteins, catalyzes a very intense production of superoxide ions $O_2^{\bullet -}$, which are transformed into other reactive oxygen species (ROS). *In vitro*, it has to be activated by addition of amphiphiles like arachidonic acid (AA). It has been shown that the membrane part of phagocyte NADPH oxidase is present in lipid rafts rich in cholesterol. Cholesterol plays a significant role in the development of cardio-vascular diseases that are always accompanied by oxidative stress. Our aim was to investigate the influence of cholesterol on the activation process of NADPH oxidase. Our results clearly show that, in a cell-free system, cholesterol is not an efficient activator of NADPH oxidase like arachidonic acid (AA), however it triggers a basal low superoxide production at concentrations similar to what found in neutrophile. A higher concentration, if present during the assembly process of the enzyme, has an inhibitory role on the production of $O_2^{\bullet -}$. Added cholesterol acts on both cytosolic and membrane components, leading to imperfect assembly and decreasing the affinity of cytosolic subunits to the membrane ones. Added to the cytosolic proteins, it retains their conformations but still allows some conformational change induced by AA addition, indispensable to activation of NADPH oxidase.

Introduction

The damaging role of reactive oxygen species (ROS) in cardiovascular diseases (atherosclerosis, vascular inflammation, and endothelial dysfunction) leading to coronary heart disease, stroke or *angina pectoris* has been known for some decades. NADPH oxidase Nox2 is one of the major actors at the origin of oxidative stress. Actually Nox2 is one of the main isomers involved in the cardiovascular field [1]. On the other hand, high cholesterol level is linked to an elevated risk of cardiovascular disease [2], through high concentration of LDL-cholesterol in blood [3,4]. High cholesterol has also been associated with diabetes and high blood pressure [5,6]. Interestingly, NADPH oxidases have been found in lipid rafts (LR), which are dynamic, detergent-resistant plasma membrane microdomains highly enriched in cholesterol and sphingolipids [7,8]. Cytoplasmic proteins are efficiently recruited to these raft-associated flavocytochrome b_{558} upon activation to reconstitute the active complex [9]. Moreover, distribution and regulation of NADPH oxidase by LRs were reported in murine microglial cells and bovine aortic endothelial and coronary arterial endothelial cells [10–14]. These facts prompted us to study the effect of cholesterol on the functioning of NADPH oxidase. In this paper, we investigated the consequences of the presence of cholesterol on the production of reactive oxygen species.

The NADPH oxidase catalyzes the formation of superoxide anion ($O_2^{\bullet -}$) by a single-electron reduction of the molecular oxygen using NADPH as the electron donor [15–17]. $O_2^{\bullet -}$ is considered to be the starting point for the generation of a vast assortment of reactive oxidants since it is subsequently transformed into hydrogen peroxide, hypochlorous acid, hydroxyl radical and peroxynitrite [18,19]. Deregulation of NADPH-oxidase activity is linked with a large panel of pathologies in addition to cardiovascular ones, involving inflammatory processes, renal damage, central nervous system diseases, immune system disorders, induction of apoptosis after irradiation by low doses of ionizing radiations etc., [20–31].

Abbreviations: AA, arachidonic acid; PBS, phosphate buffer saline; Cyt b_{558}, cytochrome b_{558}; Cytc, cytochrome c; DTT, dithiotreitol; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; FMLP, formyl-methionyl-leucyl-phenylalanine; GTP, guanosine-5′-triphosphate; HEPES, [4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid; IPTG, isopropylthiogalatoside; LB, Luria Bertoni; LDL, low density lipoprotein; LR, lipid raft; MF, membrane fractions; MβCD, methyl-β-cyclodextrin; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; PMSF, phenylmethanesulfonyl fluoride; PtdIns(3)P, phosphatidyl-inositol3-phosphate; PtdIns(3,4)P2, phosphatidylinositol(3,4)-bisphosphate; PX, phox homology domain; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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The functionally competent oxidase complex consists of a membrane-bound flavocytochrome *b*558 (Cyt b_{558}), comprising two subunits (Nox2 also known as gp91^{phox}, and p22^{phox}) and four cytosolic components. Nox2 harbors the redox carriers (bound FAD and two hemes) and the NADPH binding site. The cytosolic components include p47^{phox}, p67^{phox}, p40^{phox}, and a small GTPase Rac1 or Rac2 [32]. Because of the high toxicity of the reactive oxygen species (ROS), the NADPH-oxidase activity is tightly regulated spatially and temporally. In resting phagocytes, the components of the complex exist as separated entities but upon cell activation by pro-inflammatory mediators, the cytosolic subunits undergo posttranslational modifications such as phosphorylation [33,34] and migrate to the membrane bound Cyt b_{558} to constitute the activated NADPH–oxidase complex [35]. Actually this process involves a complicated set of protein–protein and protein–lipid interactions to conduct to oxidase assembly [36–39].

Studies on binding between the different soluble subunits p47^{phox}, p67^{phox}, p40^{phox} performed *in vitro* suggested that these three cytosolic subunits are preassembled [40–42]. Recently, different constructions of chimeras were designed, in which individual cytosolic subunits were fused [p47^{phox}–p67^{phox}] [43] or [p67^{phox}–Rac1] [44–48] and supplemented by the missing third subunit (Rac1 and p47^{phox}, respectively). Another strategy was to construct a trimera which consisted of the following domains [p47^{phox} (aa 1–286), p67^{phox} (aa 1–212) and a full length Rac1 (aa 1–192)] in which interactions among cytosolic subunits were replaced by fusion. This trimera was found to act as potent amphiphile-dependent oxidase protein activator upon assembly to native phagocyte membrane or purified Cyt b_{558}[49]. The subsequent change from this construction was performed by adding isoprenyl group to the C-terminus of Rac1 part, mimicking *in vivo* reality, where Rac is found exclusively in the prenylated form. Further modification was the introduction of Q61L mutation in the Rac part of the trimera, making Rac constitutively in the GTP-bound form. It ensures that in the trimera an intramolecular bond was built between Rac1 and p67^{phox} which is essential for oxidase activity ability of trimera [50,51].

The development of a cell-free oxidase activation system was a great help in the understanding of the mechanism of NADPH oxidase activation. This system was designed to mimic *in vivo* oxidase activity under *in vitro* conditions. In cell-free systems, the activation process is bypassed by the introduction of an activator, an anionic amphiphile such as arachidonic or other fatty acids or surfactants [52–57]. We took advantage of this system, which permits strict quantification of the components of interest, and modifications of membrane composition. In addition, for simplicity, we have replaced the cytosolic subunits by the trimera [49,50]. A precondition for using the trimera was to ascertain that it is functionally comparable with the separated cytosolic subunits. We have verified that the rates of production of superoxide anions were similar (supplementary material) and that the dependences of the activity in function of AA concentration were also comparable with the cytosolic fractions and the trimera [58]. In addition, the presence of two states in the activation process, a sensitive one followed by a resistant one against ROS damages, observed with the separated cytosolic subunits [59] was also found with the trimera (data not shown). Consequently, we have chosen the trimera instead of the separated subunits in order to diminish the number of independent parameters to consider and to facilitate the interpretation.

Material and methods

Materials

Equine heart cytochrome c (cyt *c*), arachidonic acid (*cis*-AA), phenylmethanesulfonyl fluoride (PMSF), isopropylthiogalatoside (IPTG), cholesterol, Dulbecco PBS and methyl-β-cyclodextrin (MβCD) were from Sigma (Saint-Quentin Fallavier, France). Reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) was from Acros. Ni-sepharose, superdex 75 and Ficoll-Paque Plus was from GE Healthcare, France.

Neutrophil membrane preparation

The neutrophils were prepared from human blood from healthy donors (ESF, Paris, France) as described in [60]. Briefly, 500 mL of blood was sedimented in 2% dextran solution for 40 min. PBS was added to the pellets, and then the neutrophils were separated from lymphocytes and the red cells by centrifugation for 30 min at 400*g* on Ficoll. The red cells were further eliminated after their lysis by centrifugation for 8 min, 400*g*, 4 °C. The pellet resuspended in PBS pH 7.4 containing 340 mM sucrose, 7 mM magnesium sulfate, 1 mM PMSF, 0.5 mM leupeptin was sonicated in the 30% pulse mode at power pulses (6) in an ice-cooled beaker 6 times during 10 s with interval of 1 min between the sonications (sonicator XL, Misonix Inc.). Neutrophil membranes and cytosol were separated by centrifugation for 1 h 30 min at 200,000*g* at 4 °C. The membrane fractions were resolubilized, aliquoted and stored at −80 °C for further experiments.

Expression of the trimera

The plasmid of the trimera was a generous gift from Prof. E. Pick. Trimera (p47^{phox} aa 1–286, p67^{phox} aa 1–212, and RacQ61L full length) was expressed and isolated from *Escherichia coli* BL21-DE3-plys. A stock culture of *E. coli* (glycerated, stored at −80 °C) expressing the trimera was used to inoculate a Petri dish of Luria Bertani (LB) agar, supplemented with kanamycin and chloramphenicol and incubated at 37 °C for 16 h. A colony was then cultured in 60 mL of LB medium supplemented with 50 mg/L of kanamycin and 34 mg/L of chloramphenicol, incubated at 37 °C for about 16 h. 20 mL of this culture were added to 1.5 L of Terrific Broth medium (TB) supplemented with 50 mg/L of kanamycin and 34 mg/L of chloramphenicol. The flask was incubated in shaking condition at 37 °C until it reached an absorbance of 0.9 at 600 nm, then 0.5 mM IPTG were added to induce the synthesis of protein and the culture was incubated overnight at 30 °C. The culture was pelleted and placed in the freezer at −20 °C until use.

Extraction of the trimera from bacteria

The bacterial pellet, containing the trimera obtained previously, was dissolved in a buffer containing 50 mM HEPES (pH 7.5), 200 mM NaCl and 1 mM EDTA to which was added 1 mg of DNase, 1 mM PMSF, 1 mM DTT and 1 mM benzamidine. The bacteria were sonicated during 4 times 2 min in a 50% pulse mood at power pulses (6) in an ice-cooled beaker with pauses of 2 min. The bacterial lysate was centrifuged at 160,000*g* for 1 h 30 min at 6 °C. The cleared cell-free supernatant was filtered to remove all traces of debris and bacteria.

Purification of the trimera

The trimera was expressed as fusion protein. Thus it was purified by metal chelate affinity chromatography. The above supernatant was applied to nickel affinity column after being diluted