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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

twice with buffer (0.5 M NaCl, 30 mM Na_2HPO_4, 20 mM imidazole and 1 mM PMSF, pH 7.4). The mixture was loaded for 1 h 30 min so that the proteins of interest effectively cling to the nickel resin. Then the column was washed with the same buffer to remove unwanted bound proteins. The proteins bound to the beads were eluted from the resin with elution buffer (0.1 M NaCl, 30 mM Na_2HPO_4 and 300 mM imidazole, pH 7.4). Then size exclusion chromatography was carried out to better purify the trimera. Proteins concentration was determined using a NanoDrop2000 spectrophotometer (Thermo scientific, France) and the extinction coefficient of 1.5 mg^{-1}/mL cm. The purities of all proteins were checked by migration on 10% BisTris-NuPAGE SDS gels (Invitrogen), stained with Coomassie Brilliant Blue (Fig. S1 in Supplementary material).

Dialysis and storage of the trimera

Trimera fractions were pooled in a dialysis tube whose membrane was of 10 kDa porosity. The tube was placed in 2 L dialysis buffer (100 mM NaCl and 30 mM Na_2HPO_4, pH 7.5) at 4 °C overnight. The dialyzate was recovered and trimera was stored at −80 °C.

Quantification of intrinsic cholesterol

Intrinsic cholesterol concentration in human neutrophil membrane fractions was measured by the Amplex Red Cholesterol Assay Kit purchased from Invitrogen [61,62]. The intrinsic cholesterol concentration was estimated by three independent measurements on different blood donors.

Depletion of cholesterol from neutrophil membrane

Methyl-β-cyclodextrin (MβCD) is a well established cholesterol depleting reagent of phospholipidic membrane without affecting their permeability and it is the most commonly used reagent [63–66]. The neutrophil membranes were incubated for 1 h, at 4 °C, in presence of 10 mM MβCD. The mixture was centrifuged at 148,000*g* for 1 h 30 min at 4 °C. Neutrophil membranes were found in the pellet and the cyclodextrin–cholesterol complexes were in the supernatant. The neutrophil membranes were resolubilized in PBS.

Measurement of superoxide production in cell-free assays

Superoxide anion production rates were quantified by the initial rate of cytochrome *c* (Cyt*c*) reduction, as described before [67]. The reaction is as follows:

$$\text{Cyt } c_{ox} + O_2^{\bullet -} \rightarrow O_2 + \text{Cyt } c_{red}$$

Unless indicated, the components of the cell-free system were added as follows: (2–5 nM Cyt b_{558}) membrane fractions, (100–200 nM) trimera and (40 μM) arachidonic acid in 500 μL Phosphate Buffer Saline supplemented with 10 mM $MgSO_4$ for 4 min of incubation at 25 °C in order to allow the Nox complex assembly. The production was initiated by addition of (250 μM) NADPH and the rate of $O_2^{\bullet -}$ was quantified by the reduction of cytochrome *c* (50 μM). The rate was measured at 550 nm in a Thermo evolution500 Spectrophotometer. The amount of superoxide was calculated using a molar extinction coefficient ($\Delta\varepsilon$ of the reduced *minus* oxidized form of Cyt*c*) of 21 mM^{-1} cm^{-1}. 20 mM stock solution of cholesterol was prepared in ethanol. Further dilutions have been done in ethanol to get a concentration range of cholesterol (0.1–16 mM), which was mixed with AA 65 mM. 3 μL of each mixture was introduced in the cell free system to finally have cholesterol 0.25–40 μM in a mixture with 40 μM AA in the final reaction volume. This allowed keeping the volume constant.

2.10. Determination of enzymatic parameters and curve plotting

The enzymatic parameters EC_{50} and V_{max} were calculated by non-linear least square fitting of the curves of superoxide rate of production *vs*. protein concentration using the following expression.

$$v = \frac{v_{max}[P]}{EC_{50} + [P]} \quad (1)$$

where [*P*] is the concentration of the considered protein (trimera). Plotting and calculation were performed using Graph Pad Prism Version 6.

Intrinsic fluorescence assays

Steady-state fluorescence spectra were performed on a Photon Technology International scanning fluorimeter at 25 °C. Various concentrations of AA and/or cholesterol were added as indicated to a final volume of 3 mL of buffer (phosphate buffer saline supplemented with 10 mM $MgSO_4$) containing trimera (60 nM) in a quartz cuvette. The tryptophan fluorescence spectra of trimera were obtained by exciting the samples at 290 nm (2 nm bandwidth) and recorded between 300 and 400 nm (5 nm bandwidth). The excitation wavelength was chosen at 290 nm to optimize the signal to noise ratio and to reduce the contribution of tyrosine residues to the signal [68].

Results

Intrinsic cholesterol concentration and effect of cholesterol depletion by methyl-β-cyclodextrin on superoxide production rate

We first measured the intrinsic cholesterol concentration in human neutrophil membrane fractions. We found 3 ± 1 μM cholesterol in the final reaction volume of cell free system assay (2 nM Cyt b_{558}, 40 μg/mL membrane proteins) in three independent measurements. Then to investigate the role of cholesterol that is naturally present in the neutrophil membrane on NADPH oxidase, 10 μM MβCD was used to disrupt lipid rafts by removing cholesterol from membranes [66,69,70]. The rate was measured as described in Material and methods. We found that cholesterol depletion decreased superoxide production rate relative to the non-treated membrane neutrophil to (44 ± 7)%.

In the following studies, the level of cholesterol was increased to a range corresponding to hypercholesterolemia (up to *ca*. 33% of the normal concentration) and above, to enlighten the effects of this addition.

Cholesterol as an activating molecule?

Having established the most propitious conditions for activation (see Materials and methods), we aimed at determining whether cholesterol could have an activator effect on NADPH oxidase. The results expressed as percentages of NADPH oxidase activity as a function of cholesterol are displayed in Fig. 1. Addition of cholesterol in the range 0.2–1 μM (*ca*. 7–33% increase compared to the intrinsic value) provoked slight but significant activation of NADPH oxidase complex, but not at an equivalent level to AA. The rate of production of $O_2^{\bullet -}$ stayed at around 15% of the rate value obtained with AA for concentrations above 1 μM. Comparable results were obtained using the separated subunits where a

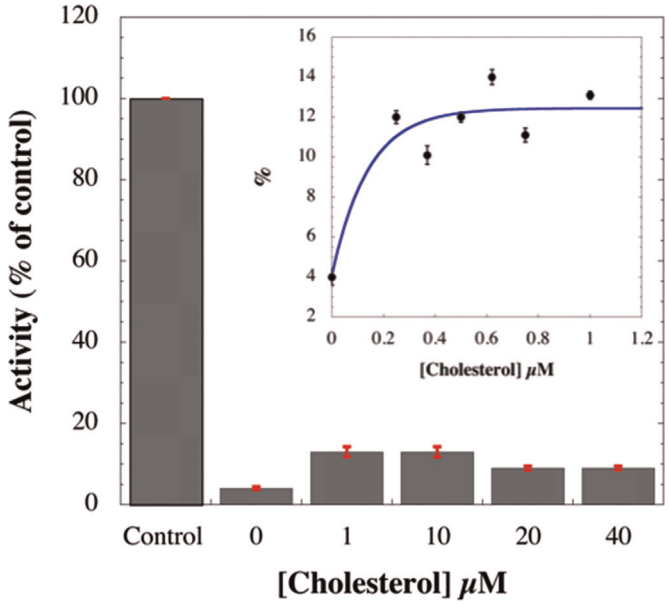

Fig. 1. Dependence of NADPH oxidase activity as a function of cholesterol concentration in the absence of arachidonic acid. Membrane fractions (2 nM Cyt b_{558}) with trimera 200 nM were incubated 4 min in presence of different concentrations of cholesterol. Control experiment representing 100% (68 mol $O_2^{\bullet -}$/s/mol Cyt b_{558}) of the activity was realized in presence of 40 μM AA and in absence of cholesterol. The rates of superoxide production were measured as described in Materials and methods. The data are the average of 3 independent measurements ± SEM.

maximum activity of (20 ± 2)% of AA-dependent activity was reached (data not shown).

Superoxide production in the presence of AA plus added cholesterol

NADPH oxidase activity was inhibited by the addition of cholesterol

Since cholesterol alone does not activate NADPH oxidase, we have tested the cholesterol effect on activation by AA.The AA induced NADPH-oxidase activity was followed upon increasing concentrations of cholesterol (Fig. 2). To avoid an increase of solvent volume, a mixture of cholesterol added with AA for each cholesterol concentration was prepared. The rate of production with AA alone was considered as 100%. Surprisingly, when membrane fractions and trimera were incubated together with 0.25 μM cholesterol and 40 μM AA, the activity dropped to about 86 ± 5%. The AA-induced NADPH oxidase activity was thus reduced by addition of less than 10% of the intrinsic cholesterol amount. The decrease of the activity could be fitted by a two inhibitory sites equation (Fig. 2) and the parameters of the fit are given in Table 1.

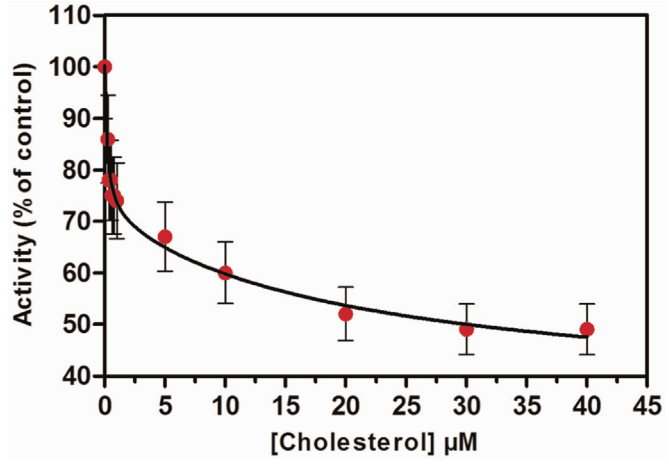

Fig. 2. NADPH-oxidase activity inhibition by cholesterol. Neutrophil membrane fractions and trimera were incubated together in the presence of a mixture of 40 μM AA plus cholesterol. The oxidase activity was expressed as the percent of activity measured in the absence of cholesterol (79 mol $O_2^{\bullet -}$/s/mol Cyt b_{558}) as described in Materials and methods. The values are an average of 3 independent measurements ± SEM. Table 1 shows the kinetic parameters of the fit.

Table 1

*Parameters of the fits of Fig. 2. $Y=Y_{min}+\frac{Y_1}{(1+[chol]/K_{i1})}+\frac{Y_2}{(1+[chol]/K_{i2})}$

	Value	Error
Y_{min} (%)	35	9.6
Y_1 (%)	30	4.6
K_{i1} (μM)	0.19	0.09
Y_2 (%)	35	7.3
K_{i2} (μM)	21.2	16.7

Effect of cholesterol on AA activation profile

To probe the effect of cholesterol on the NADPH activation profile by AA, we performed titrations of the activity *vs.* AA concentration in the absence and in the presence of two concentrations of cholesterol (0.5 and 20 μM). In this experiment cholesterol was added at the same time as Arachidonic acid (Fig. 3).

In the presence of cholesterol, the $O_2^{\bullet -}$ production was lower on the full range of concentrations of AA, which confirms the inhibitory effect of cholesterol in a concentration-dependent manner. However the maximum activity was achieved with the same AA concentration and the bell-shape curve was kept.

Modification of kinetic parameters in the presence of added cholesterol

The absence of effect of cholesterol on the AA activation profile described above raised the question of the mechanism by which addition of cholesterol decreases the activity of the complex. In that purpose, the rate of superoxide production for increasing concentrations of trimera with and without cholesterol was determined. This dependence could always be fitted using a Michaelis–Menten-like equation (see materials and methods) and from each curve one could determine EC_{50} and V_{max} values. There was a marked loss in the oxidase activity in the presence of cholesterol for the whole range of concentrations of trimera, with important variations for both the EC_{50} and V_{max} values (Fig. 4, Table 2). In the

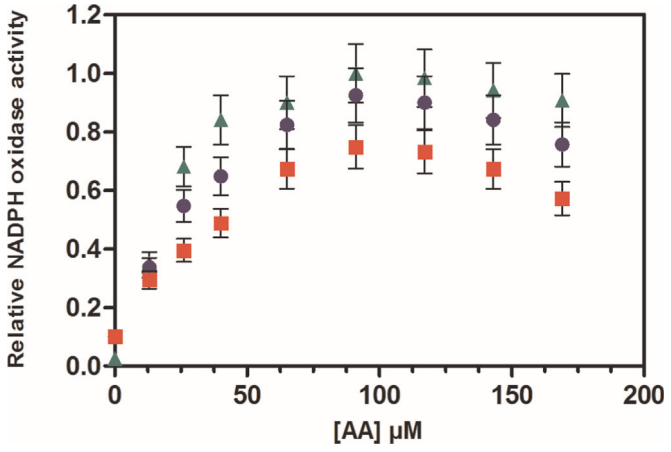

Fig. 3. Effect of cholesterol on the AA-dependent activation profile. Neutrophil membrane fractions and trimera were incubated together in the presence of a mixture of varying amounts of AA plus cholesterol. The cholesterol concentration was as follow, ▲: no cholesterol; ●: 0.5 μM cholesterol; ■: 20 μM cholesterol. Oxidase activities were expressed relative to the maximum activity (119 ± 12 mol $O_2^{\bullet -}$/s/mol Cyt b_{558}). The rate of $O_2^{\bullet -}$ production was achieved as described in Materials and methods.

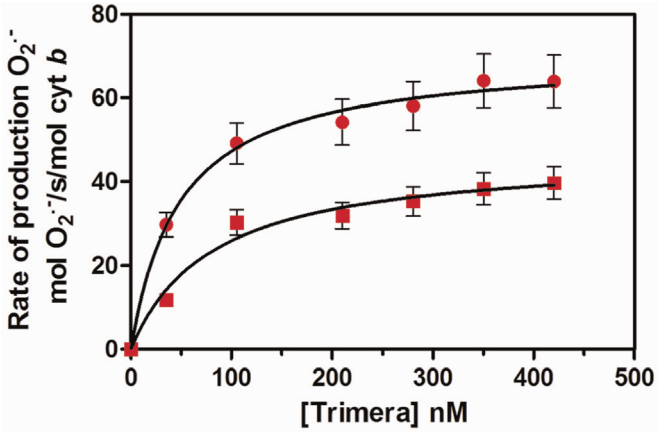

Fig. 4. Effect of cholesterol on the trimera dependence NADPH oxidase activity. The assays mixtures consisted of trimera at varying concentrations (from 0 to 420 nM) and membrane fractions of human neutrophils (3 nM Cyt b_{558}) to which were added • 40 μM AA or ▪ a mixture of 40 μM AA and 10 μM cholesterol. The superoxide formation was measured as indicated in Materials and methods. Each curve was fitted by Michaelis–Menten like equation leading to the determination of the EC_{50} and V_{max} values related to the trimera. The values of these kinetic parameters in the presence of AA alone or AA plus cholesterol are in Table 2. The values are an average of three independent measurements ± SEM.

Table 2
Kinetic parameters of NADPH oxidase activation by trimera.

Assay enriched with	*Vmax* (*mol* $O_2^{\bullet-}$/*s/mol cytb*)	EC_{50} (nM trimera)
AA 40 μM	70.3 ± 2	48.6 ± 3
AA 40 μM, cholesterol 10 μM	46.6 ± 6	79.8 ± 19

presence of cholesterol, the complex exhibited 1.5 times lower V_{max} value and the EC_{50} was 1.6 times higher than its values measured in absence of cholesterol, which might reflect a decrease of affinity of trimera for the Cyt b_{558}.

Effect of cholesterol addition during assembly phase

To examine whether cholesterol had an effect on the oxidase assembly process, 10 μM of cholesterol were added at different times during the 4-min phase of assembly (Fig. 5). Depending on the time at which cholesterol was added, various levels of

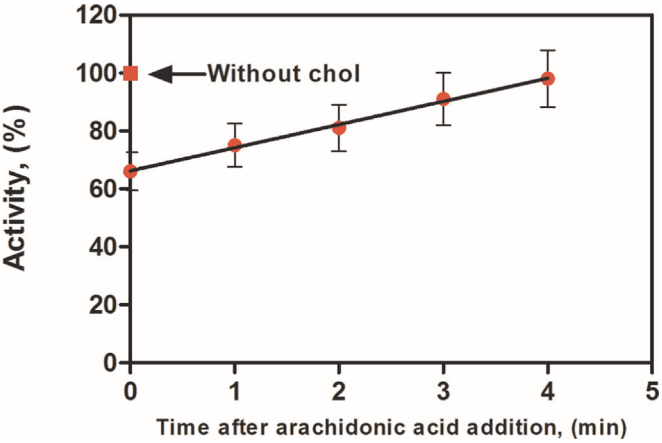

Fig. 5. Effect of the addition time of cholesterol on NADPH-oxidase activity. Membrane fractions (MF), trimera, and 40 μM AA were incubated 4 min in reaction buffer. 10 μM cholesterol were added at various times after the addition of AA. Conditions are as described under Materials and methods. Results are expressed as percentages of the control activity (without cholesterol addition). The values represent the means ± SEM of three independent experiments.

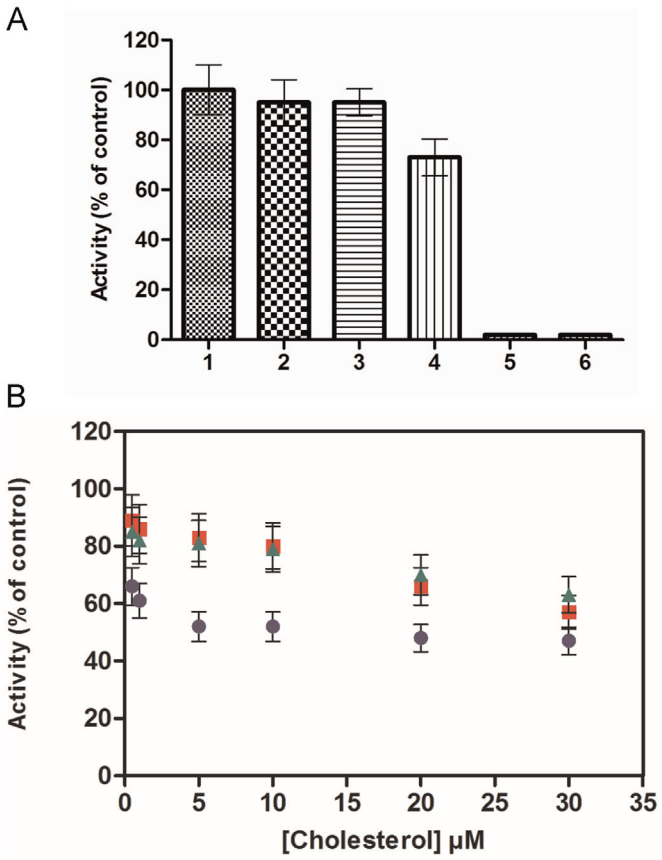

Fig. 6. Effect of cholesterol on AA activation of membrane fraction and trimera. (A) AA activation of membrane fraction and trimera (1) In the control experiment, membrane fraction and the trimera were incubated together during 4 min in the presence of 40 μM AA. The rate obtained corresponds to the 100% value (75 mol $O_2^{\bullet-}$/s/mol Cyt b_{558}). (2–4) Membrane fractions (MF) and trimera were separately preincubated during 10 s and mixed together for 4 min as follow: (2) both were preincubated with 40 μM AA, (3) only MF was preincubated with 40 μM AA, (4) only the trimera were preinicubated with 40 μM AA. (5) The rate was measured in absence of trimera. (6) The rate was measured in absence of MF. (B) Membrane fractions (MF) and trimera were separately preincubated during 10 s and mixed together for 4 min as follow: ▪ only MF was preincubated with 40 μM AA plus cholesterol as indicated, ▲ only the trimera was preincubated with 40 μM AA plus cholesterol as indicated and • both were preincubated with 40 μM AA plus cholesterol. Activities in Fig. 6B were expressed as the percent of activities measured of Fig. 6A corresponding to each state to assess the cholesterol effect only.

inhibition were noticed. $O_2^{\bullet-}$ production was drastically lowered to ~66% of the control when cholesterol was added immediately after all the NADPH-oxidase components. The inhibition was less and less important (up to 100% activity) for times longer than 1 min. If cholesterol addition took place 4 min after the mixing, the oxidase activity remained comparable to that of the control. Both results (Figs. 4 and 5) suggest that cholesterol interferes in the interaction between trimera and Cyt b_{558}.

Structural effects of added cholesterol

Effect of cholesterol on soluble and membrane proteins

To evaluate the sensitivity of each component, the membrane part and the trimera, to cholesterol, superoxide anion production rate was measured after pre-incubation of membrane components or trimera or both, either with AA alone (Fig. 6A) or with a mixture of cholesterol and AA (Fig. 6B). After 10 s of separate pre-incubations of the membrane and of the trimera, both solutions were mixed and left for a second incubation for 4 min. We chose to pre-incubate for 10 s because a preincubation for longer time (30 s) led

to a drastic decrease of the activity. This was also observed in the case of separated subunits [71].

First, when both membrane fractions and trimera were pre-incubated separately with AA, the activity was maintained to about 95% of the observed one in standard conditions. The same level of activity (95%) was measured when AA was incorporated only to the membrane fractions. On the other hand, when AA was added only to the trimera, the activity dropped to 73% (Fig. 6A).

The experiment with AA (Fig. 6A) served as control for the ones with cholesterol. In all cases, addition of cholesterol+AA either to the membrane or to the trimera or to both separately led to a decreased rate of superoxide production (Fig. 6B). Incorporating cholesterol+AA only to the membrane fractions or only to the trimera gave comparable effect, indicating that cholesterol could act on both partners. When cholesterol was added to both counterparts, the activity drop was more important, the addition of 5 μM or more of cholesterol to both fractions led to a decrease of the activity down to ~50%. The inhibition always increased by increasing cholesterol concentration, in agreement with the results presented Fig. 2.

Effect of AA and cholesterol on the tryptophan intrinsic fluorescence of the trimera

To probe the effect of AA and cholesterol on the trimera conformational state, we followed the variation of intrinsic tryptophan fluorescence spectra upon addition of AA and/or cholesterol (Fig. 7). Trimera contains a total of 13 tryptophan residues. It was previously shown that addition of AA to the cytosolic subunits p67phox and p47phox induced changes reflected by measurable decrease of the intrinsic tryptophan fluorescence level [68,72].

By increasing AA concentration up to 140 μM, the fluorescence yield of the trimera underwent a concentration dependent decrease (Fig. 7A). A small blue shift up to 5 nm was observed, consistent with a slightly more hydrophobic tryptophan environment.

Surprisingly, the addition of cholesterol had no effect on the fluorescence yield of the trimera but a similar blue shift up to 5 nm was detected by increasing cholesterol concentration to 50 μM (Fig. 7B), indicating that addition of cholesterol does not induce conformational changes in the trimera, as AA does. When cholesterol was added to the trimera treated with 40 μM AA, the initial quenching of 25% due to the presence of AA still was visible, but there was no further lowering due to cholesterol (Fig. 7C). In addition to that the same blue shift up to 5 nm was observed after the addition of both AA and cholesterol.

4. Discussion

In this work we have explored the effect of added cholesterol on the activity of the phagocyte NADPH oxidase. This study is related to events that could happen to Nox2 following hypercholesterolemia. It is also related to the hypothesis of modulation of NADPH oxidase activity by lipid rafts rich in cholesterol. In fact, some studies have reported the distribution and regulation of Nox proteins and oxidase subunits in LRs, (most of the studies were performed in non-human cells [11–14] except those in human neutrophils) [9,73]. Given that, NADPH oxidase activation demands many partners to work together, LRs can provide a useful platform for Nox2 and its subunits to aggregate and then function as an active enzyme complex that produces $O_2^{\bullet -}$ [13,14,74–78]. In particular, it has been demonstrated that NADPH oxidase was assembled and activated in LR of neutrophils, producing $O_2^{\bullet -}$, causing respiratory bursts and killing bacteria [9]. The integrity of LRs would play a crucial role in the regulation of NADPH oxidase activity and cholesterol would be an essential component in LRs for NADPH oxidase in agreement with previous reports [11,79].

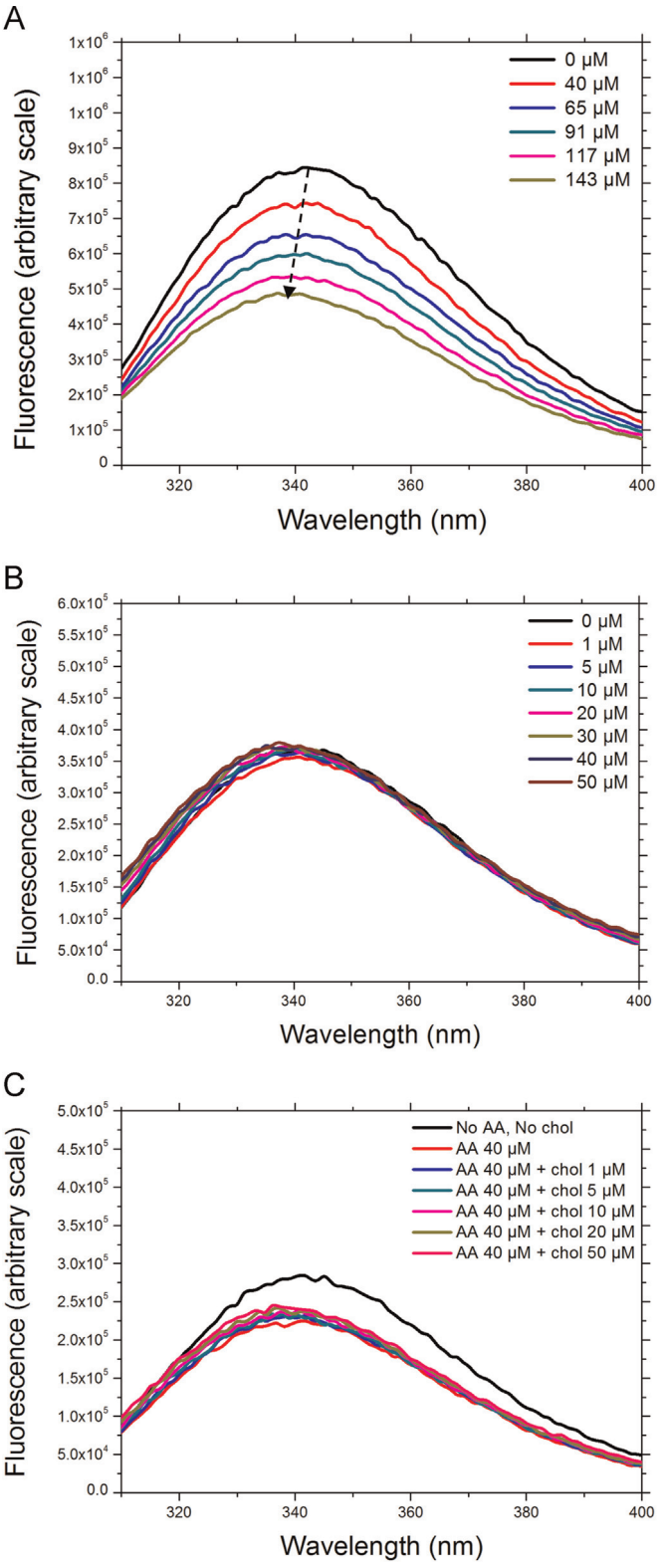

Fig. 7. Intrinsic fluorescence of trimera treated with either AA or cholesterol or both. The emission spectra were measured using an excitation wavelength of 290 nm as described in Materials and methods. Results are representative of at least three independent experiments. A: trimera 60 nM, AA as stated in the figure legend. B: trimera 60 nM, cholesterol as stated in the figure legend. C: trimera 60 nM, AA 40 μM, cholesterol as stated in the figure legend.

Actually, the role of specific protein–lipid interactions in oxidase assembly has been studied in the last decades [32,36,38]. It has been shown that the interaction of the PX domains of $p40^{phox}$ and

p47^{phox} with phospholipids constitutes an essential mechanism to orchestrate the assembly of the cytoplasmic components with the phagosomal membrane. The PX domain of p40^{phox} interacts selectively with phosphatidyl-inositol3-phosphate, (PtdIns(3)P) [80,81], while the PX domain of p47^{phox} preferentially recognizes (PtdIns(3,4)P2) [80,82].

On the required cholesterol concentration in neutrophils

The regulatory role of LR was rationalized by the fact that cholesterol exerts a stabilizing role in LRs by filling the void space between sphingolipids and forming hydrogen bonds with them. Thus, cholesterol depletion by MβCD would lead to the breakdown of LRs as it suppresses the glue effect of cholesterol on sphingolipids [83,84]. In addition to that, removal of raft cholesterol leads to dissociation of most proteins from the rafts, rendering them nonfunctional [85]. Shao et al. showed that incubation of the cells with MβCD resulted in a loss of association of gp91^{phox} with the LR fractions [9] while Vilhardt et al. reported that cholesterol depletion by MβCD reduced significantly $O_2^{\bullet -}$ production in both intact cells and a cell-free reconstituted system and MβCD effect was joined with a parallel reduction of the translocation of cytosolic components to the membrane [11]. Later, Fuhler et al. further demonstrated that treatment of neutrophils with the MβCD, abrogated fMLP-induced ROS production and activation of protein kinases ERK1/2 and B/Akt in both unprimed and primed neutrophils, further assisting the opinion that LR-associated NADPH oxidase produces ROS and contributes importantly to the onset of phagocytic respiratory bursts [86]. Our results about removal of cholesterol by MβCD are in agreement with the preceding findings since the oxidase activity was decreased to (44 ± 7)%.

On the effect of cholesterol

Our results indicated slight but significant activation of NADPH oxidase complex in cell free system by addition of cholesterol alone at physiological concentrations (*ca.* 10–30% above the normal level), without AA. Conversely, addition of cholesterol in this range has an inhibitory effect on AA activation of NADPH oxidase activity. Similar amplitude was also observed at 37 °C (data not shown). The effect of cholesterol did not interfere with that of AA (same profile), indicating different binding sites for both compounds. Several facts indicate the presence of two independent inhibitory binding sites. Effectively, in Fig. 2, the curve could be fitted only with a two-site inhibition equation. In addition, cholesterol effect has been observed when it was preincubated either with the membrane or with the trimera alone. When cholesterol (0.2–10 μM) was added to one component (membrane or trimera) (Fig. 7B) a small inhibition was observed (~20%), but when both components were preincubated with the same concentration, a higher inhibition was measured (~50%). It strengthens the idea that cholesterol affects not only the membrane fraction but also the cytosolic ones.

The kinetic parameters in the presence of cholesterol revealed that V_{max} for trimera is lower while EC_{50} is higher, which points out a less stable and imperfect assembly of the complex. Furthermore, cholesterol acts before assembly (Fig. 5), which might reflect that, one of the cholesterol binding sites is in the interaction region between membrane and cytosolic components, in the region hindered after assembly. Indeed, once the complex is formed, cholesterol cannot have access to it and makes no inhibitory effect. This effect has to be related to the observation that the depletion of cholesterol by MβCD also reduced the translocation of cytosolic proteins [10]. The cholesterol concentration found in membrane neutrophil seems to be optimal for NADPH oxidase activity.

4.3. On the conformation of the cytosolic partner

We have shown recently that AA modified the environment of tryptophan residues in the separated cytosolic subunits: both p47^{phox} and p67^{phox} underwent fluorescence decrease, which would be related to structural modifications necessary for their interaction with Cyt b_{558}[58,68]. A similar tryptophan fluorescence decrease was observed for the trimera upon addition of AA. No comparable effect of cholesterol on the trimera was observed, indicating that cholesterol cannot adapt the protein to the membrane subunit.

In conclusion, while the presence of cholesterol at physiological concentration (*e.g.* in lipid rafts) is important for the NADPH oxidase function, an increase of cholesterol amount might have several consequences: (i) a slight but significant activity in the absence of the usual pro-inflammatory signals such as AA with the possibility of a permanent mild inflammatory state and (ii) an inhibition of the activity of NADPH oxidase in the presence of pro-inflammatory signals. In both cases there would be a modification of the response to the signaling regulation.

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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.redox.2014.07.004.

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