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trans Arachidonic acid isomers inhibit NADPH-oxidase activity by direct interaction with enzyme components

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abstract

NADPH-oxidase is an enzyme that represents, when activated, the major source of non-mitochondrial reactive oxygen species. In phagocytes, this production is an indispensable event for the destruction of engulfed pathogens. The functional NADPH-oxidase complex consists of a catalytic membrane flavocytochrome *b* (Cyt_{b558}) and four cytosolic proteins p47^{phox}, p67^{phox}, Rac and p40^{phox}. The NADPH-oxidase activity is finely regulated spatially and temporally by cellular signaling events that trigger the translocation of the cytosolic subunits to its membrane partner involving post-translational modifications and activation by second messengers such as arachidonic acid (AA). Arachidonic acid in its natural *cis*-poly unsaturated form (C20:4) has been described to be an efficient activator of the enzyme *in vivo* and *in vitro*. In this work, we examined in a cell-free system whether a change of the natural *cis* geometry to the *trans* configuration, which could occur either by diet or be produced by the action of free radicals, may have consequences on the functioning of NADPH-oxidase. We showed the inability of mono-*trans* AA isomers to activate the NADPH-oxidase complex and demonstrated the inhibitory effect on the *cis*-AA-induced NADPH oxidase activation. The inhibition is mediated by a direct effect of the mono-*trans* AA which targets both the membrane fraction containing the cyt_{b558} and the cytosolic p67^{phox}. Our results suggest that the loss of the natural geometric feature (*cis*-AA) induces substantial structural modifications of p67^{phox} that prevent its translocation to the complex.

1. Introduction

NADPH-oxidase is well known as a major source for non-mitochondrial superoxide radical in cells. Superoxide anion is rapidly transformed into toxic oxygen species such as hydrogen peroxide, hypochlorous acid, hydroxyl radical and peroxynitrite. In phagocytes, the generation of superoxide anion (O₂^{•-}) initiated by the NADPH oxidase complex is triggered by the activation of these cells by

inflammatory stimuli (for a review see [1,2]). Superoxide anion and its derived Reactive Oxygen and Nitrogen Species contribute efficiently to the destruction of phagocytized infectious agents [3]. In other cells, NADPH oxidase-generated superoxide is recognized to play other crucial roles in events such as cellular communication [4]. A deregulation of NADPH-oxidase activity is associated with several pathologies involving inflammatory processes, cardiovascular diseases, renal damage, central nervous system diseases and immune system disorders (for a review see [5–9]).

The phagocyte NADPH oxidase complex consists of a heterodimeric, integral membrane protein, namely flavocytochrome *b*₅₅₈ (Cyt_{b558}, composed of p22^{phox} and a heavily glycosylated gp91^{phox} subunits) and four cytosolic subunits (p40^{phox}, p47^{phox}, p67^{phox}, GTP-binding protein Rac). The Cyt_{b558} serves as the catalytic core of the NADPH oxidase complex allowing the electron transfer across the membrane. To prevent the high toxicity of reactive oxygen species under physiological conditions, the NADPH-oxidase activity is highly regulated spatially and temporally. Following receptor-mediated activation of phagocytes, the regulatory subunits p40^{phox}, p47^{phox}, p67^{phox} and Rac translocate to the membrane bound Cyt_{b558} to form the active enzyme [10–13]. This activation process is tightly controlled by intracellular signaling

Abbreviations: cyt, cytochrome; AA, arachidonic acid; Ag-TLC, argentation thin layer chromatography; PMSF, phenylmethanesulfonyl fluoride; SOD, superoxide dismutase; bMF, bovine membrane fractions; yMF, yeast membrane fractions; Me AA, AA methyl ester; GC/MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance; BSA, bovine serum albumin; Cyt_c, cytochrome c; SH3, Src Homology 3; SDS, sodium dodecyl sulfate

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mechanisms that regulate phosphorylation events of the oxidase components (for a review see [14,15]) and the action of lipid second messengers [16,17]. Lipid mediators like arachidonic acid (5c,8c,11c,14c-C20:4, *cis*-AA), participate in the phosphorylation processes of the regulatory subunits through kinase activation, but it has been also shown that long-chain polyunsaturated fatty acids are directly involved in the NADPH oxidase assembly process and elicit superoxide anion production, with arachidonic acid being the most potent activator [18].

The understanding of the mechanism and activation of NADPH oxidase has been greatly facilitated by the development of a cell-free system in which the enzyme in the resting state is activated by anionic amphiphiles, such as arachidonic or other fatty acids as well as SDS [18–22]. Evidence exists that the anionic amphiphiles induce conformational changes in the cytosolic subunits which are analogous to those induced *in vitro* by phosphorylation [23–26], leading to unmasking the SH3 regions interacting with *Cytb*₅₅₈ [27,28]. Anionic amphiphiles impact also the integral membrane *Cytb*₅₅₈ by eliciting conformational changes, which were proposed to be relevant for the activation of superoxide production [29–32]. Moreover, it has been proposed that *cis*-AA may facilitate the accessibility of the *Cytb*₅₅₈ hemes to cyanide derivatives allowing the transition of the spin state of the heme iron [33,34].

In eukaryotic cells, the prevalent geometry displayed by unsaturated fatty acids is the *cis* configuration. The change of the *cis* geometry to the corresponding geometrical *trans* isomer is an unnatural event which influences cellular responses [35]. This can happen in an exogenous manner, with the intake of *trans* fatty acids through foods containing partially hydrogenated or deodorized vegetal/fish oils or processed at high temperature [36,37]. In addition to the uptake from food, an endogenous pathway has been identified in trans-free models and in humans through the occurrence of a free radical-induced *cis-trans* double bond isomerization [38–40].

Due to the connection of NADPH-oxidase activity with arachidonic acid and free radical production, it is important to address a first question on the relationship between the fatty acid geometrical configuration and the functioning of the NADPH-oxidase. In this paper, we explored the events induced by free long unsaturated fatty acid, that occur during NADPH oxidase activation using a cell-free system approach that permits to strictly quantify the system components. We used in parallel two cell free systems: i) the most frequent “canonical” semi-recombinant cell-free system in which mixed native *Cytb*₅₅₈ was contained in membrane fractions (isolated from neutrophils) with purified cytosolic recombinant components, and ii) an entire recombinant cell-free system in which the native neutrophil membranes are replaced by recombinant *Cytb*₅₅₈ expressed in yeast (*Pichia pastoris*) membrane [41]. In both systems, we compared the NADPH oxidase activating ability of arachidonic acid (*cis*-AA) with that of mono-*trans* arachidonic acid isomers (*trans*-AA, Fig. S1). We demonstrated a specific inhibitory effect of the *trans*-AA on the *cis*-AA-induced NADPH oxidase activation and performed experiments to decipher the target(s) of the inhibition mediated by a direct effect of *trans*-AA.

2. Materials and methods

2.1. Materials

Superoxide dismutase (SOD), reduced β -nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome c (*Cytc*), arachidonic acid (*cis*-AA), phenylmethanesulfonyl fluoride (PMSF), and tert-butylisocyanide were from Sigma (Saint Quentin Fallavier, France).

2.2. Synthesis of mono *trans*- and methyl ester arachidonic acid

Synthesis of *trans*-AA was performed following the protocol for free radical geometrical isomerization procedure [42]. Briefly, a

2.2 mL solution of 15 mM *cis*-AA in propan-2-ol (i-PrOH) was placed in a quartz photochemical reactor and bubbled with nitrogen for 20 min. The thiol (2-mercaptoethanol) in a 50% mole equivalent with respect to mole of *cis*-AA was added and the mixture was exposed to low pressure mercury lamp (5.5 W) at 25 °C under a slow flow of nitrogen. Reaction was monitored by the argentation-thin layer chromatography (Ag-TLC) that showed the formation of the *trans*-AA fraction in the first 15 min. The reaction was stopped and the mixture was dried under vacuum, followed by purification by Ag-TLC. The remaining fraction containing the starting material, the pure *cis* isomer, was used to reiterate the isomerization protocol, in order to increase the *trans*-AA yield up to an 85% (purity > 97%). The purity was checked by proton nuclear magnetic resonance (¹H NMR) as well as by GC/MS after transformation of the fraction to the corresponding fatty acid. The *trans*-AA fraction was isolated as a mixture of four mono-*trans* isomers in equal percentages as described [40] (Supplementary Fig. S1). Methyl ester fatty acids were obtained by treatment with an ethereal solution of diazomethane, as previously described [40]. 20 mg/mL stock solutions of *cis*-AA, *trans*-AA and methyl ester AA (*me*-AA) were prepared in ethanol.

2.3. Isolation of native membranes from neutrophil cells

Membrane fractions from bovine neutrophils were prepared as described [43]. Briefly, isolated neutrophils were extracted from 8 to 10 L of bovine blood by two successive hemolysis followed by Percoll sedimentation, and then they were disrupted by brief sonications. The membrane fraction (BMF) was separated from the cytosolic fraction and other cell contents on sucrose gradient and was collected by centrifugation at 150,000 $\times g$ for 160 min at 4 °C. Pellets were resuspended in phosphate buffer saline (PBS) pH 7.8 containing 1 mM EDTA, 1 mM PMSF, and 0.5 mM leupeptin before storage at –80 °C for further experiments. The protein concentration of each sample was estimated using the Pierce BCA protein assay (Thermo Scientific, Courtabœuf, France) with BSA as standard. Between 0.5 and 0.8 mg proteins/L of blood were usually obtained.

2.4. Isolation of recombinant *Cytb*₅₅₈-enriched plasma membrane from yeast cells

Recombinant *Cytb*₅₅₈ was expressed in *P. pastoris* and yeast membrane fraction (yMF) was obtained as previously described [41]. Briefly, cell pellets from a 72 h yeast culture were thawed and resuspended in cell breaking buffer (CBB) containing 50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA and 5% glycerol. All steps are carried out at 4 °C. An equivalent volume of glass beads was added and cells were disrupted by 8 cycles of 30 s vortexing/30 s ice bath. The clear supernatant containing the crude cell lysate was separated from the cell debris and unbroken cells by centrifugation at 500 $\times g$ for 10 min at 4 °C. The membrane fractions were collected by centrifugation at 100,000 $\times g$ for 120 min and resuspended in 50 mM Tris/HCl pH 8, 120 mM NaCl, 10% glycerol, and 1 mM PMSF and loaded onto a discontinuous sucrose gradient comprising 60, 40 and 20% sucrose. After overnight centrifugation at 110,000 $\times g$, the essential pure plasma membranes were removed from the 60 and 40% interface, diluted 4-fold and pelleted at 30,000 $\times g$. The *Cytb*₅₅₈-enriched membrane fraction was resuspended in 1 mM EDTA, 30% sucrose, and 20 mM Tris/HCl pH 8.0 and stored at –20 °C.

The protein concentration of each sample was estimated using the Pierce BCA protein assay with BSA as standard. About 6 mg proteins/L of yeast culture was usually obtained.

2.5. Production of recombinant cytosolic proteins

Recombinant p47^{phox}, p67^{phox} and Rac1-Q61L (Rac) were expressed and purified as described [44]. Here, the mutant Rac1Q61L was used

because it is constitutively in the active form, the GTP-bound form and thus greatly facilitates the superoxide anion production in vitro [45]. The cDNA plasmids were a generous gift from Dr. M.C. Dagher (Laboratoire TheREx, TIMC-IMAG, Grenoble, France) and Dr. M. Quinn (University of Montana, USA).

2.6. Cell-free NADPH-oxidase activity assay

Superoxide anion production was quantified by the initial rate of cytochrome c (Cytc) reduction, as described before [46]. The components of the cell-free system were added as follows: 5–8 nM Cytb₅₅₈-enriched yeast or bovine membrane fractions (estimated from difference absorption spectra), 100 nM p67^{phox}, 160 nM p47^{phox} and 80 nM RacQ61L so that the membrane protein/cytosolic protein ratio was kept at around 1/20, and the chosen free fatty acid (*cis*-AA or *trans*-AA). After 5 min of incubation at 25 °C, 0.1 mM Cytc was added and the O₂^{•−} production was initiated by the addition of 200 μM NADPH. The linear portion of the curves was used to determine the maximal rates of O₂^{•−} production. The reaction buffers contain PBS pH 7.4 and 10 mM MgSO₄ for bovine cytb₅₅₈ activity assay or 20 mM Tris/HCl pH 8, 1 mM EDTA and 120 mM NaCl for recombinant yeast cytb₅₅₈ activity assay. The SOD-inhibitable reduction of Cytc was followed at 550 nm on double beam Uvikon UV946 spectrophotometer. The amount of superoxide was calculated using a molar absorption coefficient ($\Delta\epsilon$ of the reduced minus oxidized form of cytochrome c) of 21 mM^{−1} cm^{−1}. Control experiments were performed in the presence of 350 U mL^{−1} of SOD. Negative control experiments were performed replacing the *cis*-AA by *me*-AA. Activation and inhibition curves were fitted using GraphPad Prism 5 following the equation:

$$A \frac{1}{4} A \frac{\Delta A}{1 + 10^{\log \frac{[AA] - [AA]_{50}}{[AA]_{50} - [AA]}} n} \text{ or } A \frac{1}{4} A \frac{\Delta A}{1 + 10^{\log \frac{[AA]_{50} - [AA]}{[AA] - [AA]_{50}} n}}$$

where A is the activity, ΔA is the difference between maximal and minimal activity, [AA] is the arachidonic acid concentration and n is the Hill constant.

2.7. Intrinsic fluorescence assays

Steady-state fluorescence measurements were performed on a Photon Technology International scanning fluorimeter at 25 °C. The tryptophan fluorescence spectra of protein were obtained by exciting the samples at 280 nm (1 nm bandwidth) and recorded between 300 and 450 nm (2 nm bandwidth). Although measurement of tryptophan fluorescence is usually performed using an excitation wavelength of 295 nm, we found that, as previously described [23], using an excitation wavelength of 280 nm optimized the signal to noise ratio without adding significant tyrosine fluorescence interference.

Various concentrations of *cis*-AA, *trans*-AA or *me*-AA were added as indicated to a final volume of 1 mL of buffer (10 mM potassium phosphate, pH 7.2, 130 mM NaCl) in a quartz cuvette containing a magnetic stir bar, and recombinant p47^{phox} (125 nM), or p67^{phox} (45 nM) proteins. The measured fluorescence intensity was corrected for dilution effects or for inner-filter effect.

2.8. Spectral analysis of cytb₅₅₈

Absorption spectra were recorded at room temperature using a Uvikon dual-beam spectrophotometer. The sodium dithionite-reduced minus oxidized difference of absorption spectra was recorded between 400 and 600 nm.

Yeast or bovine membrane fractions were suspended in 200 μL phosphate buffer, incubated at 25 °C for 5 min and supplemented with optimal concentrations of *cis*-AA, *trans*-AA or *me*-AA. The dithionite reduced membranes were treated with 5 mM or 30 mM tert-butyl-isocyanide for yeast or bovine membranes, respectively.

2.9. Co-sedimentation assays

Cytb₅₅₈ from bovine or yeast membrane fractions was mixed with the recombinant purified cytosolic subunits (same ratio as for activity assays) in the presence of 200 μM *cis*-AA, *trans*-AA, or *me*-AA (600 μM in the case of yeast membrane fractions). After 5 min incubation at 25 °C, the protein mixtures were layered onto a discontinuous gradient composed of 1.5 mL of 10% (w/v), 6.5 mL of 15% (w/v) and 2.0 mL of 50% (w/v) sucrose, all in 10 mM Tris-HCl pH 7.8, 1 mM EDTA, and 10 mM NaN₃ buffer, and centrifuged at 150,000 ×g for 30 min at 15 °C in a SW32Ti rotor. After centrifugation, the essential soluble proteins were collected in the 0.5 mL aliquots from the top of the gradient (“supernatant”) while the membrane or membrane-associated proteins were retrieved from the bottom of the gradient (“pellet”).

2.10. SDS PAGE and immunoblotting

“Supernatant” and “pellet” from the co-sedimentation experiments were diluted in Laemmli buffer, loaded onto a 10% ClearPAGE SDS-polyacrylamide precast gel (VWR International, Fontenay-Sous-Bois, France), and transferred to a nitrocellulose membrane. Nitrocellulose membranes were then immunoblotted with anti-p47 (1:500) or anti-p67 (1:500) polyclonal antibodies. Immune complexes were visualized with secondary peroxidase-conjugated antibodies (1:10,000 dilution), using a chemiluminescent kit (GE Healthcare, Saclay, France).

2.11. Membrane lipid composition

Fatty acid composition of yeast and bovine membrane fractions were determined after lipid extraction followed by trans-esterification to obtain fatty acid methyl esters. The methyl ester composition was analyzed by gas chromatography and compared to standard reference compounds profiles, as previously described [47]. Peaks are expressed as relative percentages of the total peak areas of the chromatograms (peak covering >99.9%). The main composition is described in Fig. 2.

3. Results

3.1. Activation of the NADPH oxidase complex in semi and total recombinant cell-free system

The NADPH oxidase activation by *cis*-AA was investigated in semi-recombinant and fully recombinant cell-free system. In both assays, the activity follows a characteristic bell shape activation profile (Fig. 1). As a negative control, the oxidase activities were quantified with *me*-AA that has been shown to competitively counteract *cis*-AA-mediated oxidase activation. In agreement with previous findings [18,34,48,49], *me*-AA was not able to activate NADPH-oxidase complex in both cell-free assays, supporting the evidence that the negative charge present in *cis*-AA is indispensable for the NADPH oxidase activation.

Similar to *me*-AA, the addition of *trans*-AA (an equimolar mixture of four mono-*trans* isomers) could not induce O₂^{•−} production neither in the recombinant (Fig. 1A) nor in the semi-recombinant cell-free system (Fig. 1B). This result indicates that *trans*-AA is unable to activate the NADPH oxidase complex although it contains the negative charge similar to the *cis* form.

In the semi-recombinant cell free system, the maximal O₂^{•−} production occurred at a *cis*-AA concentration of 200 μM (Fig. 1B), while in the yeast cell-free system, this value is significantly higher (600 μM, Fig. 1A) and the bell-shape is wider. The optimal *cis*-AA concentration for the oxidase activity in the semi-recombinant assay is in good agreement with several reported results [32,50,51], whereas lower values were also reported by other authors [20,23,52]. However no significant difference of the maximal activity values induced by *cis*-AA is observed when bovine or yeast membrane fractions were used as source of NOX. Both cell free systems produce about 20 mol Q^{•−}/s/mol heme.

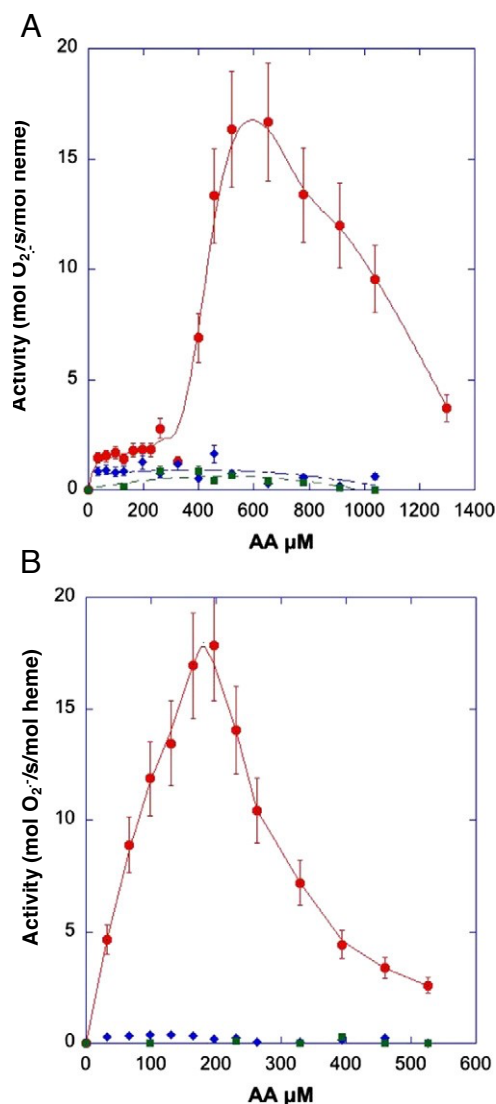


Fig. 1. Activation of NADPH-oxidase complex by different arachidonic acid isomers. The superoxide production was measured spectrophotometrically by the rate of cytochrome *c* reduction at 550 nm as described in the **Materials and methods** section. In the reaction mixture, yeast (A) or bovine (B) membrane fractions were incubated 5 min in the presence of recombinant cytosolic proteins, and different concentrations of *cis* arachidonic acid (circles), *trans* arachidonic acid (diamonds) or methyl ester arachidonate (squares). The reaction was initiated by the addition of 200 μM NADPH in the presence of 50 μM cytochrome *c*. Results are presented as the mean \pm SD of four independent experiments. Visual fit of activation curves was performed using KaleidaGraph, Synergy Software, (Reading, PA, USA).

The maximal activity obtained herein was lower compared to well characterized cell-free-AA-stimulated NADPH-oxidase activity [18] probably due to the presence of contaminant Ca^{2+} . Actually, we did not add EGTA in the NADPH-oxidase assays, working under contaminant Ca^{2+} conditions (estimated to be lower than 30 μM). It was previously demonstrated that the presence of Ca^{2+} inhibits the stimulatory action of fatty acid on NADPH-oxidase [18,53], with an IC_{50} around 100 μM . This inhibitory effect is correlated with the raise of the fatty acid Krafft point in the presence of Ca^{2+} [53].

3.2. Membrane lipid composition

Fatty acid compositions of the yeast and neutrophil membranes are also very different. As shown in Fig. 2, a prevalent presence of the saturated fatty acid C16:0 (palmitic acid) was observed in *P.*

pastoris plasma membrane. In neutrophils the C18 family was predominant, with the saturated fatty acid C18:0 (stearic acid) and the polyunsaturated omega-6 fatty acid 9c,12c-C18 (linoleic acid) as the major components, with the relative percentages of 32% and 30% of the total composition, respectively. In *P. pastoris* membrane, only 3% of linoleic acid was detected. Thus the *P. pastoris* membrane is enriched in saturated fatty acids compared to neutrophil membranes.

3.3. NADPH-oxidase activity was inhibited by the addition of *trans*-AA

The *cis*-AA induced NADPH-oxidase activity was followed upon increased concentration of either *trans*-AA or *cis*-AA (Fig. 3A). The latter experiment serves as control in order to distinguish the inhibitory action of *trans*-AA from the concentration-related inhibition itself. As NADPH-oxidase activity follows a bell shape activation profile (Fig. 1), the NADPH oxidase activities decrease upon the addition of exogenous fatty acids. The *cis*-AA-induced NADPH-oxidase activity is reduced by a threefold upon the addition of *trans*-AA in a dose-dependent manner and only twice when *cis*-AA was added. These results indicated that *trans*-AA inhibitory effect resulted from two synergic actions: an unspecific, concentration-related inhibition and a specific inhibitory effect. The fits of the curves described in Fig. 3A give IC_{50} values of $14.9 \pm 2.8 \mu\text{M}$ and $47.1 \pm 1.2 \mu\text{M}$ for *trans*-AA and *cis*-AA, respectively (Supplementary material Table S1). A residual activity of 25% remains even for higher amount of *trans*-AA. Comparable results were obtained using bovine membrane fraction (Supplementary material, Fig. S2).

A complementary experiment was performed in which activity was followed in the presence of constant 100 μM *trans*-AA concentration and variable *cis*-AA concentration up to 600 μM (Fig. 3B and Supplementary Table S2). The maximal NADPH-oxidase activity induced by *cis*-AA reached only 40% of the value obtained in the control experiment in which 100 μM *cis*-AA was added instead of 100 μM *trans*-AA. In that case, the *cis*-AA EC_{50} value (Supplementary material Table S1B) is not affected by the presence of *trans* isomer. It might suggest that inhibition is competitive.

3.4. Deciphering of the NADPH oxidase target(s) of *trans*-AA

It is likely that *trans*-AA affects differently the membrane and cytosolic components. To investigate the targets of *trans*-AA, superoxide anion production rate was measured in recombinant cell-free assay in which membrane and cytosolic components were simultaneously or separately incubated with *cis*- or *trans*-AA isomers (Fig. 4A). Activity induced by simultaneous *cis*-AA incubation was considered as the 100% value.

When membrane and cytosolic fractions were incubated separately with *cis*-AA and then mixed, the activity dropped to about 71% (Fig. 4B). Surprisingly, when *cis*-AA was added only to the cytosolic or to the membrane fraction, the activity measured was similar (~79%, Fig. 4B). It should be noted that the initial activity was measured immediately after fractions were mixed in the presence of NADPH, reducing but not eliminating the possibility of a cross activation phenomena. Indeed the activity measured in the presence of *cis*-AA but without pre incubation time (simultaneously added with NADPH to a whole mixture) did not result in a significant activity (Fig. 4A). This underlines the fact that when the NADPH oxidase components are separately incubated with *cis*-AA and subsequently mixed, they partially lose their capacity to efficiently assemble afterwards. It is consistent with the importance of the numerous protein-protein and protein-phospholipid interactions for the assembly of a functional oxidase complex during the period of incubation prior to the onset of oxidase activity.

Consistent with our above results, incubation of both fractions with *trans*-AA (Fig. 4A) was unable to activate the NADPH oxidase complex leading to a very small $\text{O}_2^{\bullet-}$ production rate (less than 10% of that in the presence of *cis*-AA, similar to baseline activity in the absence of AA, or in the presence of *cis*-AA but without pre incubation

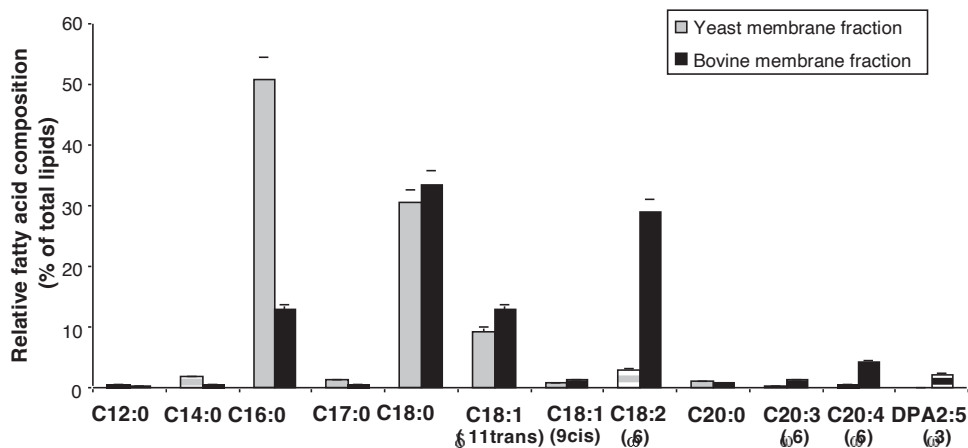


Fig. 2. Fatty acids composition in the lipids extracted from yeast strain *P. pastoris* (gray bars) and bovine neutrophil (black bars) membrane fractions. Lipid identification was performed using reference compounds. Peaks are expressed as relative percentages of the total peak areas of the chromatograms (peak covering > 99.9%).

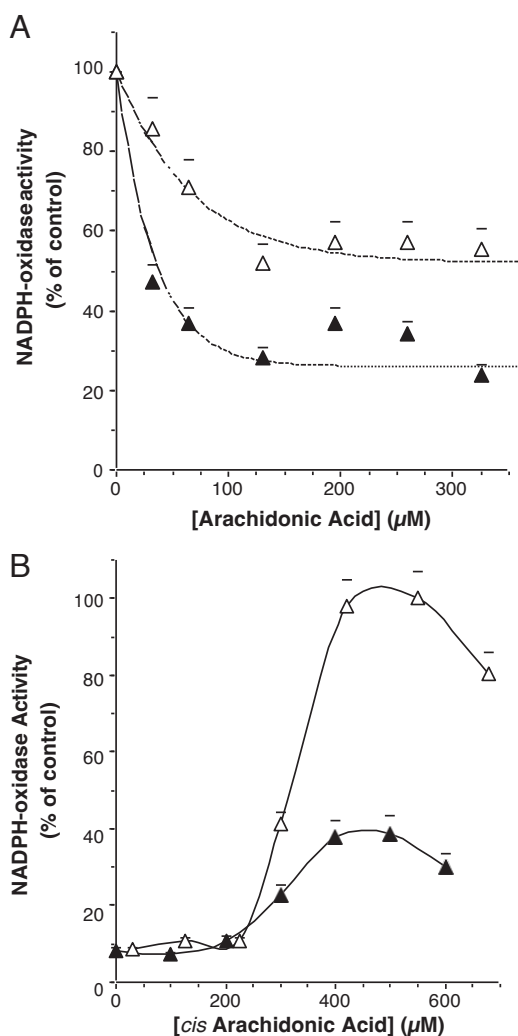


Fig. 3. NADPH-oxidase activity inhibition by *trans* arachidonic acid using the yeast membrane fractions. Yeast membrane fractions together with cytosolic proteins pre-incubated in the presence of (A) 600 μM *cis*AA supplemented with increasing concentrations of *trans*AA (filled triangles) or *cis*AA (open triangles), or (B) 100 μM *trans*AA (filled triangles) or *cis*AA (open triangles) supplemented with increasing concentrations of *cis*AA. Oxidase activity was expressed as the percent of activity measured in the absence of *trans*AA (20.3 mol O₂^{•−}/s/mol heme). Results are presented as the mean ± SEM of three independent experiments.

time). In order to identify whether *trans*-AA affected the membrane and/or cytosolic components, we performed cell-free NADPH-oxidase activity assays where cytosolic and membrane fractions were pre-incubated separately (Fig. 4B). Adding *trans* isomers resulted in a lower level of O₂^{•−} production rate (below 12%). When added only to the membrane fraction, the production of superoxide was almost undetectable. To summarize, these results let us hypothesize that *trans*-AA, in contrast to *cis*-AA, did not act as oxidase activator at the level of the membrane component of NADPH-oxidase complex. Moreover, a major result of these experiments is a decreased O₂^{•−} production of 45% when one of the components was activated with *cis*-AA and the counterpart with *trans*-AA. These decreased activities not only confirm the inability of *trans*-AA to activate the NADPH oxidase complex but also show a capacity of *trans*-AA to inhibit the oxidase activation process, through its binding on both the cytosolic and the membrane fraction. Comparable results were obtained using bovine membrane fraction (Supplementary Fig. S3).

3.5. Effect of the *trans*AA on the tryptophan intrinsic fluorescence of p47^{phox} and p67^{phox}

To probe the effect of AA isomers on the protein conformational state, we followed the evolution of intrinsic tryptophan fluorescence emission spectra when AA isomers were added to the regulatory proteins p47^{phox} and p67^{phox} (Figs. 5 and 6). Five from the seven total tryptophan residues of p47^{phox} are located in the protein region containing Src Homology 3 (SH3) domains [28,54]. It was previously demonstrated that activation of the NADPH oxidase induced changes in the micro-environment of these tryptophan residues, reflected by measurable variations of the intrinsic tryptophan emission fluorescence level [23,24]. p67^{phox}, a potential target for AA, contains a total of eight tryptophan residues and was described as a highly sensitive protein to the action of oxidant agents during the assembly process [55,56].

As was previously reported [23], the addition of *cis*-AA isomer induced a final decrease of 72% of the tryptophan fluorescence

(λ_{max} = 331 ± 1 nm) for p47^{phox} (Fig. 5A) and of 58% for p67^{phox} (Fig. 6A). The fluorescence decrease was dependent of *cis*-AA concentration up to 50 μM.

For both proteins, λ_{max} was independent of *cis*-AA concentration and no spectral shift was observed up to the maximal concentration of fatty acid used (328 μM).

Addition of *trans*-AA produced a similar effect, resulting in a decrease of the fluorescence level of about 65% for p47^{phox} (Fig. 5B)

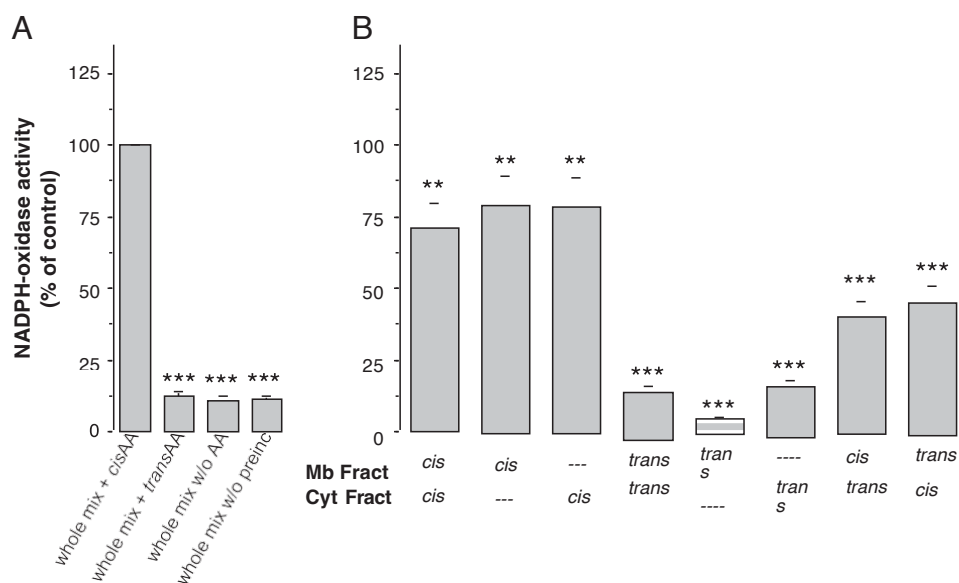


Fig. 4. Oxidase activity in cell free system with different preincubation conditions. (A) The whole mix of yeast membrane fraction and cytosolic proteins was preincubated for 5 min in the presence of 600 μM *cis*-AA or *trans*-AA or without the addition of AA. An additional control experiment was performed adding 600 μM *cis*-AA to the whole mixture and measuring activity immediately after NADPH addition without pre-incubation time (B) Yeast membrane fraction and cytosolic proteins were separately preincubated in the absence or in the presence of *cis* or *trans* arachidonic acid as indicated. Activities were expressed as the percent of control activity (16.7 mol $\text{O}_2^{*-}/\text{s}/\text{mol}$ heme, measured when whole mix was incubated in the presence of 600 μM *cis*-AA, left bar, panel A). Results are presented as the mean \pm SEM of three independent experiments. * $P < 0.05$; ** $P < 0.01$.

and 60% for $p67^{\text{phox}}$ (Fig. 6B). Addition of *me*-AA diminished the λ_{max} peak intensity to a lower extent. The fluorescence levels of $p47^{\text{phox}}$ and $p67^{\text{phox}}$ dropped only 40% (Fig. 5C) and 32% (Fig. 6C) of the initial peak value, respectively. Although we cannot exclude that the carboxylate groups of *cis* and *trans* AA interact with Trp residue(s) resulting in a quenching of their fluorescence, we can conclude that the absence of the anionic charge on the *me*-AA significantly reduces their impacts not only on $p47^{\text{phox}}$, as was previously described [23], but also with $p67^{\text{phox}}$.

Surprisingly, in the case of $p67^{\text{phox}}$ but not for $p47^{\text{phox}}$, the decrease of fluorescence intensity was associated with a red-shift from 331 nm in the absence of *trans*-AA to 348 nm upon the addition of a concentration of 50 μM *trans*-AA (Fig. 6B and D), indicating a significant change in the tryptophan environment. This can be explained by structural changes in extremely buried tryptophan(s) to fully exposed to solvent.

Analysis of the curves according to the standard Stern–Volmer relationship indicates a static quenching in the presence of an inaccessible population of tryptophan residues. The deduced K_d ($1/K_{\text{sv}}$) values and the proportion of accessible tryptophan (f_a) are shown in Table 1. *me*-AA presents lower affinity for both cytosolic proteins compared to *trans*-AA and *cis*-AA. Interestingly $p67^{\text{phox}}$ has a smaller K_d value for *trans*-AA than for *cis*-AA while for $p47^{\text{phox}}$, it is the opposite. Tryptophans of $p67^{\text{phox}}$ are equally accessible for *cis*- and *trans*-isomers, while $p47^{\text{phox}}$ presents higher accessibility for *cis*- than for *trans*-AA isomers.

3.6. Effect of the *trans*-AA on the membrane component *Cytb*₅₅₈ of the NADPH oxidase complex

Here we probe the direct effect of *trans*-AA on the *Cytb*₅₅₈. Anionic amphiphiles have been shown to influence the conformational changes during the transition from the inactive to the active form of *Cytb*₅₅₈. It has been demonstrated that *cis*-AA facilitated the accessibility of the hemes to tert-butyl-isocyanide and the transition of the spin state of the heme iron [34]. A binding site for *cis*-AA on *Cytb*₅₅₈ was suggested [57]. It has also been reported that the structural changes induced in *Cytb*₅₅₈ not only by *cis*-AA (but also by SDS and lithium dodecyl sulfate), at concentrations where the maximal

activity of NADPH oxidase is observed, are specific and might participate in oxidase activation [30,32]. To directly test the hypothesis that *trans*-AA does not target the membrane component, we used the same protocol as the one validated by Doussière's group to discriminate the spin heme iron transition using tert-butyl-isocyanide. The dithionite reduced minus oxidized absorption spectra were measured on native *Cytb*₅₅₈-containing neutrophil membrane fraction and recombinant *Cytb*₅₅₈-containing yeast membrane fraction. The direct effect of the AA isomers on the reduced minus oxidized absorption spectrum of both membrane fractions was tested (data not shown). All AA isomers failed to modify the difference absorption spectrum. In the same way, no spectral modification was observed by the addition of tert-butyl-isocyanide (at the indicated concentration) in the absence of any AA isomer. As previously observed [34], the addition of *cis*-AA (Fig. 7, trace "a") to the dithionite-reduced membrane fraction treated by tert-butyl isocyanide shifted the Soret peak from 429 to 432 nm (yeast membrane fraction, Fig. 7A) and from 427 to 434 nm (bovine membrane fraction, Fig. 7B). In contrast to *cis*-AA, neither the addition of *trans*-AA (trace "b") nor that of *me*-AA (trace "c") changed the absorption difference spectra. These results indicate that, in contrast to *cis*-AA, *trans*-AA and *me*-AA cannot modulate the tert-butyl isocyanide binding to the membrane *Cytb*₅₅₈.

3.7. Effect of the *trans*-AA on the NADPH-oxidase complex assembly

Although the assembly merges with oxidase activity, we analyzed the impact of *cis*-, *trans*- and *me*-AA on steps that bring to assembly completion. It has been previously described that ca. 5 min period of preincubation is necessary to allow the cytosolic and membrane component to completely assemble and to remain stable for several minutes [48]. We found also that 5 min incubation time was necessary to assure a constant rate of production of superoxide, i.e. full activation in our cell-free systems, indicating a complete assembly [56]. We performed yeast membrane and cytosolic component co-sedimentation by ultracentrifugation in the presence of the different AA isomers and analyzed the distribution by immunoreaction using antibodies against anti- $p47^{\text{phox}}$ or anti- $p67^{\text{phox}}$. Cytosolic proteins that had translocated to the membrane were co-localized on a sucrose gradient in the membrane pellet, while those not assembled remained in the

supernatant of the gradient. In the pellet compartment, only samples incubated in the presence of *cis*-AA presented positive signal to anti-p47^{phox} (Fig. 8A) and anti-p67 (Fig. 8B). Concomitantly, the signals in the corresponding supernatant compartments were diminished. These results are consistent with the notion that *cis*-AA is a potent activator by favoring the assembly step. In contrast, preincubation with *trans*-AA, *me*-AA or without any fatty acid leads to anti-p47^{phox} and

anti-p67^{phox} recognitions mainly in the supernatant compartment indicating that no translocation to the membrane of these proteins occurred in those cases.

4. Discussion

Polyunsaturated fatty acids such as *cis*AA are usual targets for a variety of free radicals [39,58,59] leading to generation of endogenous *trans* fatty acids by radical-induced *cis-trans* isomerization [36,38,60–64]. It has been shown that *trans* unsaturated fatty acids were ineffective on the release of superoxide from human neutrophils [65], but other authors working with cell-free system, have suggested that both *cis* and *trans* unsaturated fatty acids might activate NADPH-oxidase [66]. Although the necessity for anionic amphiphiles such as arachidonic acid to trigger the NADPH oxidase activation in cell-free system is well documented, the precise role played by arachidonic acid is still uncertain. Evidences have been reported on amphiphile-induced conformational changes in p47^{phox} similar to those required to induce oxidase activity by phosphorylation [23,24] favoring the binding of the SH3 domain of p47^{phox} to the p22^{phox} subunit of the Cytb₅₅₈ [27]. *Cis*AA, SDS and lithium dodecyl sulfate (LDS) have also been shown to induce conformational changes in flavocytochrome *b*₅₅₈ [30,32,34]. All activating molecules possess similar characteristics that define the requirement for effective action: an anionic head group and either an unsaturated or a restricted length of the saturated fatty acid part [18,19,30,67]. Thus, it was of particular interest to clarify whether *trans*-AA isomers are able to activate the NADPH-oxidase complex in a cell free system with similar efficiency to *cis*-AA and whether its targets are among the cytosolic and membrane oxidase components.

The major result that we have shown here is that, in contrast to *cis*-AA, *trans*-AA was not able to activate the NADPH-oxidase complex but moreover can inhibit the *cis*-AA-elicited superoxide production by interacting directly with the cytosolic component p67^{phox} and with the membrane fraction, probably indirectly by modifying the membrane physical properties.

4.1. Effects on cytosolic proteins

We have followed the changes in the intrinsic tryptophan fluorescence of isolated p47^{phox} and p67^{phox} treated with either *cis*-AA, *trans*-AA or *me*-AA. Both anionic AA isoforms quenched the tryptophan fluorescence signal of p47^{phox} down to about 70–65%. The effect of *me*-AA is undoubtedly different and indicates that the conformational changes associated with the methyl ester of fatty acid are only partial. The absence of major differences between the *cis*- and *trans*-isomers suggests that inhibition of the NADPH-oxidase activity is not due to a direct effect of *trans*-AA on p47^{phox}. It is postulated that *cis*-AA mimics phosphorylation events from the cellular signalization by adding negative charges. It is known that in vivo p47^{phox} is phosphorylated sequentially. Three serine residues (S345, S359, S370; [68]) are first phosphorylated followed by additional phosphorylations on seven other serines, all situated on the C-terminal domain. The phosphorylation of these residues is necessary for the translocation to the membrane, in unveiling the SH3 domains-mediated binding of p47^{phox}, which contain five of the seven tryptophan residues. Because of the absence of negative

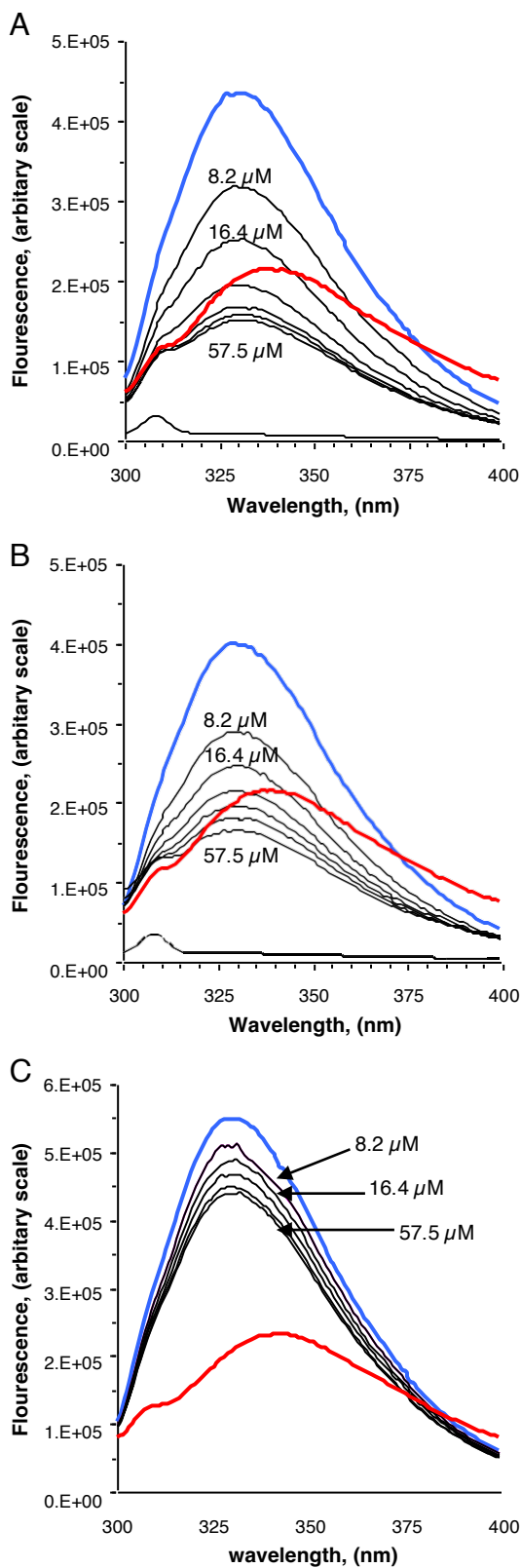


Fig. 5. Intrinsic fluorescence of p47^{phox} treated with different arachidonic acid (AA) isomers. 125 nM p47^{phox} was treated with raising concentration of *cis*-AA (panel A), *trans*-AA (panel B) or *me*-AA (panel C), and the emission spectrum was measured using an excitation wavelength of 280 nm as described in the **Materials and methods** section. In seek of clarity, only the first 7 concentrations were included in the figure. They correspond from top to bottom to the following concentrations of the 3 different AA isomers: 0 (blue thick line), 8.2; 16.4; 24.6; 32.8; 41.1 and 57.5 μ M. Background buffer signal (black thin line) and 8 M urea treated protein spectrum (red thick line) were also included. Results are representative of at least three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

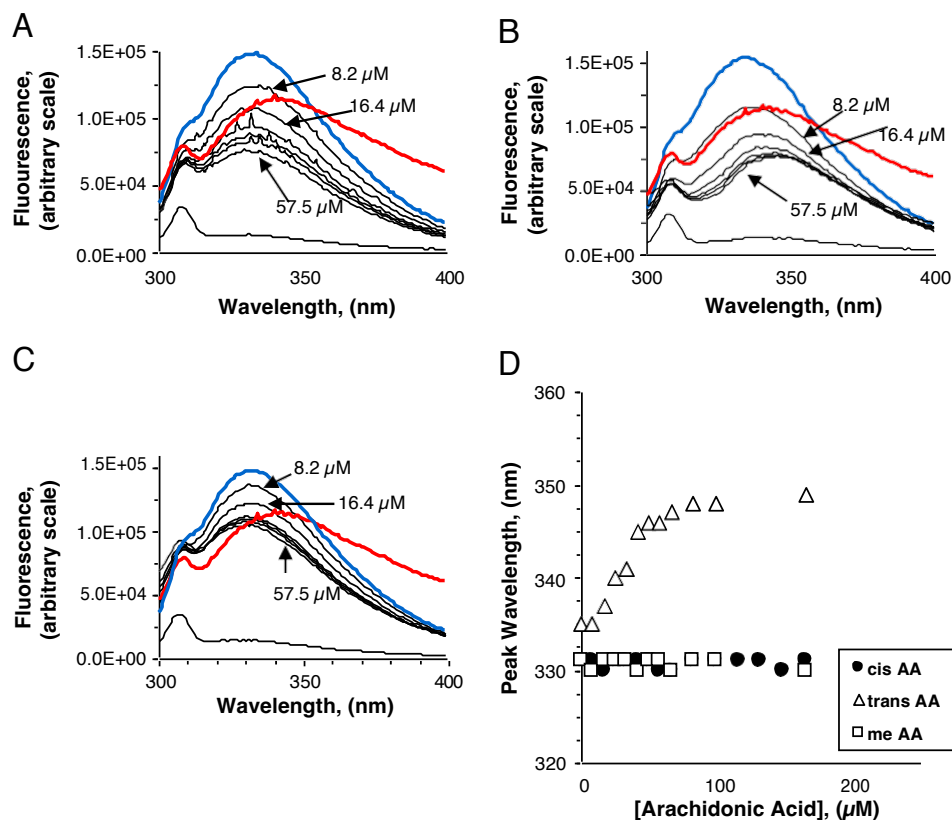


Fig. 6. Intrinsic fluorescence of p67^{phox} treated with different arachidonic acid (AA) isomers. 45 nM p67^{phox} was treated with increasing concentrations of *cis*-AA (panel A), *trans* AA (panel B) or *me* AA (panel C), and the emission spectrum was measured using an excitation wavelength of 280 nm as described in the Materials and methods section. In seek of clarity, only the first 7 concentrations were included in the figure. They correspond from top to bottom to the following concentrations of the 3 different AA isomers (in μM): 0 (blue thick line), 8.2; 16.4; 24.6; 32.8; 41.1 and 57.5. Background buffer signal (black thin line) and 8 M urea treated protein spectrum (red thick line) were also included. Peak wavelength value (panel D), was plotted as a function of arachidonic acid concentration. Results are representative of at least three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

charge, *me*-AA cannot contribute to the repulsive effect known to induce the active form of p47^{phox} protein and therefore is unable to totally mimic the phosphorylation events. In the case of *me*-AA addition, the structural changes are limited to hydrophobic interactions involving the fatty acyl chain.

The effects of AA isomer addition on isolated p67^{phox} were surprising. Indeed, the tryptophan fluorescence intensities were lowered as observed with p47^{phox}. Conversely, in addition to the fluorescence quenching, *trans*-AA led to a progressive red shift (up to 20 nm) of the maximum fluorescence wavelength of p67^{phox} close to the wavelength maxima determined for denaturated protein. This indicates that the environment of tryptophan residues was strongly affected by the addition of *trans*-AA, bringing tryptophans from non polar to polar environment. *Trans*-AA is able to induce very large structural changes in p67^{phox} that *cis*-AA cannot perform. These results

underline the importance of the conformation of the acyl chain of fatty acids given by different double bond geometries (Fig. S1). The *trans*-AA induced “denaturation” of p67^{phox} could be the major cause of the inhibition of *cis*-AA-induced NADPH-oxidase activity. It means that the hydrophobic part of amphiphilic molecules contains the capacity to inhibit the system whereas the anionic part is crucial to modify the soluble proteins to their active forms.

4.2. Effects on protein translocation

Our results show that *trans*-AA can bind p47^{phox} and p67^{phox} but does not allow the NADPH complex assembly. The co-sedimentation experiments showed that no translocation to the membrane occurs in the presence of *me*-AA and *trans*-AA. If the absence of co-translocation to the membrane with *me*-AA is due to the absence of

Table 1
Characteristics of p47^{phox} and p67^{phox} intrinsic fluorescence quenching by arachidonic acid isomers.

	p47 ^{phox}			p67 ^{phox}		
	1/Ksv (μM)	<i>f</i> _a	F _{min} (%)	1/Ksv (μM)	<i>f</i> _a	F _{min} (%)
<i>cis</i> AA	21.93 ± 0.63	0.87 ± 0.03	29.79 ± 0.73	42.74 ± 0.30	0.69 ± 0.02	42.41 ± 0.61
<i>trans</i> AA	31.45 ± 1.28	0.71 ± 0.01*	37.63 ± 0.72*	23.98 ± 0.39**	0.63 ± 0.03	42.53 ± 0.87
<i>me</i> AA	204.08 ± 38.22**	0.45 ± 0.10**	60.92 ± 2.54**	79.37 ± 4.17**	0.39 ± 0.02**	67.99 ± 0.83**

Quenching data from Figs. 5 and 6 were analyzed according to the standard Stern–Volmer relationship, $F_0/F = 1 + K_{sv}[AA]$, where F_0 is the intensity of fluorescence at 335 nm in the absence of AA, F is the intensity of fluorescence at the same wavelength in the presence of different AA isomer concentrations, and K_{sv} is the Stern–Volmer quenching constant obtained from the slope of a plot of F_0/F vs $[AA]$ (Supplementary Fig. S4A and B). To estimate the accessible fraction f_a , modified Stern–Volmer plots were used, according to the relation $F_0/(\Delta F) = 1/f_a + 1/(f_a K_{sv} [AA])$. The f_a values were extrapolated from the plot of $F_0/(\Delta F)$ (Supplementary Figs. S4C and D).

◆ Pb 0.05.
** Pb 0.01.

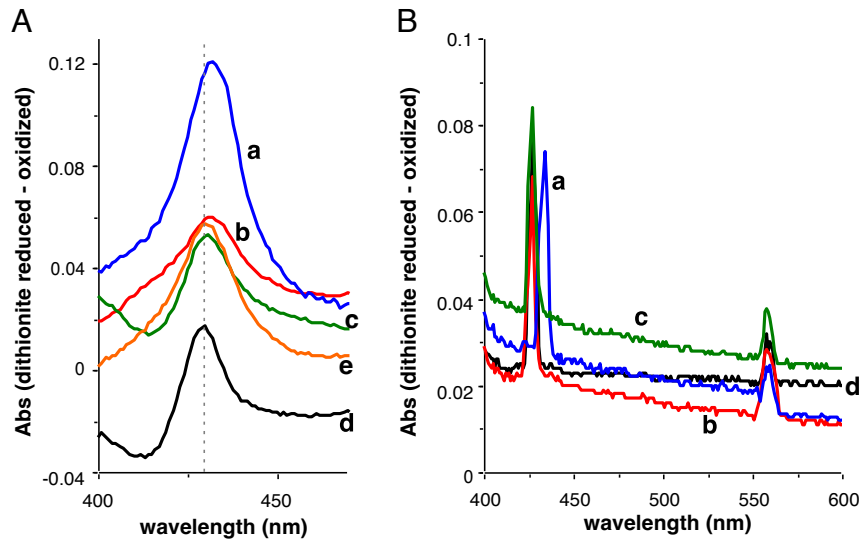


Fig. 7. Effect of arachidonic acid isomers and tert-butyl isocyanide on the dithionite-reduced minus oxidized absorption spectra of membrane fraction. Yeast (panel A) or bovine (panel B) membrane fractions supplemented with 600 μ M (yeast) or 200 μ M (bovine) *cis*-AA (a, blue), *trans*-AA (b, red) or *me*-AA (c, green) were suspended in 200 μ L phosphate buffer and incubated at 25 $^{\circ}$ C for 5 min. The dithionite reduced membranes were treated with tert-butyl-isocyanide as indicated in the Materials and methods section. Control spectra were performed in the absence of arachidonic acid isomers and with (e, orange) or without (d, black) tert-butyl-isocyanide. Results are representative of at least three separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the anionic part, thereby impeding the formation of the active form of cytosolic proteins, the effect of *trans*-AA is more subtle. The absence of co-translocation to the membrane fraction in the presence of *trans*-AA can be attributed to the dramatic structural changes of p67^{phox}.

4.3. Effects on the membrane fraction

The effect of *trans*-AA isomers on the NADPH-oxidase membrane fraction is more complex. In contrast to *cis*-AA, neither *trans*-AA isoforms nor *me*-AA facilitates the accessibility of the hemes to tert-butyl-isocyanide as revealed by the unmodified dithionite reduced minus oxidized absorption spectra of *cytb*₅₅₈ (Fig. 7). It is likely that the spin transition does not occur correctly in the presence of *trans*-AA, supporting the hypothesis that the activation process of the NADPH-oxidase is dependent of the heme iron spin state.

As was previously demonstrated the change of the natural geometrical configuration of lipid unsaturations could also affect NADPH-oxidase activity by modifying basic membrane physical properties [65]. This indirect effect of AA isomers depends on membrane

lipid composition since the NADPH oxidase activity could be modified by the constitution of the lipid environment [41,69].

Indeed we found that the optimal *cis*-AA concentration for the oxidase activity is different depending on the source of *Cytb*₅₅₈ (bovine vs. yeast membrane fraction), which present different lipid compositions and *Cytb*₅₅₈/total protein ratios.

At one hand, yeast membranes contain lower proportion of *Cytb*₅₅₈ compared to neutrophil membranes (0.4% vs 7% of total membrane proteins, respectively). Since activation experiments were performed using similar amounts of *Cytb*₅₅₈, yeast membrane assays contribute by higher quantities of membrane lipids into the enzymatic reaction mixture than neutrophil assays. The consequence is that the added arachidonic acid was distributed proportionally to the amount of lipid content leading to a decrease of the effective concentration of *cis*-AA for yeast membranes compared to neutrophils.

It is known that the difference of fatty acid is due to the very different lipid biosynthesis pathways in yeast and mammalian. However, if a strictly biophysical comparison is concerned, the results also indicate that saturated fatty acids in yeast contribute to a membrane environment with properties such as permeability and fluidity

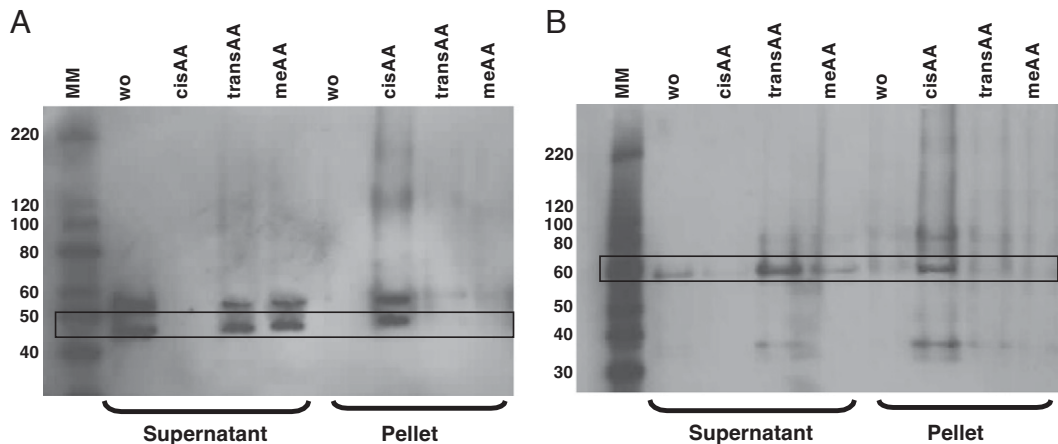


Fig. 8. Effect of arachidonic acid isomers on NADPH-oxidase complex assembly. Recombinant *Cytb*₅₅₈-enriched yeast membrane fraction, p47^{phox}, p67^{phox} and RacQ61L were pre-incubated together 5 min in the absence (w/o) or in the presence of *cis* (*cis*-AA), *trans* (*trans*-AA) or methyl ester (*me*-AA) arachidonic acid. Samples were then centrifuged 30 min at 150,000 *g* and supernatant and pellet were separated. SDS-PAGE and western blotting were performed using anti p47^{phox} (A) or anti p67^{phox} (B) polyclonal antibodies. Molecular weight markers (MM) were seeded as indicated.

significantly lower compared to neutrophil membranes. In addition, yeast membranes contain ergosterol instead of cholesterol present in mammal cells that leads to a more condensed and thicker membrane and lipid domain formation as computed by molecular dynamics simulation studies [70]. Taken as a whole, the different membrane properties furnish here a coherent explanation to the differences observed for the optimal fatty acid concentrations that have to be added to activate the complex. Higher concentration of arachidonic acid is necessary to activate the highly ordered yeast membrane fraction, rich of saturated fatty acids, than to activate bovine membrane fraction. However it is still not clear how *cis*-AA activates the NADPH oxidase complex at a concentration above its critical micellar concentration (60 μ M; [71]). Taken as a whole, our results will need additional studies to separate direct effects on *cytb*₅₅₈ from effects on membrane properties.

Further studies should be performed to better understand the role of different fatty acid compositions, which can vary also in correlation with dietary intakes. The relative abundance of *cis* and *trans* arachidonic acid isomers has been described in models, such as cells and animals, as well as in human with pathological conditions and the relative abundance of *cis*-AA and its mono-*trans* isomers can vary from model to model. For example, in rat erythrocytes fed a *trans*-free diet it was found to be ca. 2% (0.4% relative to *cis*-AA). The presence of *trans*-AA in humans has been reported in the case of dermatological diseases (around 1% relative to *cis*-AA in [72]) or smokers [73], however an extrapolation to the in vivo impairment of NADPH oxidase activity is premature.

5. Conclusion

Taken as a whole, our results underlined the importance of the chemical characteristics of the activating molecule and especially its *cis*-*trans* configuration for the activation of the NADPH oxidase components. The role of AA and its geometrical transformations under cellular stress deserves further investigations taking into account that, from the effects found on the NADPH oxidase, this isomerization appears to contribute to a down regulation of oxidative stress by lowering the superoxide production. The nature of fatty acids ingested in food might thus have consequences at the level of non-specific immune defenses, which could be considered for potential pharmacological applications. It has been reported that free radical *cis/trans* isomerization of membrane lipids is connected with inflammation and allergy process [72–75]. Our results (if extrapolable to in vivo conditions) suggest that the *trans* fatty acids would act on these processes also by other mechanisms (at the level of membrane protein regulation). It is likely that the massive production of free radicals generated by the activated NADPH oxidase could contribute to the in situ concentration increase of *trans*-AA isomers and this aspect is worth further in vivo investigations.

Overall, the present work intends to highlight the importance of the lipid geometry in living systems, which can be considered either as a signal or damage, and to stimulate further interest for this relevant molecular aspect.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamem.2012.04.018>.

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