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Polyunsaturated fatty acids modulate NOX 4 anion superoxide production in human fibroblasts

ADRIEN ROSSARY^{*}, KHELIFA ARAB[†], JEAN-PAUL STEGHENS^{*}

Corresponding author:

Dr Jean-Paul Steghens

UF 21455 Stress Oxydant et Vitamines

Fédération de Biochimie

Hôpital E. Herriot

5, Place d'Arsonval

F-69437 Lyon France

Tel. : +33 4 72 11 06 23

Fax.: +33 4 72 11 06 75

Email. : jean-paul.steghens@chu-lyon.fr

* UF 21455, Oxidative Stress and Vitamins, Biochemistry Federation, E. Herriot Hospital, Lyon, France and EA 3090, Claude Bernard University Lyon 1, Lyon, France.

† Division of Toxicology and Cancer Risk Factors, German Cancer Research Center (DKFZ), Neuenheimer Feld 280, 69120 Heidelberg, Germany.

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Abbreviations :

ROS: reactive oxygen species; NOX: NADPH oxidase; DUOX: dual oxidase; PUFA: polyunsaturated fatty acid; DPI: diphenyl iodonium; SOD: super oxide dismutase; XO: xanthine oxidase; AA: arachidonic acid (20:4 n-6); DHA: docosahexaenoic acid (22:6 n-3); DHA met: docosahexaenoic methyl ester ; CLA: conjugated linoleic acid (18:2 [9Z, 11E]); EPA: eicosapentaenoic acid (20:5n-3); HE: hydroethidine; E⁺: ethidium; E_{OH}⁺: hydroxyethidium; ESI⁺: positive electrospray ionization mode; TIC: total ion current; qPCR: quantitative PCR.

Summary

The strong reactive oxygen species (ROS) production, part of an antioxidant response of human fibroblasts triggered by docosahexaenoic acid, served as a model for deciphering the relative contribution of NADPH oxidase (NOX) to ROS production, as the role of this enzymatic system remains controversial. Using hydroxyethidium fluorescence for fibroblast ROS production, RT-PCR for NOX 4 mRNA quantification, and mRNA silencing, we show that ROS production evolves in parallel with the catalytic activity of NOX and is suppressed by siNOX 4 silencing. Apocynin and plumbagin, specific inhibitors of NOX, prevent ROS production in this cellular model and confirm the role of NOX 4 for this production. Furthermore we show that, in cell lysates, NOX 4 activity can be modulated by polyunsaturated fatty acids (PUFAs) at the micro molar level in the presence of calcium: NOX 4 activity is increased by arachidonic acid (around 175% of control), and conjugated linoleic acid is a potent inhibitor (50% of control). Unexpectedly, intracellular superoxide dismutase (SOD) does not participate in the modulation of this ROS production and the opposite effects of some PUFA, described in our experiments, could suggest another way of regulating NOX activity.

Introduction

Several novel enzymes, homologs of gp91^{phox}, the catalytic unit of the protein complex responsible for the oxidative burst in polymorphonuclear cells, have been identified in mammals [1-4]. The identification of new members of this family of enzymes has been an important step in understanding the role of reactive oxygen species (ROS) in the regulation of many biological processes, although their detailed involvement has not yet been fully defined. They are able to produce O₂⁻, which more and more seems implicated in cell signal transduction and communications [5]. For instance, NADPH oxidases (NOXs) may serve as an oxygen sensor [6], may be required for cell proliferation [7, 8], may participate in vascular reactivity or smooth muscle contractility [9, 10], regulate the expression of some proteins [11, 12], and play a role in signal transduction [13]. NOXs and Dual oxidases (DUOX) generate ROS in a regulated manner in response to different signals such as growth factors, cytokines, calcium, etc. Surprisingly, overproduction of ROS due to NOX dysfunction is now suggested to be associated with several diseases, leading to increased bioavailability of ROS and subsequent oxidative damage [14-17].

Recently we described the induction of glutathione synthesis by human fibroblasts triggered with docosahexaenoic methyl ester (DHA met) [18]. The antioxidant response enhanced by human fibroblasts triggered with DHA met was associated with strong production of ROS. While fatty acids are frequently described as potential NOX activators in endothelial cells or neutrophils [19-23], and more and more data report on NOX activity in fibroblasts [24-27], few results confirm the effect of fatty acids on fibroblasts and the type of NOX homolog involved in this ROS production. We were therefore interested in deciphering the relative contribution of NOX to ROS production in our cellular model triggered by polyunsaturated fatty acids (PUFAs).

Materials and methods

Chemicals

Chemicals were obtained as follows: diphenyl iodonium (DPI), super oxide dismutase (SOD), xanthine, plumbagin and apocynin from Sigma-Aldrich, (Sigma-Aldrich, Saint Quentin Fallavier, 38297, France); arachidonic acid (AA, 20:4 n-6), docosahexaenoic acid (DHA, 22:6 n-3) and methyl ester (DHA met), conjugated linoleic acid (CLA, 18:2 [9Z, 11E]), eicosapentaenoic acid (EPA, 20:5n-3), from Cayman Chemical (Interchim, Montluçon, 03103, France); xanthine oxidase (XO), NADPH from Roche (Roche, Meylan, 38242, France); hydroethidine (HE) from Fluoprobe (Interchim, Montluçon, 03103, France). All other reagents were of analytical grade. All concentrations are final concentrations in incubation or culture medium.

Cell culture

Human dermal fibroblasts, a gift from Dr Damour, were grown in DMEM containing 10% fetal calf serum (Gibco, Pontoise, 95613, France), fungizone 2.5 µg/ml, penicillin 98 U/ml and streptomycin 98 µg/ml (Cambrex, Emerainville, 77184, France). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂. Cells were harvested after trypsinisation and three phosphate buffer saline (PBS) washes. The total cell lysates were obtained by two successive thawing-freezing cycles in Tris HCl 25 mM buffer (buffer A) pH 7.4 containing Tween 20 0.1%, with 15-second periods in an ultra-sound bath. The total cell lysates were stored at -80°C until analysis.

Protein measurement

Proteins were quantified by the bicinchonic acid method (BCA from Pierce, Interchim, Montluçon, 03103, France) according to the manufacturer's instructions.

HPLC analysis of hydroxyethidium (E₂OH⁺)

Hydroethidine (HE), ethidium (E⁺) and hydroxyethidium (E₂OH⁺), the oxidation product of HE by O₂⁻, were separated by HPLC according to a method modified from Zhao *et al.* [28]. The HPLC system (Agilent HP 1100, Agilent technologies, Massy, 91745, France) was equipped with a fluorescence (Kontron SFM25, Paris 75012, France), or UV (Thermo Spectra system UV 6000 LP, Thermo Electron Corporation, Cergy-Pontoise,

95615, France), or mass spectrometer (Agilent LC/MSD SL) single quadrupole detector. The mobile phase was ammonium acetate 15 mM, adjusted to pH 4 with acetic acid and 35% (v/v) of methanol. The stationary phase was a C18 reverse-phase column (Atlantis™, 2.1X150 mm, dC18, 5µm, Waters SAS, Guyancourt, 78280, France).

Samples were positive controls of superoxide anion production obtained by incubation at 37°C of xanthine (500 µM) and xanthine oxidase (0.2 UI/ml) or cells and cell culture medium. One sample volume was mixed with 2 volumes of methanol chilled on ice and then centrifuged before injection. HE, E⁺, and E_OH⁺ were separated by gradient analysis with a linear increase in methanol concentration from 35% to 90% over 13 min at a flow rate of 0.2 ml/min, with a 5 µl injection volume. In a first step, the elution was monitored by a variable UV detector at 284 and 360 nm and a fluorescence detector with excitation and emission wavelengths at 465 nm and 575 nm, respectively. For the mass spectrometer detector, detection was optimized in a positive electrospray ionization mode (ESI⁺) with E_OH⁺ and set at 350°C (drying gas), fragmentor at 175 V and capillary voltage at 3 kV. Chromatograms were recorded as total ion current (TIC) and in single ion monitoring mode at $m/z^+ = 314$ for ethidium, 330 for E_OH⁺, and 301 for the main fragment obtained after cleavage of E_OH⁺ [28] as a control. HPLC peak areas were normalized to protein concentration.

Cell culture and measurements on fibroblasts grown with DHA methyl ester or other PUFAs

Fibroblasts were grown either for 4, 8, 24 or 48 hours in DMEM with 15 µM of DHA methyl ester (final concentration) or for 4 hours with AA, CLA and EPA at the same concentration.

In order to confirm the implication of NOX in ROS production, fibroblasts were grown in the presence of apocynin 2.5 µM or plumbagin 1 µM or DPI 4 µM, with and without DHA methyl ester 15 µM. Culture controls were carried out with ethanol and DMSO in the culture medium.

For each culture condition, O₂⁻ production was measured either by LC/MS or by flow cytometry on intact cells. mRNA quantification by quantitative PCR were carried out on total cell lysates.

SOD activity

SOD activity was measured by a colorimetric assay, the RANSOD kit (RANDOX laboratories Ltd., Montpellier Fréjorgues, 34131, France); $O_2^{\cdot-}$, generated by a xanthine/XO reaction, oxidizes 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) into a red formazan, and this oxidation is inhibited by SOD. Measurements were performed according to the manufacturer's instructions on a Delta Kone automatic analyser.

mRNA quantification by quantitative PCR

Total RNA was isolated from fibroblasts by the total RNA isolation kit (Rneasy® mini kit from Qiagen, Courtaboeuf, 91974, France). Reverse transcription was performed with 1 µg of total RNA for each condition with a first-strand cDNA synthesis kit (Amersham, Orsay, 91898, France). Quantitative PCR (qPCR) was carried out on a Light Cycler® (Roche) by normalizing all cDNA to β actin.

Sequences and fragment sizes of human-specific primers used for β actin, NOX 1, 2, 4, p22^{phox}, and SOD 1 are shown in table I.

Silencing of NOX 4

To confirm the involvement of NOX expression in DHA treatment, we inhibited NOX 4 expression with small interfering RNA (siRNA). Twenty-one nucleotide doublestranded siRNAs (from Qiagen HP genome wide siRNA databank, cat number : SI00117663 www.qiagen.com; 5 nM), targeting the NOX 4 sequence, were used. Nonsilencing fluorescein-labeled control (5 nM) was used as the negative siRNA control (scrambled siRNA).

SiRNA transfection to fibroblasts was performed according to the manufacturer's instructions and was monitored with the control siRNA labeled with fluorescein (scrambled siRNA). Quantitative RT-PCR was performed to compare NOX 4 mRNA degradation in the presence and absence of siNOX 4.

After 36 hours silencing, fibroblasts were triggered by DHA for 4 hours and cellular ROS production on whole cells was performed by flow cytometry.

Evaluation of cellular ROS production by flow cytometry

In order to assess the production of ROS and superoxide anion, fibroblasts were loaded with hydroethidine (HE). After 0, 4, 8, 24, and 48 hours of culture, cells were harvested

and washed twice with PBS, incubated with 2 μM of HE in PBS at 37°C for 30 min. Then the cell suspension was submitted to fluorescent flow cytometry analysis (FACScan flow cytometer, BD Biosciences) on a log scale for 10,000 events (cell counts). The oxidation of HE was measured, with the excitation set at 488 nm, as an increase in fluorescence at 585.

Statistical analysis

Statistical analysis was carried out by GraphPad analysis (version 2.1). All data are expressed as means \pm SE. Comparisons among groups were performed by the Student's *t*-test or one-way ANOVA, with Dunnett's multiple range tests. The significance level was 0.05.

Results

Effects of inhibitors and fatty acids on whole cell fluorescence at 4 hours

We investigated the fluorescence of ROS production of fibroblasts triggered for 4 hours with DPI (data not shown), apocynin, plumbagin and/or with fatty acids. The vehicle controls - DMSO for DPI, ethanol for apocynin, plumbagin and DHA met - had no significant effect on ROS production. DPI increased production of ROS at 4 hours to $143 \pm 4\%$ of control, and this increase was cumulative with that due to DHA, reaching $185 \pm 1\%$.

On the other hand, E_{OH^+} fluorescence induced by DHA alone was $165 \pm 18\%$ of control and that of the inhibitors, alone or with DHA, was between 104 and 112 % of control (fig 1A).

The effects of fatty acids on fluorescence produced by whole cells (through NOX activity) are shown on fig 1B: both DHA and EPA significantly increased cell fluorescence ($148 \pm 23\%$ and $143 \pm 16\%$) and only AA increased signal up to $202 \pm 10\%$. Fluorescence of fibroblasts triggered by CLA for 4 hours was lower than of control, but not significantly.

HPLC analysis of hydroxyethidium (E_{OH^+})

As described by Zhao *et al.* [28], hydroethidine (HE) is known to produce a specific oxidation product, named hydroxyethidium, in the presence of superoxide anion. In order to demonstrate the production of superoxide by fibroblasts triggered by fatty acids,

we performed LC/MS analysis for hydroxyethidium on different samples including culture media and cells.

As a positive control for superoxide anion production, we used a xanthine-xanthine oxidase assay for which typical chromatograms are shown in figure 2A. The TIC chromatogram is shown on the upper graph, and specific ion current chromatograms are displayed for m/z^+ 314 = E⁺ (fig 2A2), 330 = E_{OH}⁺ (fig 2A3), and 301, the main fragment of 330 (fig 2A4). Obviously m/z^+ 330 and 301 have the same retention time (RT) which is different of that both of HE (RT = 11.04) and E⁺ (RT = 8.85). Figure 2B shows similar chromatograms obtained for fibroblasts triggered for 2 hours with AA 15 μ M and HE 5 μ M. Figure 2C displays the results (mean of triplicate) for culture media, in the same conditions, with a 2.4-fold increase in E_{OH}⁺ production and a production similar to ethanol control if SOD (15UI/ml) is added. These experiments are a further confirmation of superoxide anion production by NOX of fibroblasts triggered by different PUFAs.

Time course analysis of cell culture with DHA methyl ester

We grew human fibroblasts with 15 μ M of DHA methyl ester (DHA met) up to 48 hours. Fluorescent measurements of O₂⁻ production were performed at 0, 4, 8, 24, and 48 hours. At the same time points, different mRNAs were quantified by quantitative RT-PCR.

During this time period, neither SOD 1 mRNA expression nor total SOD catalytic activity were significantly different from control (data not shown).

O₂⁻ production measured by fluorescence of E_{OH}⁺ (fig 3A) peaked at 4 hours (174 \pm 28%) and then decreased until 48 hours. Cell culture controls (fibroblasts at time 0 and fibroblasts grown with ethanol for 48 hours) were not significantly different from each other.

Quantification of mRNA for NOX 4 and p22^{phox}

First, we investigated the mRNA expression of the different NOX homologs (NOX 1, 2 and 4) in human fibroblasts by agarose gel electrophoresis and RT-PCR. Amplification was detected only with NOX 4 and p22^{phox} primers on fibroblasts whereas NOX 2 primers were able to detect mRNA expression in white blood cells as a control.

Second, the time course of mRNA expression for NOX 4 showed a significant decrease at 48 hours (fig 3B). During the same time period, p22^{phox} mRNA expression decreased

gradually (fig 3C), and the absence of mRNA expression for NOX 1 and 2 was confirmed.

Silencing of NOX 4

To confirm the involvement of NOX 4 expression in response to DHA treatment, we inhibited NOX 4 expression with small interfering RNA (siRNA).

After 36 hours silencing, quantitative RT-PCRs were performed to compare NOX 4 mRNA degradation in the presence and absence of siNOX 4 (fig 4A). Expression remained only 6% of basal level.

Fibroblasts treated by siNOX 4 were triggered by DHA for 4 hours. Neither NOX catalytic activity on total cell lysates (data not shown) nor fluorescent ROS production performed on whole cells (fig 4B) showed any activation under DHA and siNOX 4.

Discussion

NOXs, cellular sources of reactive oxygen species, use O_2 and NADPH as substrates and FAD as coenzyme. As sources of ROS, they are detectable by lucigenin luminescence, a fast tool for screening of NOX catalytic activity. However lucigenin specificity is often questioned and we were strongly advised to use hydroxyethidium analysis either by fluorescence or LC/MS for $O_2^{\cdot-}$ production. NOXs, particularly NOX 1 and 2, are claimed to be activated by some fatty acids [19-23]. In human fibroblasts, Dhaunsi *et al.* reported the effect of only very long-chain fatty acids on NOX 2 activity [24]. In our model, with fibroblasts grown with DHA met (22:6 n-3), an increase in ROS production at 4 hours was observed [18]. This model therefore seemed appropriate for deciphering the relative contribution of NOX isoenzymes to this ROS production and for investigating the potential modulation of this catalytic activity by fatty acids.

Preliminary results showed that ROS production obtained with cell lysates incubated with NADPH was totally inhibited by DPI (a well known NOX inhibitor acting on FAD coenzyme) and with added SOD, the enzyme responsible for physiological $O_2^{\cdot-}$ degradation. As demonstrated by nitrogen bubbling, this signal was also dependent on O_2 . The latter inhibition was within a very close range of those obtained with DPI or SOD, suggesting that a unique catalytic activity, dependent on a FAD coenzyme, required NADPH and O_2 for $O_2^{\cdot-}$ production, as these ROS were SOD-sensitive.

Moreover, inhibition was in the same range for specific NOX inhibitors (plumbagin [29] and apocynin) as well as for non specific inhibitors (DPI or SOD, specific for $O_2^{\cdot-}$ but not for NOX). These results strongly suggested that this ROS production was due essentially to NOX activity.

To confirm this hypothesis, we investigated the action of NOX inhibitors such as apocynin, plumbagin or DPI on whole cells triggered by DHA. As shown in figure 1A, apocynin, a specific NOX inhibitor which prevents NOX subunit assembly, totally inhibited ROS production under DHA, thus confirming the participation of NOX in the production of ROS at 4 hours. Moreover, plumbagin, described as a NOX 4 inhibitor [29], also totally inhibited ROS production under DHA, thus defining NOX 4 as the NOX homolog of our fibroblasts.

Unlike apocynin, DPI acts by covalent binding to FAD; in this manner, it is a general inhibitor of all enzymes requiring FAD as a coenzyme. As described in the literature [30, 31], DPI induces oxidative stress and apoptosis via mitochondrial $O_2^{\cdot-}$ production. Our results confirmed these observations, and also that DPI, active on many enzymes *ex vivo*, is not relevant for the investigation of NOX activity in intact cells.

Quantification by RT-PCR of mRNA for NOXs confirmed that mRNA for NOX 4 was the only NOX homolog mRNA present to explain ROS production. After 36 hours silencing with siRNA for NOX 4, quantitative RT-PCR showed NOX 4 mRNA degradation (fig 4A). Furthermore fibroblasts silenced by siNOX 4 (for 36 hours) and triggered by DHA for 4 hours displayed no cellular ROS production (fig 4B) unlike fibroblasts silenced by the scrambled siRNA. Associated with the fact that NOX catalytic activity measurement [32] and E_{OH^+} fluorescence on whole cells show a similar pattern, these results strongly confirm the role of NOX 4 as the only NOX isoenzyme for ROS production in human fibroblasts and are in agreement with very recent publications on fibroblasts [25,27;33].

Lipids in general, and AA in particular, are known to interact with NOX in phagocytic and nonphagocytic cells [19-24], and a recent publication has implicated very long-chain fatty acids in the activation of NOX in some peroxisomal disorders of human dermal fibroblasts [24]. In a previous publication [32], we investigated the effect of DHA, free or as a methyl ester, and of arachidonic acid (AA) on fibroblast extracts: in our experiments, neither calcium, described as a NOX 5 activator [34], nor arachidonic acid (AA) alone, known as a gp91^{phox} activator [21, 22], had any effect on NOX catalytic activity of fibroblast lysates. However AA associated with calcium strongly increased

NOX activity (175% of control); this result recalls the work of Xiao-Lan Cui *et al* [19]. According to these authors, AA activates the c-jun N-terminal kinase through NOX in rabbit proximal tubular epithelial cells. In our model, NOX catalytic activity was due to NOX 4, as demonstrated by RT-PCR. In agreement with previous results on Renox, the first name of NOX 4 [19, 21], NOX 4 was responsive to arachidonic acid and required Ca^{2+} mobilization.

Unexpectedly, neither DHA (free or as a methyl ester) nor EPA, both with calcium, activated NOX on cell lysates [32], whereas they strongly induced NOX activity on whole fibroblasts triggered for 4 hours (fig 1B). Very recently we showed that, in fibroblasts triggered by DHA met, DHA increased threefold and induced a profound change in total cell lipid composition [18] with a low cellular AA increase. These results strongly suggest that, when PUFA induce a huge O_2^- production, it could be due to release of AA by membranes, with subsequent NOX activation. Thus, this increase in O_2^- production in the first 4 hours could play the role of an intracellular messenger, a role already suggested in the model by Xia-Lan Cui *et al.* in 1997 [19] after AA activation and more recently by Colston *et al.* in 2005 [25]. A general mechanism involving AA and calcium should be in agreement with the results of Bouzidi *et al.* [35], who reported this association as a NOX activator with an effect mediated by the myeloid-related proteins (S100A8/A9) which bind both calcium and AA. Furthermore, the hypothesis that AA is released from membranes could explain the similar activation of NOX obtained with many lipids: according to Rouhanizadeh *et al.* [23], “the specific mechanism(s) by which ox-PAPC induces NADPH oxidase activity and O_2^- production remain to be defined and the effect may be similar to that reported for lysophosphatidylcholine (LPS) which stimulates monocyte chemoattractant protein-1 (MCP-1) expression and O_2^- production by NADPH oxidase. Similar activation is also reported for other lysolipids such as phosphatidic acid and platelet-activating factors, which induce mitogenic signaling pathways.”

We already showed that fibroblasts triggered by DHA [18] and other PUFAs, except CLA [36], produce superoxide anion and activate an antioxidant response. Now we demonstrate that this ROS production is due to NOX 4 activation. During this antioxidant response, we were unable to detect any change either in mRNA SOD1 expression or total cellular catalytic SOD activity. For this reason, regulation of ROS production by cellular SOD seems to be excluded, at least during the relatively short

time periods of our experiments. However, during the antioxidant response triggered by different PUFAs [18, 36], we demonstrate the induction of HO-1, a typical enzyme of the antioxidant response as it contains some antioxidant responsive elements in its gene-promoter region. HO-1 produces CO through heme degradation and its induction strongly suggests that, apart from regulation at the transcriptional level [37] confirmed by a decrease of NOX 4 and p22^{phox} mRNA expression, a further factor of regulation for NOX activity, as suggested recently [32], could be through CO inhibition [38] or heme degradation [39].

As CLA is the only PUFA able to induce an antioxidant response (testified by glutathione synthesis up-regulation) without NOX 4 activation, our cellular model may be also useful to explore the different facets and steps leading to a “physiological” defense mechanism, the antioxidant response, and question the exact role of ROS for its signaling.

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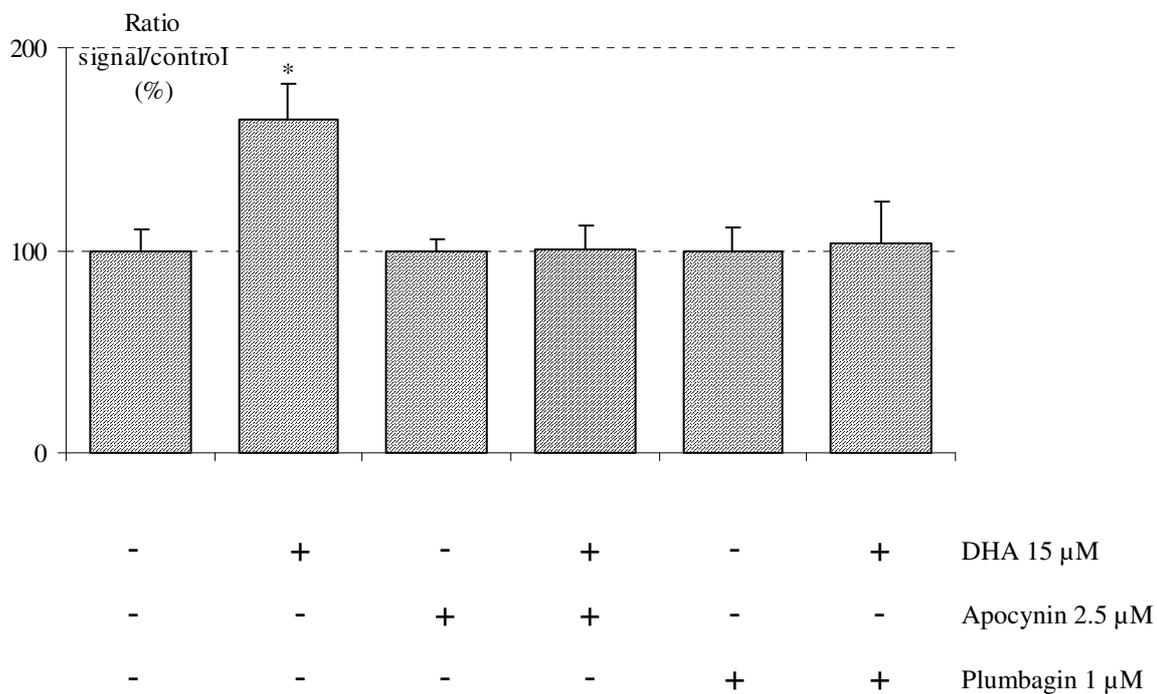
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Table 1

Name	Accession number	Primer sequences		Fragment length (pb)
β actin	NM_001101	Forward Reverse	5' -TCG-TGC-GTG-ACA-TTA-AGG-AG-3' 5' -AGC-ACT-GTG-TTG-GCG-TAC-AG-3'	262
NOX 1	AJ 438989	Forward Reverse	5' -TCG-AAC-AAC-AAT-ATT-CAC-CA-3' 5' -TGG-CCT-TGT-CAA-AGT-TTA-AT-3'	225
NOX 2	NM_000397	Forward Reverse	5' -AGA-GTT-CGA-AGA-CAA-CTG-GA-3' 5' -CCT-CCT-TCA-GGG-TTC-TTT-AT-3'	233
NOX 4	NM_016931	Forward Reverse	5' -CTT-TTG-GAA-GTC-CAT-TTG-AG-3' 5' -GTC-TGT-TCT-CTT-GCC-AAA-AC-3'	231
p22 ^{phox}	NM_000101	Forward Reverse	5' -CTT-CAC-CCA-GTG-GTA-CTT-T-3' 5' -CGA-ACA-TAG-TAA-TTC-CTG-GTA-3'	174
SOD 1	NM_000454	Forward Reverse	5' -AAG-TAC-AAA-GAC-AGG-AAA-CG-3' 5' -AGC-AAC-TCT-GAA-AAA-GTC-AC-3'	193

Figure 1

A: Effects of inhibitors



B: Effects of fatty acids (with calcium)

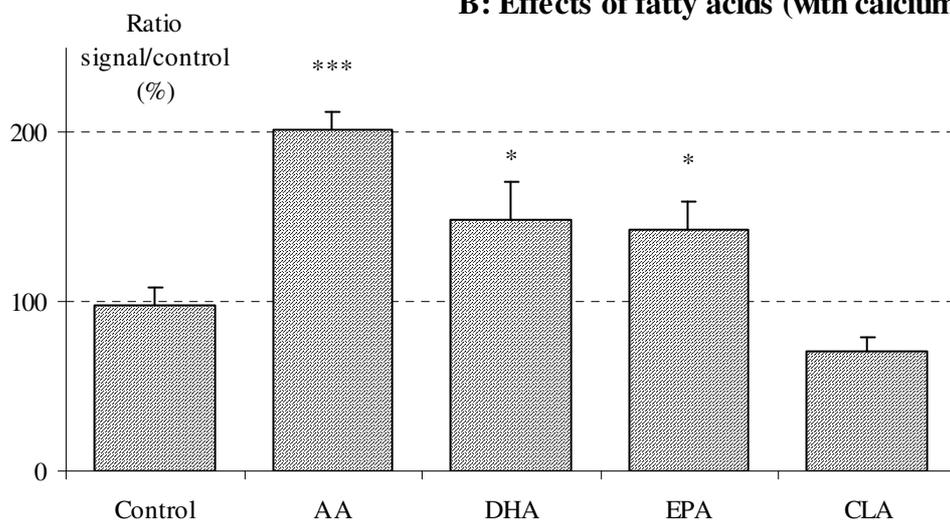
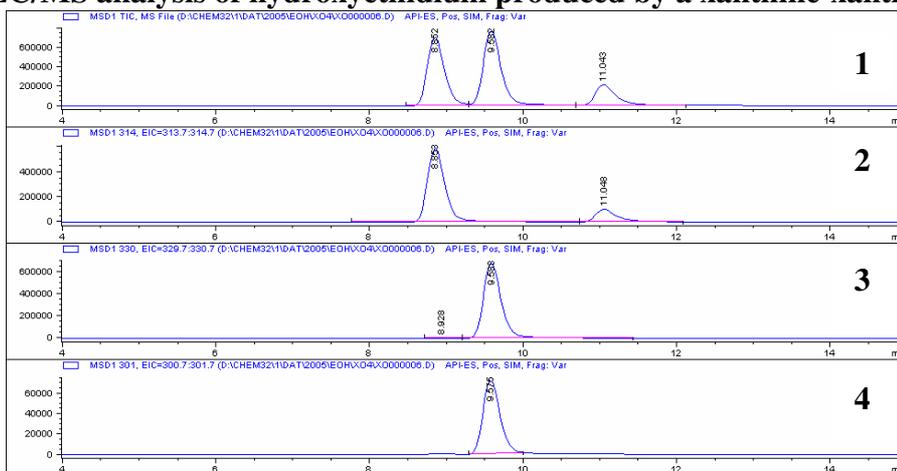
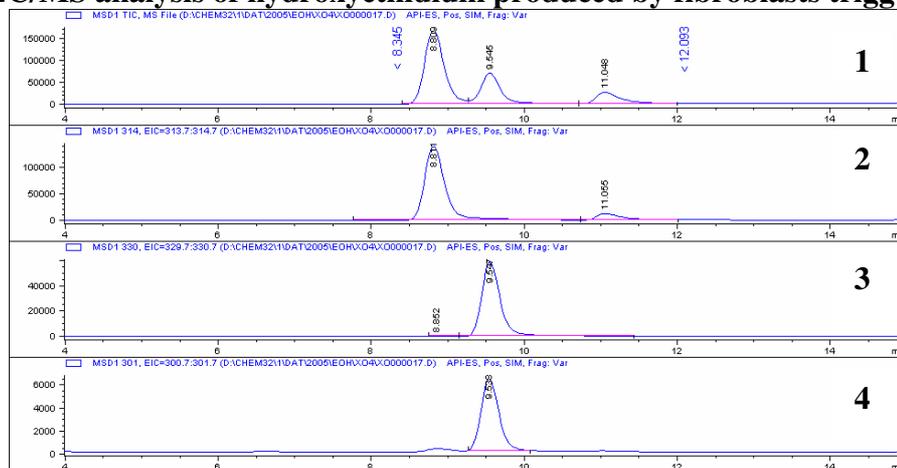


Figure 2

A : LC/MS analysis of hydroxyethidium produced by a xanthine-xanthine oxidase assay



B : LC/MS analysis of hydroxyethidium produced by fibroblasts triggered by AA



C : Hydroxyethidium produced in cell culture medium of fibroblasts triggered by AA

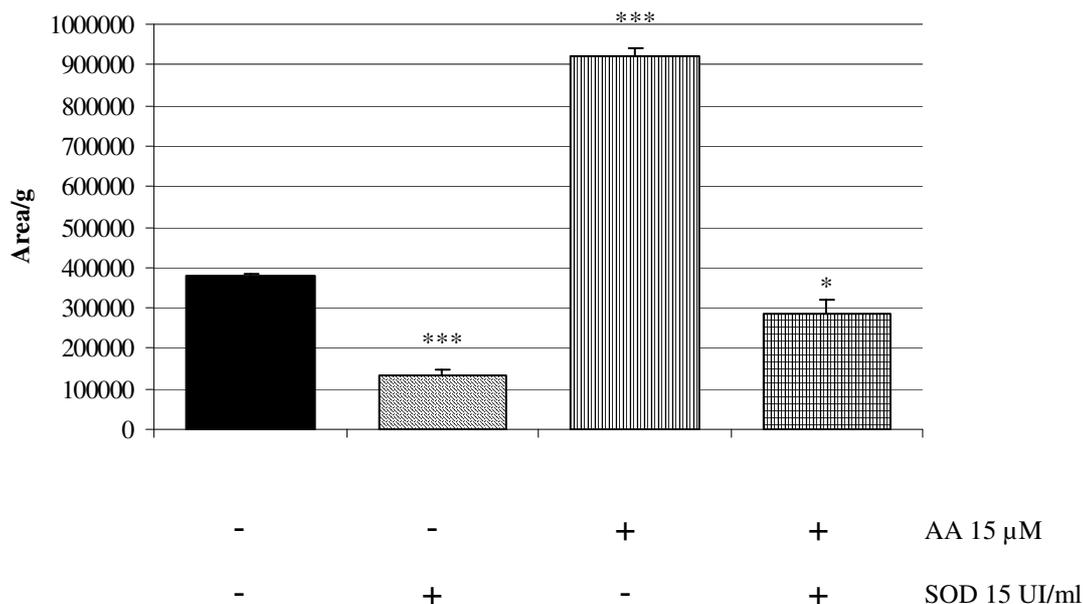


Figure 3

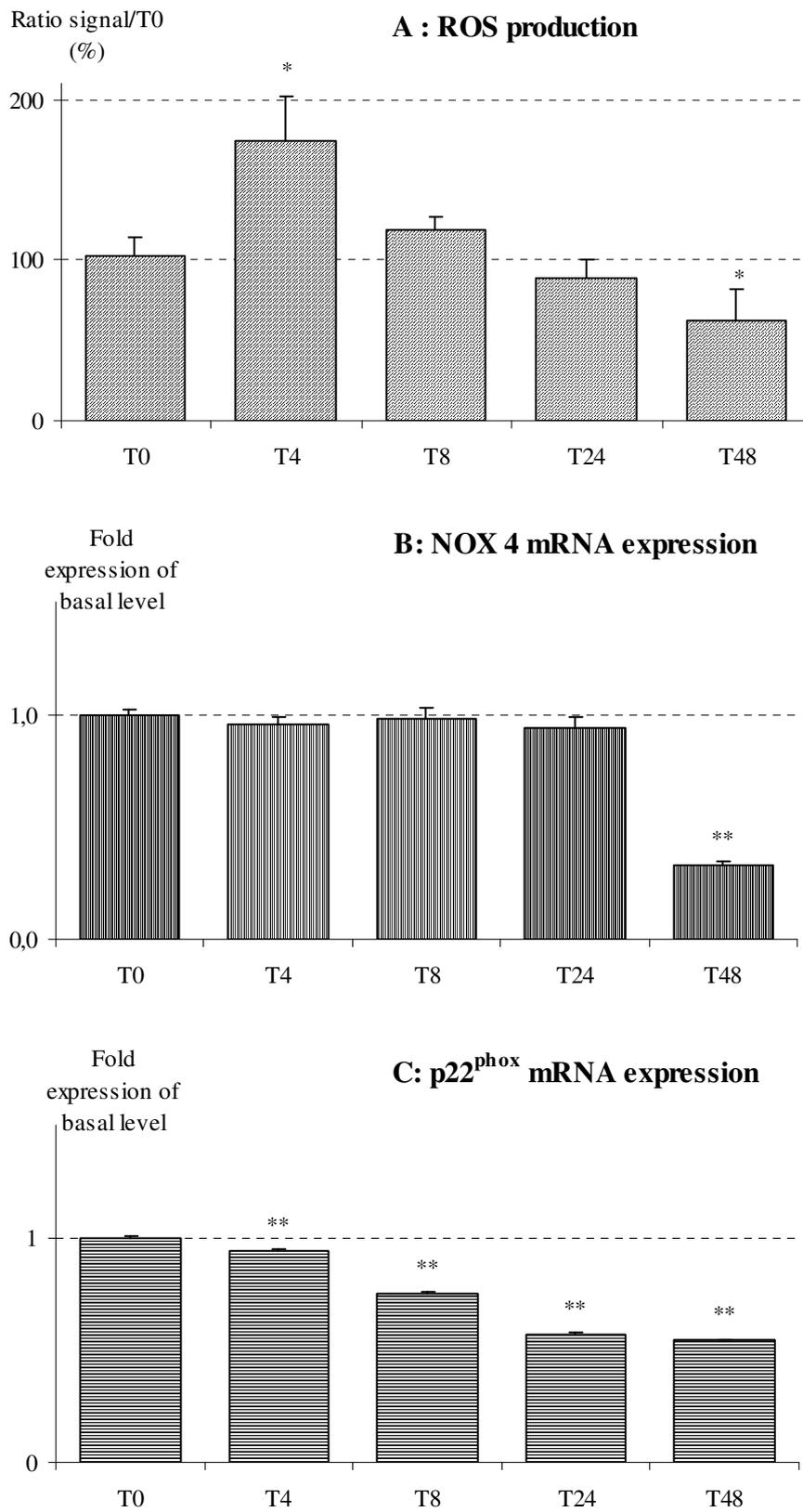
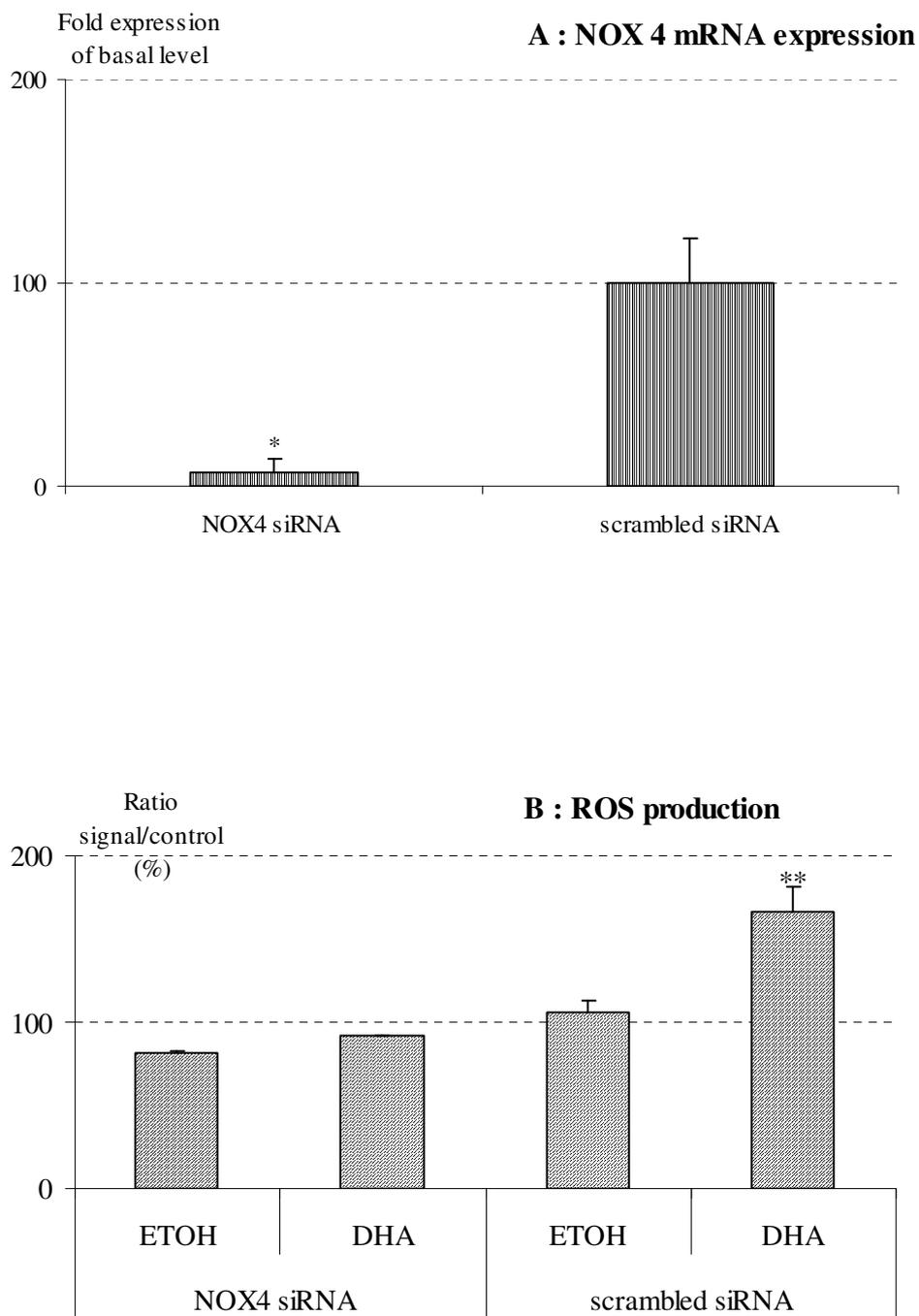


Figure 4



Legends :

Table I : Primer sequences used for qPCR.

Figure 1: Effects of specific inhibitors (A) and fatty acids (B) on whole cells (fibroblasts grown for 4 hours). Each bar corresponds to the mean of at least three assays (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

A: Apocynin or plumbagin inhibition of the fluorescence emitted by fibroblasts triggered by DHA. Controls correspond to fibroblasts grown for 4 hours with ethanol (negative), DHA (positive) or inhibitor alone.

B: Enhancement of fluorescence of fibroblasts triggered with 15 μM of AA, DHA or EPA, for 4 hours. Unlike other PUFAs, CLA decreases the fluorescence. Control corresponds to fibroblasts triggered with ethanol for 4 hours.

Figure 2: LC/MS analysis of hydroxyethidium produced from O_2^- with hydroethidine.

A and B: chromatograms of a xanthine–xanthine oxidase assay and of a total cell lysate, respectively.

Total ion current chromatograms are shown in A1 and B1. Specific chromatograms are displayed at $m/z^+ = 314$ for ethidium (at $\text{RT} = 8.8$) in A2 and B2, at $m/z^+ = 330$ for hydroxyethidium (at $\text{RT} = 9.6$) in A3 and B3 and at $m/z^+ = 301$ for the main fragment of hydroxyethidium (at $\text{RT} = 9.6$) in A4 and B4.

C: hydroxyethidium production in cell culture medium by fibroblasts triggered for 2 hours by arachidonic acid at 15 μM , with or without SOD at 15 UI/ml. Each bar is the mean of triplicates (* $p < 0.05$, *** $p < 0.001$).

Figure 3: Time course of ROS production (A) and mRNA expression (B,C) in fibroblasts grown with DHA methyl ester 15 μM , for 48 hours. Ethanol is the vehicle control at 48 hours. All data are expressed as a ratio with the reference at time 0. (* $p < 0.05$, ** $p < 0.01$).

A: Evaluation of Total ROS production by hydroxyethidium fluorescence.

B: NOX 4 mRNA expression.

C: p22^{phox} mRNA expression.

Figure 4: Silencing of NOX 4 in human fibroblasts.

A: Quantitative RT-PCR of NOX 4 mRNA after 36 hours silencing in presence and absence of siNOX 4 on fibroblasts.

B: ROS production measured by hydroxyethidium on whole cells triggered by DHA for 4 hours, after 36 hours silencing in presence and absence of siNOX 4.

Nonsilencing control (5 nM) was used as the negative siRNA control (scrambled siRNA). (* $p < 0.05$, ** $p < 0.01$).