

Activation of Human Neutrophils by Oleic Acid Involves the Production of Reactive Oxygen Species and a Rise in Cytosolic Calcium Concentration: a Comparison with N-6 Polyunsaturated Fatty Acids

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Key Words

Oleic acid • N-6 PUFA • ROS • Ca²⁺ signaling • Human neutrophils

Abstract

Background: There is a growing body of evidence showing that dietary constituents and lipids in particular, influence the function of the human immune system. However, although the beneficial effects of oleic acid (OA) are clear, its mechanism of action at the molecular level is poorly understood. **Aims:** To evaluate neutrophil activation under the influence of OA and compare this with several n-6 PUFAs. **Methods:** Two key aspects of neutrophil activation were investigated: oxygen radical (ROS) production and intracellular Ca²⁺ signaling. **Results:** OA and the n-6 PUFA arachidonic acid (AA) both induced ROS production in a dose-dependent manner, although AA was the more potent stimulus. When looking for the mechanisms behind these effects, we found that both FA induce increases in cytosolic calcium concentration ([Ca²⁺]_i), but whereas OA-induced ROS production is totally mediated through Ca²⁺ signaling, this is not the case for AA since ROS generation by AA is only partly inhibited in BAPTA-treated cells. We

also found evidence for the involvement of protein kinase C (PKC) in the OA-induced ROS generation; by contrast, other enzymes apart from PKC seem to be implicated in n-6 PUFA-induced ROS production. In addition, our results argue against the involvement of a pertussis toxin-sensitive receptor activated by OA. **Conclusions:** OA differs from the n-6 PUFA in the activation of human neutrophils and these differences may be related to their distinct immunomodulatory properties.

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Introduction

Approximately one-quarter of the total daily energy in the diet of inhabitants of developed countries is provided by fatty acids (FA). Such fatty acids can be either unsaturated or contain one or more double bonds typically positioned at the 3, 6, 7 or 9th carbon atom from the terminal methyl group [1]. There is growing evidence showing that dietary constituents influence the function of the human immune system, and clinical studies have suggested that lipids in particular may affect our defenses against microorganisms [2].

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In fact, epidemiologic and experimental studies show that changes in the source of lipid consumed in the diet may modify the FA composition of many cell types, including those involved in the development of many inflammatory and immune-mediated diseases [3-5].

Although in recent years there has been a great interest in the immune modulating effects of n-3 polyunsaturated fatty acids (PUFA), less attention has been paid to n-9 FA, such as oleic acid (OA).

The Mediterranean diet, rich in monounsaturated fatty acids (MUFA), especially OA from olive oil, has been associated with a lower risk of coronary heart disease [6, 7]. Olive oil may have favorable effects on lipid peroxidation and oxidative stress, immune and inflammatory responses relative to other frequently consumed oils. The consumption of diets rich in MUFA has also been linked to a low prevalence of atherosclerosis [8]. It has been suggested that FA of the n-9 series derived from the OA might play an important role in immunomodulatory processes [9, 10].

In addition, regarding intravenous (parenteral) nutrition, concerns around the potential adverse effects of a 100% soybean oil-based emulsion, have led to the development of lipid emulsions that replace some of the PUFAs with alternative oil sources, including olive oil [11].

The beneficial properties of olive oil often have been attributed to its high MUFA content, in the form of OA (C18:1 (n-9)) [12]. Nevertheless, although its beneficial effects can no longer be doubted, little is known about the mechanisms by which the FA elicit their effects, or of the signal transduction systems that are involved herein.

Neutrophils are the first cells that migrate to tissues in response to invading microorganisms. The antimicrobial function of these phagocytes depends on the release of lytic enzymes stored in cytoplasmic granules and on the production of reactive oxygen species (ROS) [1]. A number of metabolic changes occur when polymorfonuclear leukocytes are challenged with certain stimuli. Among others, these changes involve the generation of oxygen radicals [13], depolarization of the membrane potential [14], activation of phospholipase A₂ (PLA₂) and C (PLC) [15, 16]. Activation of neutrophils due to various stimuli has been thought to be mediated by an increase in the amount of intracellular calcium [17-19].

The above-mentioned information led us to investigate the effect of the C18 MUFA oleic acid on human neutrophil function. We designed the present study to evaluate neutrophil activation under the influence of OA and compare this with n-6 FA, which are widely

described as proinflammatory mediators in concentrations that are physiologically relevant. Two key aspects of neutrophils activation were investigated: oxygen radical production and intracellular calcium signaling.

Materials and Methods

Subjects

Informed consent from all volunteers was obtained and the human experimentation guidelines of the author's institution were followed in the conduct of this study. After overnight fasting, blood samples drawn from healthy volunteers, none of whom was on medication, were collected and processed as described below.

Reagents

Fura-2/AM was obtained from Fluka. Thapsigargin, oleic acid, linoleic acid, linolenic acid, arachidonic acid, 1,2-bis(o-aminophenoxy)-ethane-N,N,N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM), N-formyl-methionyl-leucyl-phenylalanine (fMLP), pertussis toxin, staurosporine, bisindolylmaleimide I, Phorbol 12-myristate 13-acetate (PMA), 1-[6-[[[(17 β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), aspirin, nordihydroguaiaretic acid (NDGA) and 2',7'-dichlorofluorescein diacetate (DCFDA) were purchased from Sigma.

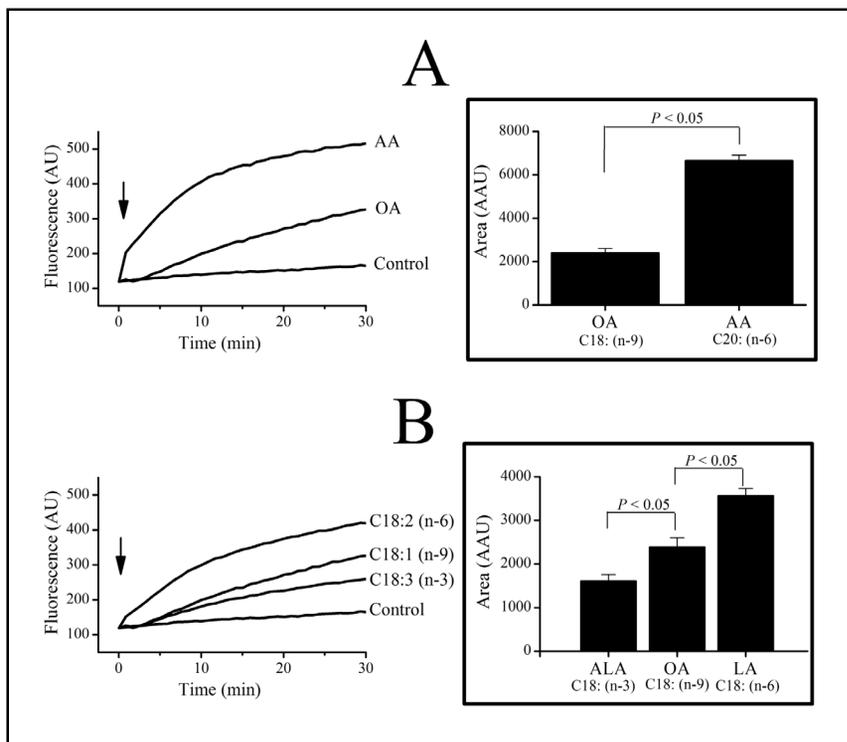
Neutrophil isolation

Neutrophils were purified from blood anti-coagulated with lithium heparin. The blood, diluted 1:1 with PBS, was placed on Percoll (p 1.076 g/mL) and centrifuged (700xg, 20 min, rT). The granulocyte-containing pellet was suspended in 50 mL ice-cold lysis solution and lysis of erythrocytes was performed on ice for 10-15 min. After centrifugation (400xg, 5 min, rT), remaining erythrocytes were lysed on ice in fresh lysis solution (155 mM NH₄Cl, 10 mM KHCO₃, pH 7.4) for another 5 min. The granulocytes were then washed twice, suspended to 8x10⁶ cells/mL in neutrophil medium and kept at rT. This method of neutrophil isolation yields > 97% pure and > 99% viable cell samples as determined by May-Grünwald/Giemsa and trypan blue staining [20].

Measurement of Intracellular Calcium Concentration ([Ca²⁺])

Neutrophils (4x10⁶ cells/mL) were loaded with 5 μ mol/L Fura-2/AM for 15 min at 37°C. Excess Fura-2/AM was removed by washing the neutrophils twice (NaCl, 145; KCl, 5; MgCl₂, 1; CaCl₂, 1; glucose, 10; Hepes, 10 (pH 7.4)). Subsequently, the neutrophils were transferred to a cuvette placed in a Cary Eclipse Fluorescence Spectrophotometer (Varian Ltd., Madrid, Spain) equipped with a magnetic stirrer and a thermostated cuvette holder (37°C). In the cuvette, baseline fluorescence was determined for 1 min before the FA and the appropriate stimulus were added. The change in Fura-2 fluorescence emission ratio at 510 nm was monitored as a measure of the average of the individual changes in cytosolic free Ca²⁺ concentration

Fig. 1. ROS production in isolated neutrophils stimulated with fatty acids. (A) Comparison between oleic acid (OA) and arachidonic acid (AA). (B) Comparison between C18 PUFAs: C18:1 (n-9)=OA, C18:2 (n-6)=LA, C18:3 (n-3)=ALA. FA were added at the time indicated by the arrows at a concentration of 10 μ M. The curves are representatives for 6 volunteers (measurements performed in triplicate). Data in histograms represent mean (SD) values of arbitrary area units (AAU), and were analyzed using the LSD test of significance. The area under the curve for each fatty acid was subtracted from the area under the curve of the control cells.



of all cells in the suspension after excitation at 340 and 380 nm [21, 22]. Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2 340/380 fluorescence ratio. The $[Ca^{2+}]_i$ was calculated using the method of Grynkiewicz et al. [23]. Increases in $[Ca^{2+}]_i$ are expressed as nM (mean \pm SD).

Oxygen radical production

DCFDA is a fluorescent probe that can be used to monitor oxidant production in living cells [24]. It passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases, releasing the corresponding dichlorofluorescein derivative. DCFDA oxidation yields a fluorescent adduct dichlorofluorescein (DCF) that is trapped inside the cell [25]. Cells were incubated at 37°C with 10 μ M DCFDA acetyl ester for 30 min, then centrifuged, and the pellet was resuspended in fresh medium. Fluorescence was recorded from 1 ml aliquots using a Fluorescence Spectrophotometer (Varian Ltd., Madrid, Spain). Similar results were obtained when we performed experiments in a 96 well plate. Samples were excited at 488 nm and the resulting fluorescence was measured at 530 nm.

Pertussis Toxin (pre-) incubation

Neutrophils (8×10^6 cells/mL in medium) were incubated with pertussis toxin (PT) (0.5 or 1.0 mg/mL) for 1.5 h by gentle headover-head turning at 37°C. Then, the cells were washed (5 min, 400xg, rT) and resuspended in medium to the desired final concentration.

Fatty acid preparations

Fatty acids were dissolved in ethanol [0.1% (v/v)] and used immediately or kept at -20°C, tightly sealed under a stream of nitrogen.

Statistical analysis

Analysis of statistical significance was performed using Statgraphics Centurion XVI. The data are represented as mean \pm SD for each group. One-way ANOVA was used to analyse the statistical significance between mean values followed by a least-significant difference (LSD) test. $P < 0.05$ was taken as the minimum level of significance.

Results

Oleic acid induces ROS production in human neutrophils

Oleic acid (OA) (C18:1 (n-9)) induced ROS production in human neutrophils. As a comparison, we included arachidonic acid (AA) (C20:4 (n-6)) as a widely recognized proinflammatory n-6 PUFA and stimulating agent. AA induced a significantly increased ($P < 0.05$) ROS production when compared with OA (Fig. 1A). To rule out that AA-derived metabolites, such as leukotrienes, prostaglandins or other eicosanoids are responsible for the observed effects we included a cyclooxygenase inhibitor (aspirin), and a lipoxygenase inhibitor (NDGA) in our experiments and found both agents did not affect ROS production by neutrophils stimulated with AA (data not shown).

Since the different effects of OA and AA might be the consequence of differences in carbon chain length we also evaluated the effect of linoleic acid (LA) (C18:2

Fig. 2. Dose-response curves of oleic acid-induced ROS production in human neutrophils. Oleic acid (OA) was added at the time indicated by the arrows. The curves are representative for 6 volunteers (measurements performed in triplicate). Data in histograms represent mean (SD) values of arbitrary area units (AAU), and were analyzed using the LSD test of significance. The area under the curve for each fatty acid was subtracted from the area under the curve of the control cells.

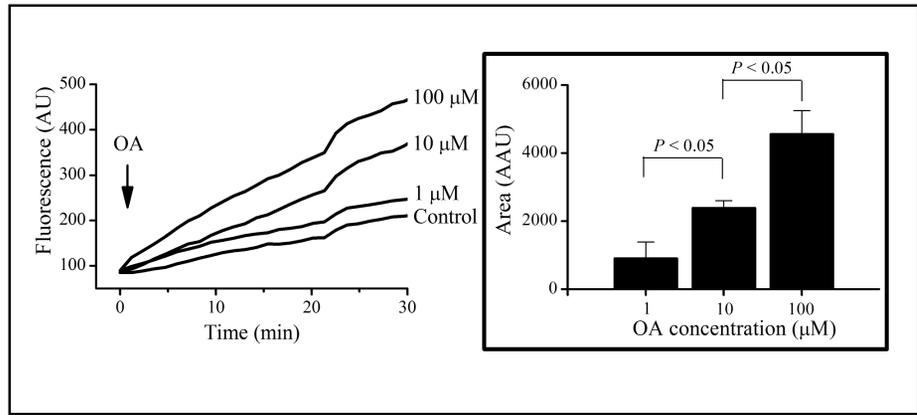


Fig. 3. Dose-dependent effect of oleic acid on $[Ca^{2+}]_i$ in isolated neutrophils in a Ca^{2+} -containing medium. Oleic acid (OA) was applied at the time indicated by the arrows. The curves, representing the Ratio (340/380), are representative for 6 volunteers (measurements performed in duplicate). Data in histograms, representing mean (SD) values of the increases in $[Ca^{2+}]_i$ (nM), were analyzed using the LSD test of significance. The calibration of the experiment was performed as described in the Material and Methods section.

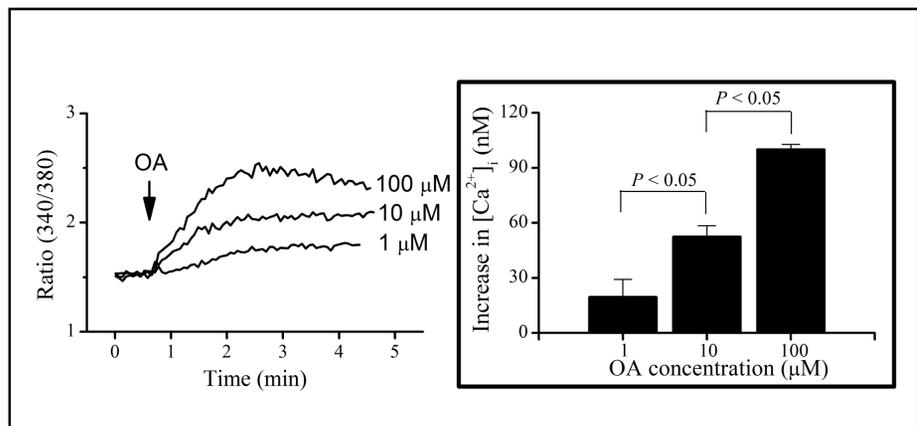
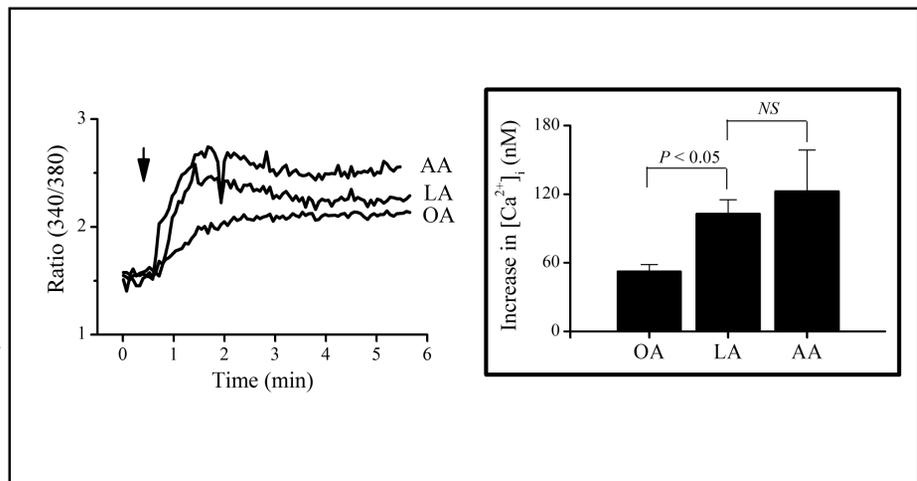


Fig. 4. Effect of 10 μM fatty acids on $[Ca^{2+}]_i$ in isolated neutrophils in a Ca^{2+} -containing medium. Oleic acid (OA), linoleic acid (LA) and arachidonic acid (AA) were added at the time indicated by the arrows. The curves, representing the Ratio (340/380), are representative for 6 volunteers (measurements performed in duplicate). Data in histograms represent mean (SD) values of the increases in $[Ca^{2+}]_i$ (nM) and were analyzed using the LSD test of significance. The calibration of the experiment was performed as described in the Material and Methods section. $NS = P > 0.05$.



(n-6): an n-6 FA with the same carbon chain length as OA. This FA behaved in a similar way as AA, displaying the potent proinflammatory effect of the n-6 family of PUFA. We also tested the n-3 family member α -linolenic acid (ALA) (C18:3 (n-3)): this PUFA was the less potent FA in inducing ROS production, in accordance with its anti-inflammatory properties (Fig. 1B).

Stimulation of ROS generation by OA occurred in a concentration-dependent manner. The effect was established at 3 different, physiologically relevant concentrations. 1 μM hardly induced any effect, but increasing the dose towards 100 μM resulted in significant ROS generation (Fig. 2). Similarly, the response to the n-6 FA AA proved to be concentration-

Fig. 5. Effect of fatty acids on $[Ca^{2+}]_i$ in isolated neutrophils in a Ca^{2+} -free medium. Traces represent the effects of 10 μ M oleic acid (OA) (A) and 10 μ M arachidonic acid (AA) (B) in experiments performed in a Ca^{2+} -containing medium “ Ca^{2+} (+)” and in a Ca^{2+} -free medium “ Ca^{2+} (-)”. Fatty acids were applied at the time indicated by the arrows. The curves are representative for 6 volunteers (measurements performed in duplicate). Data in histograms, representing mean (SD) values of the increases in $[Ca^{2+}]_i$ (nM), were analyzed using the LSD test of significance. The calibration of the experiment was performed as described in the Material and Methods section. *NS* = $P > 0.05$.

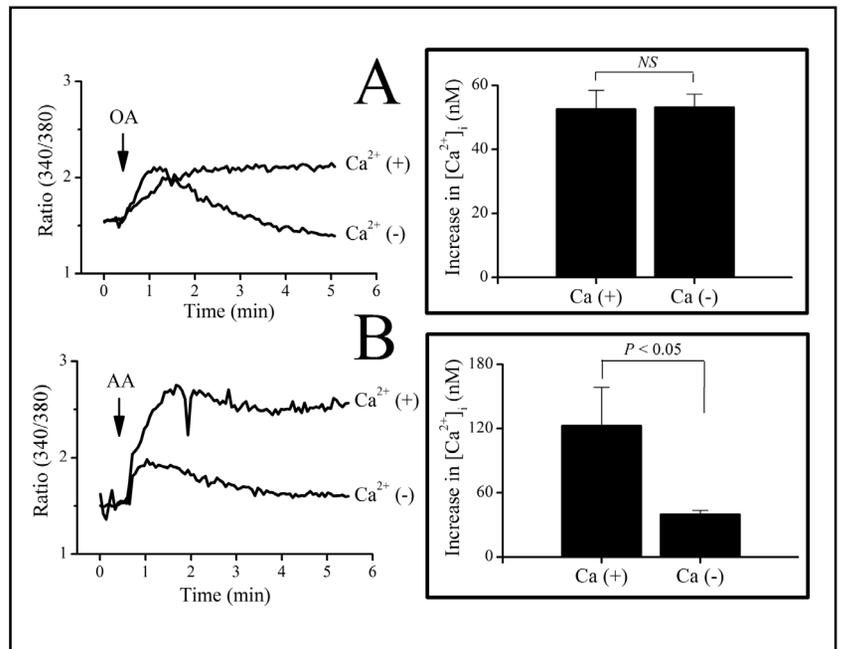
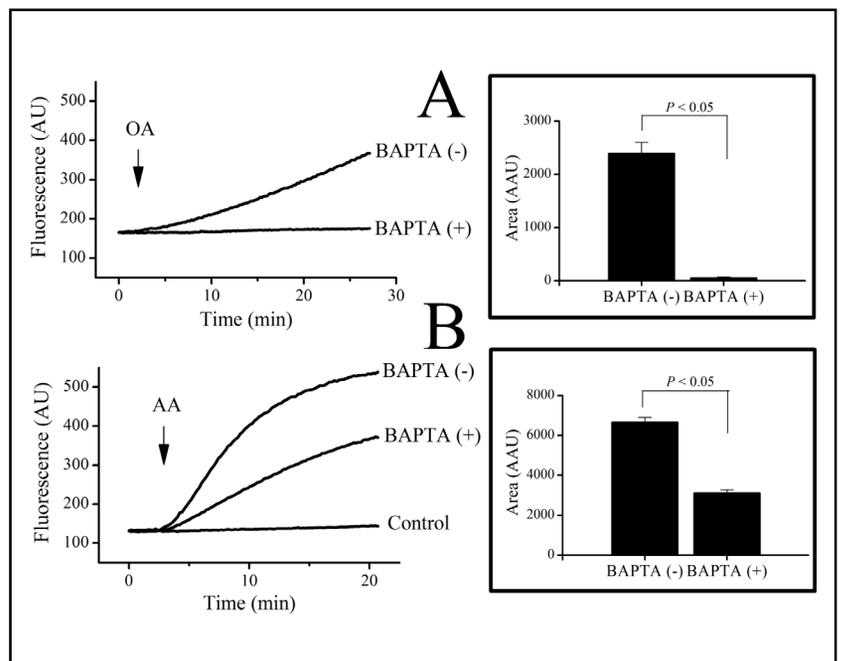


Fig. 6. ROS-production induced by 10 μ M fatty acids in BAPTA-treated neutrophils. Oleic acid (OA) (A) and arachidonic acid (AA) (B) were added at the time indicated by the arrows. “BAPTA (+)” represents cells treated with 5 μ M BAPTA-AM during 30 minutes. “BAPTA (-)” represents control cells. The curves are representative for 6 volunteers (measurements performed in triplicate). Data in histograms represent mean (SD) values of arbitrary area units (AAU), and were analyzed using the LSD test of significance. The area under the curve for each fatty acid was subtracted from the area under the curve of the control cells.



dependent (data not shown). The concentrations chosen for the study were all within range that is considered as physiological for the fatty acids tested [26].

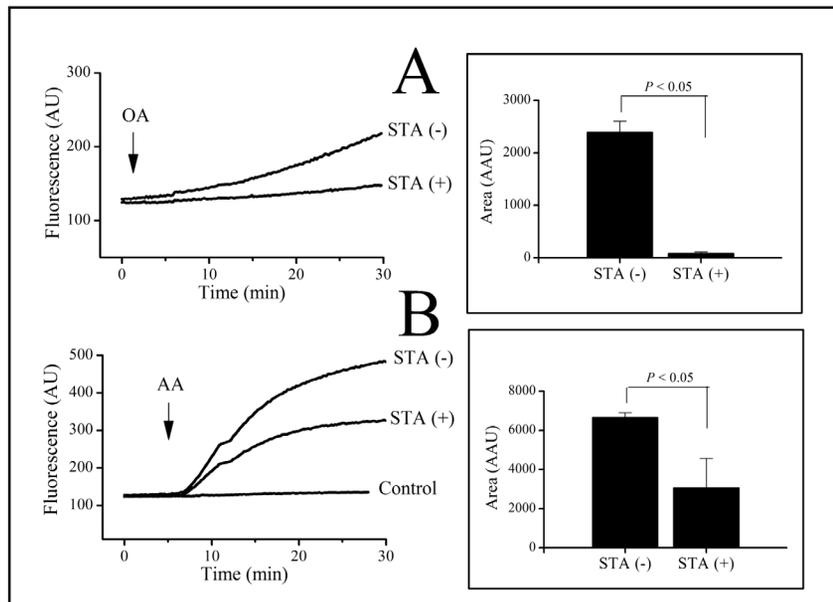
OA induces increases in $[Ca^{2+}]_i$ in human neutrophils

To investigate the mechanism behind the differences in ROS generation by OA and n-6 FA, we further explored possible mechanisms that might underlie such differences. Thus, the effects of both FA with regard to their potency

to induce changes in $[Ca^{2+}]_i$ in human neutrophils were compared, using the fluorescent probe Fura-2.

OA increased $[Ca^{2+}]_i$ in human neutrophils in a Ca^{2+} -containing medium. This increase was shown to be dose-dependent. OA at a concentration of 1 μ M hardly induced any rise, but increasing the dose towards 100 μ M resulted in a significant Ca^{2+} mobilization (Fig. 3). AA also induced a dose-dependent Ca^{2+} mobilization (data not shown). However, we observed clear differences between OA and AA effects. Fig. 4 shows the recordings of these experiments when 10 μ M FA was tested: Whereas OA

Fig. 7. ROS-production induced by 10 μM fatty acids in staurosporine-treated neutrophils. Oleic acid (OA) (A) and arachidonic acid (AA) (B) were added at the time indicated by the arrows. “STA (+)” represents cells treated with 1 μM staurosporine for 5 minutes. “STA (-)” represents control cells. The curves are representative for 6 volunteers (measurements performed in triplicate). Data in histograms represent mean (SD) values of arbitrary area units (AAU), and were analyzed using the LSD test of significance. The area under the curve for each fatty acid was subtracted from the area under the curve of the control cells.



increased $[\text{Ca}^{2+}]_i$ in a slow and gradual manner until a sustained plateau was reached, AA resulted in a biphasic response, with an immediate increase that was followed by a second slower rise. Interestingly, LA, being also a n-6 PUFA, showed the same biphasic response as AA.

The observed differences made us hypothesize that different mechanisms of Ca^{2+} mobilization are involved in the actions of both FA and, therefore, we were urged to study possible calcium sources that might contribute to the overall increase.

First, we conducted experiments in the absence of extracellular Ca^{2+} entry to explore the relative contribution of intracellular calcium pools to the overall response induced by OA. Assuming that no extracellular Ca^{2+} could enter the cells, any increase in $[\text{Ca}^{2+}]_i$ would have to arise from Ca^{2+} released from the intracellular stores. Fig. 5A shows the OA response in a Ca^{2+} -free medium. In cells treated with EGTA (an extracellular Ca^{2+} chelator), OA increased $[\text{Ca}^{2+}]_i$ in human neutrophils. No statistical differences ($P < 0.05$) were observed when the maximum peak value in both media (Ca^{2+} -containing and Ca^{2+} -free medium) was compared. However, in a Ca^{2+} -free medium no plateau was maintained after several minutes recording, and the levels in $[\text{Ca}^{2+}]_i$ immediately returned to those of the basal conditions. In contrast, 10 μM AA was also capable to induce an initial release of intracellular stored Ca^{2+} , but the $[\text{Ca}^{2+}]_i$ peak value under Ca^{2+} -free conditions in the bath was significantly lower ($P < 0.05$) when compared to that obtained in a Ca^{2+} -containing medium (Fig. 5B). This indicates that extracellular Ca^{2+} influx has also an effect on the early phase of the $[\text{Ca}^{2+}]_i$ transient induced by AA.

Effect of BAPTA on OA-induced ROS production

It is well established that Ca^{2+} -signaling is a key second messenger in the regulation of neutrophil functions [27]. Thus, assuming that the above results demonstrate the ability of OA to induce both ROS generation and increases in $[\text{Ca}^{2+}]_i$, we investigated whether these mechanisms are interconnected, and, if so, which event occurred first. We used BAPTA-AM, a cell-permeable Ca^{2+} chelator. OA failed to induce ROS-production in cells treated with 5 μM BAPTA-AM (Fig. 6A). However, although ROS generation induced by AA was curtailed ($\pm 55\%$), it was not totally abolished in cells treated with the Ca^{2+} chelator (Fig 6B). Similar results were obtained for LA (data not shown). Higher concentrations of BAPTA-AM did not result in a more potent inhibitory effect for the n-6 PUFAs (data not shown).

These results suggest that the OA-induced ROS production is mediated through a Ca^{2+} signaling pathway.

Effect of PKC on OA-induced ROS production

Once it was demonstrated that the increase in $[\text{Ca}^{2+}]_i$ was the first event to occur, we evaluated which mechanism could be the link between these two events. Because PKC is activated by Ca^{2+} [28], involvement of this enzyme in the generation of ROS was postulated. Staurosporine (STA) is a potent inhibitor of PKC. To test whether PKC also has a role in the activation of ROS generation by OA, the effect of OA on STA-treated cells was investigated. We found that OA-induced generation of ROS was totally abolished by STA (Fig. 7A). On the other hand, AA-induced ROS generation was only partly ($\pm 54\%$) but not totally abolished in cells treated with STA

(Fig. 7B). Again, similar results were obtained for LA (data not shown). Higher concentrations of STA did not result in a more potent inhibitory effect for the n-6 PUFAs (data not shown).

Although STA is the most potent inhibitor of PKC described in the literature with a half-maximal inhibitory concentration (IC₅₀) of 10 nM, this natural product is poorly selective when assayed against other protein kinases. Therefore, to further corroborate our results, we also tested the specific PKC inhibitor, bisindolylmaleimide I [29] and found similar effects compared to those obtained with STA (data not shown).

These observations suggest the involvement of PKC in the OA-induced generation of ROS in human neutrophils.

Murakami and Routtenberg demonstrated a direct activation of PKC by unsaturated FA [30]. Thus, we finally investigated the mechanism by which OA exerts its effect. Diacylglycerol is considered to be a second messenger for activation of PKC. It is a breakdown product of phosphatidylinositol by PLC. Several G protein-coupled receptors (GPCRs) have been recently identified as receptors for free FA on leucocytes and might, therefore, play a role in the OA-induced neutrophil activation [31, 32]. Some members of the G_i family are Pertussis toxin (PT)-sensitive (ADP ribosylation of their α_i -subunit by this toxin prevents coupling of the ligand bound receptor to the G_i-protein) [33]. Thus, we conducted experiments to evaluate the involvement of a PT-sensitive GPCR in the effects of OA. After treating the cells with PT (1 μ g/mL) OA still induced an increase in $[Ca^{2+}]_i$ and no differences were found when compared with control cells. Similar results were obtained for the n-6 FA (data not shown). The efficacy of PT treatment was tested by determining its effect on the fMLP-induced increase in cytosolic free Ca^{2+} concentration, shown to be abolished by this toxin in isolated neutrophils [34]. In addition, we studied the possible involvement of a G_q-protein coupled receptor, indirectly, by using the phospholipase C inhibitor U73122 [35]. This latter agent also failed to inhibit the fatty acid-response (data not shown).

Discussion

Previous reports have suggested that the use of dietary virgin olive oil should be considered as a valuable strategy in modulating the generation of inflammatory mediators [2]. However, the results from these studies could not rule out that other component than the lipid

component, such as antioxidants or free FA explain their findings. We therefore focused on the effect of the FA *per se* on an important aspect of immune cell stimulation, i.e. neutrophil activation.

The n-6 PUFA AA (C20:4) and other PUFA in the extracellular environment exert multiple effects on neutrophil functions [36-43]. However, few studies have focused on OA so far. In this paper, we addressed the question whether monounsaturated OA and proinflammatory n-6 PUFAs have distinct effect on human neutrophils activation, in order to explain their different biological actions, by investigating the effects of these FA on two major aspects of neutrophils activation, i.e. ROS production and intracellular calcium signaling.

It is well-known that the effects of PUFA on leucocyte function is highly dependent on FA structure and that the number of carbon-carbon double bonds, the carbon chain length and the position of the carboxy group is crucial in this respect [36, 40]. In accordance with other investigators [1, 44-49], we found clear differences between various structurally different FA and their ability to induce ROS generation in unstimulated neutrophils, suggesting that FA distinctively influence neutrophil-function depending on the number of double bonds. N-6 fatty acids with known proinflammatory characteristics showed a clear potency to generate the production of ROS, whereas this effect was much lower with OA.

This finding is in line with studies on parenteral lipid emulsions showing that olive oil-based lipid emulsions have a lower *in vitro* and *in vivo* impact on neutrophil functions when compared with n-6 PUFA-based lipids [50].

Triglyceride concentration is an important issue when the effect of lipid emulsions on granulocyte function is studied [51]. Our results confirm that the induction of ROS generation by the monounsaturated OA depends on the FA concentration and that, also in an OA concentration-dependent manner, this process relies on a prior activation of Ca^{2+} signaling pathways. Both intracellular release and extracellular Ca^{2+} entry contributed to the overall effect and the increase in intracellular Ca^{2+} levels resulted a prior and instrumental event in the ROS generation, in agreement with previous reports also showing that there is a critical dependence of activation of the proinflammatory activities of neutrophils on Ca^{2+} [27]. The n-6 PUFAs also increased $[Ca^{2+}]_i$, more potently than the responses obtained with OA, which correlates with the magnitude of the effect of the former FA on ROS generation and further support the key role of Ca^{2+} signaling in the oxygen radical production induced by these FA. Despite the clear differences observed between the

FA-induced Ca^{2+} mobilization, our results also show that both OA and AA release calcium from intracellular pools. It has previously been reported that free FA can mobilize calcium in several cell lines [52, 53], although AA in our study appeared to cause an earlier Ca^{2+} influx than OA since the initial phase of Ca^{2+} mobilization by AA was reduced in Ca^{2+} -free medium. In addition, intracellular calcium elevation was found to be important but not essential for the AA-induced ROS generation which supports the hypothesis of previous reports showing that Ca^{2+} mobilization induced by C20:4 (n-6) may be partly but not totally involved in ROS production [37]. It should be mentioned here that other groups have found a poor correlation between the degree of Ca^{2+} mobilization and the amount of superoxide produced [43] or even an enhanced Ca^{2+} rise in the absence of extracellular calcium [46] making it difficult to conclude the role that Ca^{2+} plays in regulating superoxide production in response to n-6 FA.

Previous authors have shown that PKC is activated by a rise in intracellular calcium concentration, and our results also indicate that PKC might be the connection between the two mentioned pathways that seem to be activated by OA since, the oxidative burst induced by OA appears to be the consequence of PKC activation induced by an elevation in intracellular Ca^{2+} concentration. These results underscore that both Ca^{2+} mobilization and PKC are implicated in the stimulation of neutrophils [54, 55] and support the hypothesis that ROS generation in neutrophils requires increases in $[\text{Ca}^{2+}]_i$ and C-kinase activity [28].

N-6 FA-induced ROS generation was partly but not entirely mediated by PKC activation. Others have shown that stimulation of PKC by arachidonate is not the unique phenomenon in this respect since several other enzymes are affected by this agent [56, 57] leaving room for the hypothesis that other enzymes are involved in the AA-induced neutrophil stimulation as well.

It has been suggested that the mechanism of C20:4 (n-6)-induced ROS generation in neutrophils is related, at least in part, to its ability to activate PLA_2 and a critical dependence of PLA_2 activation on FA structure has been reported, increasing in line with carbon chain length and with the number of double bonds [58]. These findings suggest that extracellular application of n-6 FA may be responsible for a PLA_2 activation leading towards an increased endogenous production of AA and, hence, respiratory burst as long as AA becomes present in a cellular compartment where NADPH oxidase can be

activated.

Finally, the results of our study argue against a role of PT-sensitive GPCRs in the mechanism of action of OA on human neutrophils. The study of Wang et al. showed that medium chain free fatty acids (MCFFAs) act as ligands for PT-sensitive GPCRs [59], but our results indicate that OA activates neutrophils differently from MCFFAs. We have postulated PKC activation induced by OA, suggesting a role of one or more isoforms of this kinase in the mechanism of action of the MUFA. It has been shown that each isoform can be activated in several ways, including PLC activation by PT-sensitive and PT insensitive GPCRs, among others [33, 60–62]. However, our study also argues against a PLC-dependent pathway. Some authors suggest that unsaturated fatty acids do not act as other stimulating agents do that bind to neutrophils at specific receptors. Modulation of physicochemical properties such as membrane fluidity (e.g. by fatty acids) can result in neutrophil activation [63, 64]. Apart from membrane fluidity, the induction of signaling pathways by the plasma membrane in immune competent cells may be exerted by intercalating into and disordering of specific regions of membranes, the so-called lipid rafts, i.e. microdomains characterized by a unique lipid environment [65–68]. Although clear differences of structurally different lipids on neutrophil membrane fluidity have been shown previously, further investigation of these possibilities is clearly required [69].

Taken together, our results are in accordance with previous studies demonstrating that various aspects of neutrophil activation including aggregation and degranulation are differentially modulated by OA when compared with n-6 PUFA [38]. In addition, release [70] and activity [47] of myeloperoxidase of neutrophils is reduced by OA.

In conclusion, our findings demonstrate that OA activates human neutrophils through increases in $[\text{Ca}^{2+}]_i$ and PKC activation that leads to ROS production. The observed effects in this respect clearly differ from those of n-6 FA, which might in turn explain the different effects on inflammatory pathways of the oils containing these FA.

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