Oleic Acid Versus Linoleic and α -Linolenic Acid. Different Effects on Ca^{2+} Signaling in Rat Thymocytes

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Key Words

Oleic acid • Linoleic acid • α -Linolenic acid • Ca^{2+} signaling • Rat thymocytes

Abstract

Background: Oleic acid is the principal fatty acid of olive oil composition and is reported to play a crucial role in its healthy aspects. However, the detailed mechanism of action is poorly understood. **Aims:** This study aims to elucidate the role of oleic acid in calcium signaling in rat thymocytes, in comparison to linoleic and linolenic acid. **Methods:** Fatty acids were applied to thymocytes isolated from wistar rats and loaded with Fura-2 to measure calcium signals. **Results:** The main results showed a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ induced by the 3 fatty acids. Raising the number of unsaturations resulted in greater increases. Two different pathways contributed to the increase induced by the polyunsaturated fatty acids: an IP_3 -independent release from the thapsigargin-sensitive stores and an extracellular calcium entry by econazole and nifedipine-insensitive channels. However, the OA-induced increases in $[\text{Ca}^{2+}]_i$ seemed to be due mostly to the Ca^{2+} recruited from the intracellular stores. **Conclusion:** This study

demonstrates that the fatty acids tested induce increases in $[\text{Ca}^{2+}]_i$ in rat thymocytes, with differences in close relation to the degree of unsaturation. Such differences could be responsible for their different physiological action.

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Introduction

Epidemiological studies show that increased dietary intake of olive oil has protective effects against cardiovascular disease, cancer risk, and inflammatory and degenerative diseases [1-4]. A study conducted in hypertensive patients showed that a monounsaturated (MUFA)-rich diet was associated with significantly reduced resting blood pressure and daily antihypertensive medication requirements compared with a polyunsaturated (PUFA)-rich diet [5].

Olive oil may have favorable effects on lipid peroxidation and oxidative stress, immune and inflammatory responses, and hepatobiliary function relative to other consumable oils [6]. In addition, the incidence of obesity in people who were not obese at baseline was very high in those who consumed sunflower oil [7].

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Identification of the main constituents of olive oil is crucial for the formulation of appropriate dietary guidelines. Thus, regarding its lipid composition, olive oil is low in saturated fatty acids (SFA), widely associated with adverse effects both acutely and chronically [8]. In fact, the low-PUFA, high-MUFA, low-saturated fatty acids (SFA) content of olive oil most closely matches international dietary recommendations for fatty acid ratio, which aim to limit the intake of both PUFA and saturated fat.

Studies have shown that the n-6 PUFA linoleic acid may have suppressive effects on cell-mediated immunity [9, 10]. In contrast, oleic acid has been found to induce few or no immunomodulatory effects. Moreover, n-6 PUFA rich oils have been implicated in the promotion of inflammation, primarily via the production of pro-inflammatory eicosanoids (eg, prostaglandins and leukotrienes) by the metabolism of arachidonic acid [6, 11]. However, oleic acid is not associated with the production of eicosanoids and may further reduce the pro-inflammatory properties of olive oil by reducing the n-6 PUFA content in the oil and, thus, in cell membranes.

In conclusion, the beneficial effects of olive oil on human health have been often attributed to its high monounsaturated fatty acid content, namely in the form of oleic acid (18:1n-9) [3]. Although its implications in health can no longer be doubted, the exact mechanism of action of this fatty acid (FA) is poorly well understood.

Being calcium signaling an essential tool for the development of different cellular responses, as gene expression, cell proliferation or apoptosis [12-15], in the present study, we investigated the role of oleic acid (OA) in calcium signaling in rat thymocytes, in comparison to linoleic acid (LA) and α -linolenic acid (ALA).

Materials and Methods

Materials

Wistar rats were procured from the University of Valladolid (UVA). Fura-2/AM was obtained from Fluka. Thapsigargin, oleic acid, linoleic acid, linolenic acid, methyl oleate, methyl linoleate and methyl linolenate, bovine serum albumine (free fatty acids) and econazole were purchased from Sigma (St. Louis, MO, USA). U73122 and nifedipine were bought from Calbiochem (La Jolla, CA, USA).

Cell isolation

Rat thymocytes were obtained from Wistar male rats (150 g) and handled as described previously [16]. The cells were washed twice by centrifugation at 300 x g for 5 min and resuspended in standard buffered saline containing: 150 mM

Fig. 1. Dose-dependent effect of OA, LA and ALA on $[Ca^{2+}]_i$ in rat thymocytes in a 1 mM Ca^{2+} -containing medium. Data in histograms are represented as means $\pm$ SD of nine independent experiments and analyzed by employing LSD test of significance ($P < 0.05$). White histograms represents 5 μ M FA; lined histograms represents 50 μ M FA; black histograms represents 100 μ M FA.

NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES. Prior to the experiment, cells were incubated for 30 min with 2 μ M fura-2-acetoxymethylester (Fura-2/AM). Then the cells were washed twice and placed in medium free of the dyes in a Ca^{2+} -containing medium (1 mM). When experiments were performed in a Ca^{2+} -free medium, extracellular Ca^{2+} was chelated by addition of 5 mM EGTA-Tris to the cell suspension. The experimental protocol was approved by Ethical Committees.

Measurement of Intracellular free Calcium Concentration ($[Ca^{2+}]_i$)

Fluorescence intensity was recorded from 1 ml aliquots of magnetically stirred thymocytes suspension (1 - 2% citocrite) using a Cary Eclipse Spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Baseline fluorescence was determined for 1 min before the fatty acid and the appropriate stimulus were added and readings were taken for a further 10 min. Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. The $[Ca^{2+}]_i$ was calculated using the method of Grynkiewicz et al. [17]. $[Ca^{2+}]_i$ increases are expressed as nM (mean $\pm$ SD).

Mn^{2+} measurements

Mn^{2+} was used as a tracer for Ca^{2+} entry (1 mM $MnCl_2$ was added directly to the cuvette with the cell suspension). The entry of Mn^{2+} was monitored by the quenching of fluorescence excited at 360 nm (emission 510 nm), which is insensitive to Ca^{2+} variations [18]. The rate of the fluorescence decrease provides a relative measure of the divalent cations permeability.

Fig. 2. Effect of 100 μ M FA on $[Ca^{2+}]_i$ in rat thymocytes in a 1 mM Ca^{2+} -containing medium. FA were applied at the time indicated by arrows. (A). The figure shows the single traces of observations which were reproduced nine times independently. Statistical differences ($P < 0.05$) between the 3 FA were found when the maximum peak was compared ($n=9$) (B).

Fatty acids preparation

Fatty acids (FA) were dissolved in ethanol [0.1% (v/v)] and used immediately or kept at $-20^{\circ}C$, tightly sealed under the stream of nitrogen.

Statistical analysis

Analysis of statistical significance was performed using Statgraphics 5.1 Plus. 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

OA, LA and ALA induce increases in $[Ca^{2+}]_i$ in rat thymocytes

OA, LA and ALA increased $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} in rat thymocytes. The effect of different concentrations was tested (Fig. 1). ALA had the higher effect in all concentrations tested, followed by LA and OA. The maximum effect for the 3 fatty acids was found when 100 μ M was added to the cells. It is noteworthy that the PUFAs did not present statistical differences between 50 and 100 μ M, but OA showed a little effect when 50 μ M was tested and therefore, in order to compare the 3 fatty acids at the same dose, we took 100 μ M concentrations in the whole study. Such a

Fig. 3. Effect of 100 μ M FA on $[Ca^{2+}]_i$ in rat thymocytes in a Ca^{2+} -free medium. Traces represent the effects of 100 μ M OA, LA and ALA on $[Ca^{2+}]_i$ in experiments performed in a Ca^{2+} -containing medium " Ca^{2+} (+)" and in a Ca^{2+} -free medium " Ca^{2+} (-)". The figure shows observations which were reproduced several times independently ($n \geq 6$).

concentration was not toxic to the cells. However, increasing the concentration of the PUFAs further on resulted in a membrane permeabilization (data not shown).

Figure 2 shows typical recordings of 100 μ M FA (Fig. 2A). Statistical differences ($P < 0.05$) were found when comparing the three FA maximum peaks at 100 μ M (Fig. 2B). Moreover, there is a further difference between the $[Ca^{2+}]_i$ increase produced by the three FA tested focusing on the shape of the response. Both LA and ALA induced a rapid increase in $[Ca^{2+}]_i$ immediately

Fig. 4. Effects of FA on releasing thapsigargin-sensitive store. FA were added to thapsigargin (Tg) treated thymocytes (A). The degree of filling of intracellular stores in FA-treated cells was measured by addition of thapsigargin (B, C, D). Reagents were applied at the time indicated by arrows. The concentration of reagents were 400 nM thapsigargin and 100 μ M FA. The figure shows the single traces of observations which were reproduced several times independently ($n \geq 6$).

Fig. 5. Effects of OA, LA and ALA on the increases in $[Ca^{2+}]_i$ in U73122 treated thymocytes (black line). Before starting the measure, cells were incubated 5 minutes with 3 μ M U73122. The arrow heads indicate the time when 100 μ M FA was added into the cuvette. Grey lines represent non-treated cells. The figure shows the single traces of observations which were reproduced several times independently ($n \geq 6$).

after the addition and a sustained plateau was maintained for several minutes. In contrast, the rising intensity of the Ca^{2+} signal was slower in response to OA, which increased $[Ca^{2+}]_i$ in a low gradually way until a slight plateau was reached.

OA, LA and ALA releases Ca^{2+} from Thapsigargin-sensitive stores

We conducted experiments in a Ca^{2+} -free medium, to evaluate the sources of the increase in $[Ca^{2+}]_i$ induced by the FA. OA, LA and ALA evoked increases in $[Ca^{2+}]_i$ in a Ca^{2+} -free medium (Fig. 3), suggesting that the FA recruited Ca^{2+} from the intracellular stores.

To further investigate the origin of the calcium recruited by the 3 FA, an inhibitor of intracellular Ca^{2+} stores was used. Thapsigargin (Tg), without acting on IP_3 receptors, increases cytosolic Ca^{2+} by inhibiting the Ca^{2+} -ATPase present on the endoplasmic reticulum (ER) [19].

Application of 400 nM of Tg, in a Ca^{2+} -free medium, caused an increase in $[Ca^{2+}]_i$ that comprised an initial rise and a gradual decrease toward baseline; after

Fig. 6. Effects of 100 μM OA, LA and ALA on Mn^{2+} permeability of the plasma membrane. The quenching of 360 nm fluorescence was recorded. Dotted line represents control cells. The figure shows observations which were reproduced several times independently ($n \geq 6$).

depleting the endoplasmic reticulum Ca^{2+} store with Tg, addition of 100 μM FA (OA, LA or ALA), did not induce any Ca^{2+} increase (Fig. 4A). Moreover, addition of 400 nM Tg after treating cells with either LA and ALA, also failed to induce a $[\text{Ca}^{2+}]_i$ rise (Fig. 4B and 4C). This signal most likely reflected endoplasmic reticulum Ca^{2+} release induced by these FA. In contrast, results with OA-treated cells were different: a significant ($P < 0.05$) $[\text{Ca}^{2+}]_i$ rise could be seen after addition of 400 nM Tg to OA-treated cells (Fig. 4D).

Inhibition of IP_3 production does not decrease FA-induced increase in $[\text{Ca}^{2+}]_i$

Previous studies have shown that in several T-cells, stored Ca^{2+} can be released by pathways dependent or independent on PLC-induced IP_3 formation [20, 21]. In order to evaluate whether OA, LA and ALA evoke increases in $[\text{Ca}^{2+}]_i$ via production of IP_3 , we used U73122, an agent known to inhibit the generation of free IP_3 [22, 23]. Addition of 3 μM U73122, altered neither basal $[\text{Ca}^{2+}]_i$ nor the response induced by the FA (Fig. 5).

Extracellular Ca^{2+} influx also contribute to the FA-induced rise in $[\text{Ca}^{2+}]_i$

To further study the implication of Ca^{2+} influx, we planned further experiments on Mn^{2+} quenching. Thus, in order to probe directly the effects of OA, LA and ALA on Ca^{2+} permeability of the plasma membrane, the unidirectional uptake of Mn^{2+} into Fura-2 loaded thymocytes was measured. Mn^{2+} , widely used as a probe for Ca^{2+} influx pathways in different non excitable cells, enters the unstimulated thymocytes as well [16, 24]. We

Fig. 7. Effects of ALA on the increases in $[\text{Ca}^{2+}]_i$ in econazole treated thymocytes (solid line). Before starting the measure, cells were incubated 5 minutes with 10 μM econazole. The arrow heads indicate the time when ALA was added into the cuvette. Dotted line represents non-treated cells. The figure shows the single traces of observations which were reproduced several times independently ($n \geq 6$).

Fig. 8. Effects of LA on the increases in $[\text{Ca}^{2+}]_i$ in nifedipine treated thymocytes (solid line). Before starting the measure, cells were incubated 5 minutes with 10 μM nifedipine. The arrow heads indicate the time when LA was added into the cuvette. Dotted line represents non-treated cells. The figure shows the single traces of observations which were reproduced several times independently ($n \geq 6$).

compared the effects of 100 μM on the influx of Mn^{2+} entry (Fig. 6). All of the PUFA accelerated Mn^{2+} entry, confirming the extracellular Ca^{2+} entry induced by the FA as previously suggested. Such acceleration was in clearly relation to the number of double bonds; those cells stimulated with ALA had a higher entry. However, the monounsaturated FA hardly induced any entry.

These results are consistent with those found in Figure 3. Removal of extracellular Ca^{2+} nearly abolished the plateau of the Ca^{2+} response. Once reached the peak, $[\text{Ca}^{2+}]_i$ returned to basal levels. However, in OA-treated cells these differences cannot be established, suggesting that the OA-induced increases in $[\text{Ca}^{2+}]_i$ was mostly due to the Ca^{2+} recruited from the intracellular stores.

Fig. 9. Effects of methyl-OA, methyl-LA and methyl-ALA on $[Ca^{2+}]_i$ in rat thymocytes. The arrow heads indicate the time when methyl-fatty acids (Methyl-FA) were added to the cells. Dotted line represents non-stimulated cells. The figure shows the single traces of observations which were reproduced several times independently ($n \geq 6$).

In order to probe the role of Ca^{2+} channels implicated in the influx of extracellular Ca^{2+} , we used econazole, an inhibitor of store operated channels (SOC) in various cell types [25–27]. Figure 7 shows that econazole, able to inhibit Tg-induced Ca^{2+} entry (data not shown), failed to abolish the Ca^{2+} entry induced by ALA. Similar results were obtained for LA (data not shown). We also tested nifedipine, an inhibitor of L-type channels. Colucci et al. [28] showed that nifedipine, inhibits the influx of Ca^{2+} induced by a variety of treatments each of which depleted intracellular Ca^{2+} stores. Figure 8 shows that preincubation of rat thymocytes in the presence of 10 μ M nifedipine failed to curtail the $[Ca^{2+}]_i$ increase by the LA. Similar results were found for ALA (data not shown).

OA, LA and ALA act extracellularly in rat thymocytes

Finally, we wanted to assess the mechanism by which the FA exert these effects, whether they act extracellularly or not. We used the fatty acid-free bovine serum albumin (BSA) at a final concentration of 0.3%, as at this concentration, BSA has been shown to compete with PUFA bound to the plasma membrane [29]. Addition of BSA during the peak response of OA, LA, ALA, abruptly diminished the FA-induced rise in $[Ca^{2+}]_i$ in rat thymocytes. Addition of BSA alone exerted no significant perturbation in the fura-2 fluorescence (results not shown).

Moreover, we used Methyl-OA, Methyl-LA and Methyl-ALA, which have a similar structure to OA, LA and ALA respectively, without the free carboxylate group. All of them failed to induce an increase in $[Ca^{2+}]_i$ (Fig. 9).

Discussion

During the course of recent years, olive oil has been considered as one of the main contributors to the healthy aspects of the Mediterranean diet. OA is the major FA of olive oil, thus reported to play a crucial role as regards its effects are concerned. However, the detailed mechanism of action of OA is poorly understood. In the present study, we focus on the role of OA in calcium signaling in rat thymocytes, in comparison to LA and ALA. It is important to point out that, as far as we are concerned, there is no early study aiming to investigate such a comparison in rat thymocytes.

OA, LA and ALA caused an increase in $[Ca^{2+}]_i$ in rat thymocytes. Similar results were obtained for different FA, as docosahexaenoic acid (DHA), arachidonic acid (AA) or LA, in several cell lines [30–34]. In addition, according to previous reports, such an increase was found to be concentration-dependent for the three FA tested [30, 31].

The $[Ca^{2+}]_i$ increase observed was in accordance to the degree of unstauration of the FA. ALA induced higher $[Ca^{2+}]_i$ increases, nearly followed by LA and further by OA. This is consistent with earlier studies suggesting that n-3 PUFA are very potent agents in regulating intracellular calcium levels [35]. Moreover, not only the net value, but also the shape of the response was enough to differentiate between OA and the effects induced by the PUFAs. We observed that LA and ALA caused an immediate rise in $[Ca^{2+}]_i$ followed by a sustained phase whereas OA showed a slower response, increasing $[Ca^{2+}]_i$ in a low gradually way until a slight plateau was finally reached. We could hypothesize that the FA behave qualitatively in the same way and only their potencies are simply different, but in any case, previous studies also demonstrated different sensitiveness of isolated murine thymocytes to FA depending on the number of double bonds [36].

The $[Ca^{2+}]_i$ increase induced by LA and ALA was due to both a release from the intracellular pool and an extracellular Ca^{2+} entry. Similar results were found by Teshima et al. for several n-6 PUFA in RBL-2H3 cells [37]. The Ca^{2+} recruited from the stores contributed to the first phase of the response. The sustained plateau was maintained by the extracellular Ca^{2+} influx. This is consistent with results previously reported by Bonin and Khan for DHA [33]. However, OA hardly induced any extracellular Ca^{2+} entry and the increase in $[Ca^{2+}]_i$ induced by 100 μ M OA, seemed to be caused mainly by the mobilization of Ca^{2+} from the intracellular stores.

Regarding the intracellular Ca^{2+} stores, the Tg-sensitive endoplasmic reticulum store, appears to play a dominant role, because the FA-induced Ca^{2+} release was significantly inhibited by depletion of the endoplasmic reticulum Ca^{2+} store with Tg. These results indicate that FA stimulate an increase in $[\text{Ca}^{2+}]_i$ by releasing Ca^{2+} from Tg-sensitive stores. Similar results were also obtained for LA in rat pancreatic β -cells [38]. However, although all OA, LA and ALA mobilize Ca^{2+} from the endoplasmic reticulum pool, they act in a quantitative different way. Several minutes incubation of fura-2-loaded thymocytes with the different 100 μM FA, resulted in different degrees of store filling, measured as the increase of $[\text{Ca}^{2+}]_i$, when Tg was added in a Ca^{2+} -free buffer.

How FA release Ca^{2+} from the stores is still unclear, but the mechanism seems to be independent of IP_3 , because suppression of IP_3 production with U73122, failed to affect the FA-induced Ca^{2+} release. This notion is further supported by the group of Chow, who noticed that various PUFA, including, LA and ALA, evoked increases in $[\text{Ca}^{2+}]_i$ without the production of IP_3 in Jurkat T-cells [29, 39]. Bonin and Khan obtained similar results for DHA in human T-cells as well [33]. Moreover, our results corroborate the findings of Teshima et al. for n-6 PUFA in RBL-2H3 cells [37].

Considering both, releasing from the stores and extracellular influx results all together, we could conclude with OA as the one increasing $[\text{Ca}^{2+}]_i$ in a lower degree, firstly as a consequence of its small power in the releasing pathway and secondly, and maybe subsequently, due to the low influx induced. This is consistent with previously reports indicating such a link between the degree of filling of the stores and the rate of Mn^{2+} entry [16, 40].

We were also tempted to assess the nature of the channels involved in such an influx. Our results suggest that the FA tested did not induce a Ca^{2+} entry via econazole-sensitive channels in rat thymocytes. Moreover, the FA-induced $[\text{Ca}^{2+}]_i$ rise, appears to be via a pathway independent of L-type Ca^{2+} channels as well. However, the specific channels involved and the signaling mechanism

that causes such an opening is yet unknown.

OA, LA and ALA may act extracellularly. Addition of free-fatty acid BSA caused an abrupt decrease in the peak induced by the 3 FA. This is consistent with the results that Khan and collaborators found for DHA in T-cells [33]. This group also found that BSA detached the plasma membrane bound FA thus contributing to the lowered response of the FA. The precise mechanism by which these FA deliver its signal, by acting extracellularly is still unknown. However, the free carboxylate group is required, as the methyl-analogs of the 3 FA failed to increase $[\text{Ca}^{2+}]_i$ which is consistent with results previously reported in several cell lines [38].

Collectively, this study demonstrates that OA, LA and ALA induce increases in $[\text{Ca}^{2+}]_i$ in rat thymocytes in close relation to the degree of unsaturation. The PUFAs caused Ca^{2+} signals in a concentration-dependent manner by evoking IP_3 -independent Ca^{2+} release, from the endoplasmic reticulum thapsigargin-sensitive store, and also by causing extracellular Ca^{2+} influx. Such an influx does not involve econazole-sensitive or L-type channels. However, the OA-induced increases in $[\text{Ca}^{2+}]_i$ seemed to be due mostly to the Ca^{2+} recruited from the stores. Previous reports showed that cell death in rat thymocytes was always accompanied by an increase in the levels of $[\text{Ca}^{2+}]_i$ and this authors also demonstrated that such an increase was in proportion to the potency of the PUFAs in inducing cell death, varying in clear connection with the degree of unsaturation [41]. In conclusion, the differences observed between OA and the PUFAs when a similar dose is tested could be responsible for the different physiological action of these FA.

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