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Effects of EPA and DHA on lipid droplet accumulation and mRNA abundance of PAT proteins in caprine monocytes

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ABSTRACT

The present study investigated the *in vitro* effects on caprine monocytes of two ω-3 PUFAs, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on lipid droplet formation, an emerging process of fundamental importance in innate immunity regulation. The mRNA abundance of PAT protein family (PLIN1, PLIN2 and PLIN3), involved in the formation and trafficking of the droplets, was also assessed. The effects of EPA and DHA on monocyte apoptosis were studied as well. The number of lipid droplets per cell was found to be dependent on both type and concentration of fatty acid. ω-3 PUFAs upregulated PLIN3 and PLIN2 gene expression, as well as apoptosis rate. The present findings suggest that PUFA might modify innate immune functions of goat monocytes by interfering with the formation of lipid droplets and by upregulating proteins belonging to PAT protein family.

1. Introduction

Polyunsaturated fatty acids (PUFAs) metabolism, in particular that of ω-3 PUFAs, is of fundamental importance in regulating immune-cell function (Calder, 2001). The most powerful immunomodulatory effect is achieved by two ω-3 PUFAs, namely eicosapentaenoic acid (EPA, C20:5[ω3]) and docosahexaenoic acid (DHA C22:6[ω3]) (Calder, 2006).

Long chain ω-3 PUFAs modulate immune functions in several ways. They alter the levels of phospholipid-derived second messengers (Madani et al., 2001) and the composition of lipid-raft associated proteins (Fan et al., 2003), modulating the production of inflammatory eicosanoids (Lee et al., 1985) and interfering directly with cytokine gene expression (Yaqoob and Calder, 1995). Finally, both EPA and DHA give rise to a newly discovered family of anti-inflammatory mediators called resolvins (Serhan et al., 2002). Most of these activities directly target monocyte/macrophage defensive functions by modulating phagocytosis (Halvorsen et al., 1997), chemotaxis (Schmidt et al., 1992) and pro-inflammatory cytokine expression (Babcock et al., 2002). While still not completely understood, the main biological effect of ω3

long chains fatty acids has been ascribed to their incorporation in cell membrane phospholipids, thus affecting eicosanoid synthesis and altering membrane fluidity (Das, 2006). Since ω-3 PUFA are also stored in lipid droplets, a mechanism of action to explain their modulatory activity on immune cells involving lipid droplet formation has been hypothesized in the past (Finstad et al., 1998). Lipid droplets, also named lipid bodies or adiposomes, are lipid-rich organelles, their cytoplasmic accumulation representing a common feature of innate immunity reactions and inflammatory conditions (Pacheco et al., 2002). The molecular mechanisms regulating the formation and metabolism of lipid droplets are not well understood. Structurally, lipid droplet consist of a lipid core surrounded by a phospholipid monolayer, which contains various proteins involved in lipid metabolic processes such as lipolysis or lipogenesis (Bozza et al., 2009). The major structural proteins present at the surface of lipid droplet are those belonging to the proteins of the PAT family, named after perilipin (PLIN1), adipophilin (PLIN2), and tail-interacting protein of 47 kDa (TIP47), or perilipins (Kimmel et al., 2010). These proteins have been implicated in the lipid droplet assembling and biogenesis (Bickel et al., 2009). Different from neutral lipid storing cells, monocytes have virtually no lipid droplet under resting conditions. However, increased numbers of cytoplasmic lipid droplet in monocytes are often associated with infectious and other inflammatory conditions (Das, 2006).

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While ω-3 fatty acids are long since utilized as lipid sources in goat diets with the aim to increase the proportion of nutritionally beneficial ω-3 PUFAs in milk for human consumption (Cattaneo et al., 2006), yet few studies have been carried out to investigate the effects of fish oil-derived PUFAs on leukocyte function in this species. The information available clearly evidenced an effect on leukocyte functions both *in vivo* (Agazzi et al., 2004; Thanasak et al., 2004; Bronzo et al., 2010) and *in vitro* on isolated neutrophils (Pisani et al., 2009) and monocytes (Lecchi et al., 2011).

The aim of this study was to investigate the relationship between EPA and DHA treatment and the formation of lipid droplets, in goat monocytes. The mRNA abundance of the three major perilipins, namely PLIN1, PLIN2 and PLIN 3, which are involved in lipid droplet formation, was also investigated by quantitative PCR. Finally, since both perilipins and ω-3 PUFA are involved in the regulation of cell lifespan, apoptosis rate of caprine monocytes after EPA and DHA treatment was also assessed by caspases 3/7 activity measurement.

2. Materials and methods

2.1. Materials

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), EDTA (ethylenediaminetetraacetic acid), RPMI-1640, Red Blood Cell Lysing Buffer Hybri-Max, DHA (cis-4, 7, 10, 13, 16, 19-Docosahexaenoic acid sodium salt), EPA (cis-5, 8, 11, 14, 17-Eicosapentaenoic acid sodium salt), Nile Red, PBS without Ca^{++} and Mg^{++} and endotoxin-free water were purchased from Sigma–Aldrich Co. (Milano, Italy). Fetal Bovine Serum (FBS) was provided by Biochrom AG (Berlin, Germany) and Ficoll 1077 by GE Healthcare Europe GmbH (Munich, Germany).

MACS Separation Columns and CD14 Microbeads were purchased from Miltenyi-Biotech (Calderara di Reno, Bologna, Italy), the RNeasy Mini Kit from Qiagen GmbH (Milano, Italy), iSCRIPT cDNA SYNTESIS Kit and SYBR Green mix from BIORAD Laboratories (Segrate, Milano, Italy) and Apo-ONE® Homogeneous Caspase-3/7 Assay was obtained from Promega Corporation (Milano, Italy). $MgCl_2$, dNTPs and *Taq* polymerase were purchase from Vivantis Inc. (Oceanside, Ca, USA).

Six wells sterile cell culture plates and 384 wells black sterile cell culture plates were purchased from Nunc (Rochester, NY, USA).

2.2. Preparation of PUFAs

Stock solution of DHA sodium salt and EPA sodium salt were prepared as previously described (Zhao et al., 2005). The DHA and EPA sodium salts were reconstituted in endotoxin-free water at the concentration of 14.3 mM and 15.4 mM, respectively and were stored in the dark at −80 °C to prevent peroxidation for no longer than 6 months. The doses of PUFAs used ranged from 0 to 200 μM (Thanasak et al., 2005).

2.3. Source and purification of caprine monocytes

Six clinically healthy multiparous lactating goats (Saanen) between 5 and 6 years of age and between 30 and 33 weeks after parturition were used for blood collection. Goat diet was based on alfalfa hay and a commercial concentrate mix without any ω-3 fatty acids supplement added. Blood samples were collected before feeding administration by venipuncture from the jugular vein into 10 ml K3 EDTA vacuum sterile glass tubes. Samples were delivered to laboratory within 2 h at 5 °C.

Blood was centrifuged at 1260*g* for 30 min at room temperature in order to obtain the buffy coat. Mononuclear cells were obtained after dilution of the buffy coat 1:5 (vol/vol) in cold PBS with 2 mM EDTA and 10 ml of this solution were overlaid on 3 ml Ficoll 1077 and centrifuged for 30 min at 1500*g* at 4 °C. Mononuclear cells were collected from the interface. The cells were resuspended in 50 ml cold PBS with 2 mM EDTA and centrifuged for 7 min at 400*g* at 4 °C in order to remove platelets. This step was repeated four times. The remaining erythrocytes were lysed with Red Blood Cell Lysing Buffer Hybri-Max.

Monocyte (CD14+) cells isolation was carried out using magnetic activated cellular sorting. Briefly, Peripheral Blood Mononuclear Cell (PBMC) were incubated with anti-human CD14 labeled super-paramagnetic beads (20 μl/10^8 cells) for 15 min at 4 °C and CD14+ cells were isolated from a MidiMacs column according to the manufacturer's instruction. The purity of the sorted cells (>95%) was evaluated by flow cytometry. Cell viability, as determined by Trypan Blue exclusion, was >95%.

2.4. Caprine monocyte apoptosis measurement

The concentration of monocytes was adjusted to 2×10^6/ml and 25 ωl of this solution (5×10^4 cells) were seeded in 384 wells black sterile cell culture plates and incubated for 24 h at 37 °C in humidified atmosphere of 5% CO_2. The DHA and EPA were diluted in medium (RPMI 1640 with 20 mM HEPES, 10% heat inactivated FBS, 100 μU penicillin/ml, 100 μg streptomycin/ml) to concentration of 0, 25, 50, 100 and 200 μM. All the experiments were carried out in triplicate.

Apoptosis was determined by measuring the modification of the activities of two enzymes that play effector roles in apoptosis: caspase-3 and caspase-7 as previously described in bovine (Ceciliani et al., 2007).

2.5. Measurement of intracellular lipid droplets

Caprine monocytes were seeded in number of 1.5×10^5 in 6 wells sterile cell culture plates containing sterile coverslips and were incubated for 24 h at 37 °C in humidified atmosphere of 5% CO2 in air. The DHA and EPA were diluted in complete RPMI (RPMI 1640 with 20 mM HEPES, 10% heat inactivated FBS, 100 μU penicillin/ml, 100 μg streptomycin/ml) to final concentration of 0, 25, 50, 100 and 200 μM. The coverslips were collected from the cell culture plates and washed twice in cold PBS; the cells were then fixed with 2% formalin at room temperature for 30 min and stained with 0.15 μg/ml Nile Red for 15 min. Nile Red was prepared as described by Listenberger and Brown (2007). After the first staining with Nile Red, the cover slips were washed twice in PBS and chromatin DNA was stained with DAPI (0.05 μg/ml in PBS). Nile Red fluorescence intensity was stable for several hours. All samples were mounted with an anti-fade medium (Vectashield Vector lab., Burlingame, CA, USA) and observed with a conventional epifluorescence microscope (Eclipse E 600, Nikon, Torino, Italy) at a 100× magnification. Quantification of lipid droplet number and size was carried out using ImageJ software (NIH, Bethesda, MA, USA) (Stringer et al., 2010). Ten fields were randomly selected and the cells were divided in negative cells, in which no fluorescence was detected, and positive cells with lipid droplets. For quantification of the number measurements, all lipid droplets within the ten fields were counted.

2.6. Perilipin mRNA abundance assessment

The concentration of caprine monocytes was adjusted to 3×10^6/ml and 1 ml of this solution was seeded in 6 wells sterile cell culture plates and incubated for 24 h at 37 °C in humidified atmosphere of 5% CO_2 in air. The DHA and EPA were diluted in complete RPMI (RPMI 1640 with 20 mM HEPES, 10% heat

inactivated FBS, 100 μU penicillin/ml, 100 μg streptomycin/ml) to final concentration of 0, 25, 50, 100 and 200 μM. Total RNA was extracted from stimulated monocytes using the RNeasy Mini Kit according to the manufacturer's protocol. The reverse transcription (RT) reaction was carried out on 1 μg RNA using an iScript™ cDNA Synthesis Kit. The thermal profile was according to the manifacturer's recommendations.

To perform gene testing, the cDNA derived was pooled and used as the template for PCR reactions; cDNA purified from caprine perirenal adipose tissue was used as positive control. The reactions were carried out in a 10 μL final volume containing 1× buffer, 1.5 mM MgCl$_2$, 0.2 mM each deoxynucleotide triphosphates (dNTPs), 1 μM each primer and 0.025 U Taq polymerase. The same primers were used in qualitative and quantitative PCR (Table 1). Amplifications were performed using 35 cycles of 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing) and 72 °C for 45 s (extension) (Eppendorf Mastercycler). PCR products were visualized on 1.9% agarose gels stained with ethidium bromide.

Quantitative reactions were performed in 25 μL of SYBR® Green mix and: 400 nM of SDHA primers and PLIN3 primers and 300 nM of YWHAZ primers and PLIN2 primers. In order to evaluate the PCR efficiency using a relative standard curve, series of dilution were prepared by performing 4-fold serial dilution starting from pooled cDNA sample in triplicate, obtained by mixing 2 μl of each sample. Each sample was tested in duplicate. The thermal profile used (95 °C for 90 s, 50 cycles of 95 °C for 15 s and 62 °C for 60 s; for melting curve construction, 55 °C for 60 s and 80 cycles starting to 55 °C and increasing 0.5 °C each 10 s) was the same for each target gene. The results obtained were compared using the delta–delta Cq method (Giulietti et al., 2001) after normalization of the sample using the SDHA and YWHAZ as reference genes, which were selected as housekeeping gene following previous studies on bovine White Blood Cells (De Ketelaere et al., 2006; Spalenza et al., 2011) and other tissues (Goossens et al., 2005; Lisowski et al., 2008; Bougarn et al., 2011).

2.7. Statistical analysis

Apoptosis data were analyzed by ANOVA using the General Linear Model of SAS (SAS/STAT, Version V8, 1999, SAS Inst., Inc., NC, USA). Significance was declared for $P < 0.01$ and $P < 0.05$.

3. Results

3.1. EPA and DHA modulate caprine monocyte apoptosis only when used at high concentrations

To investigate the apoptosis-modulating activity of EPA and DHA, the enzymatic activities of caspase-3 and caspase-7, the two major executioners of the apoptosis pathway, were measured. Caspase-3/7 activity was detected 24 h after monocytes medium supplementation with EPA or DHA. The results showed that

treatment with low doses (between 25 and 100 μM) of both fatty acids did not influence the apoptosis rate compared with untreated cells. On the contrary, higher concentrations of both fatty acids (200 μM) induced a significantly increase of monocytes apoptosis: in particular, EPA caused an increase of 35% ($P < 0.01$) compared with untreated cells (Fig. 1A), while DHA an increase of 24.9% ($P < 0.05$) (Fig. 1B).

3.2. Intracellular lipid accumulation was differentially modulated by EPA and DHA

In order to determine whether treatment with EPA and DHA was associated with intracellular accumulation of neutral lipids, monocytes were grown on slides and incubated for 24 h with EPA and DHA at 50 μM or 200 μM. The experimental concentration of EPA and DHA was selected based on apoptosis experiments that showed marginal differences between 25 μM and 100 μM, the higher effect being observed at a concentration of 200 μM. The results obtained are showed in Fig. 2. Panel A presents isolated monocytes after Nile Red (lipid droplets) and DAPI (nucleus) staining. The number of lipid droplets per cell was dependent on both type and concentration of fatty acid. Treatment of caprine monocytes with EPA and DHA resulted in intracellular accumulation of lipid droplets. In particular, treatment of monocytes with EPA, even when added at a low concentration (50 μM) induced an accumulation of lipid droplets in 18% of goat monocytes (Fig 2B). On the contrary, DHA had fewer effects (94% negative cells where no droplets are observed).

When cells were treated with high concentration of fatty acids, the number of intracellular lipid droplets was dramatically modified. When added at 200 μM, DHA was more effective than EPA in increasing the accumulation of lipid droplets. In particular, the number of droplets was 10-fold increased, stepping from 6% to 63%. The effects of EPA were less evident. Treating monocytes with high concentrations of EPA resulted in an increase of droplets from 20% to 40%.

3.3. PLIN2 and PLIN3 mRNA abundance was regulated by EPA and DHA

The effects of EPA and DHA on three proteins belonging to the perilipin family, namely PLIN1, PLIN2 and PLIN3, were investigated by incubating caprine monocytes for 24 h with or without fatty acids and mRNA abundance was determined by quantitative PCR.

In order to investigate the presence of perilipin in goat, mRNA from CD14$^+$ monocytes was examined by qualitative PCR. PLIN2 and PLIN3 specific bands were detected in cDNA of monocytes stimulated with EPA and DHA and positive control (adipose tissue). Monocytes did not produce detectable amount of PLIN1 mRNA, as shown in Fig. 1 supplemental.

The relative quantification of PLIN2 and PLIN3 mRNA was carried by delta–delta Cq method after normalization of the sample using the SDHA and YWHAZ as reference genes. Results are presented in Fig. 3.

Table 1
Sequences of oligonucleotide primers for perilipin.

Symbol	Alias	Sense	Antisense
PLIN1	Perilipin	5′-GTCGAGAAGATGGTAGAGTTCCTC-3′	5′-TGGAAGGTGTGTCTAGAGAGAGTG-3′
PLIN2	Adipocyte differentiation-related protein (ADPR)	5′-CTGCTGGCTGATTTCTTCTTTCTC-3′	5′-TGATGTAAGCCGAGGAGACCA-3′
PLIN3	Tail-interacting protein of 47 kDa (PLIN3)	5′-GAGCATCACATCCGTCATTAGG-3′	5′-AGGGTGTGGGATGTAAGAAC-3′
SDHA	Succinate dehydrogenase complex, subunit A	5′-TGTATAATAGCTCACGCCCG-3′	5′-TCTATCAGATGGCCTCCTCAG-3′
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	5′-GCATCCCACAGACTATTTCC-3′	5′-GCAAAGACAATGACAGACCA-3′

The PLIN2, PLIN3 and SDHA were designed on the basis of GenBank sequences (accession numbers AF541976, NM001077046.1 and NM174178.2 respectively). The PLIN1 primers sequences were from Xu et al. (2011) and the YWHAZ primers from Goossens et al. (2005).
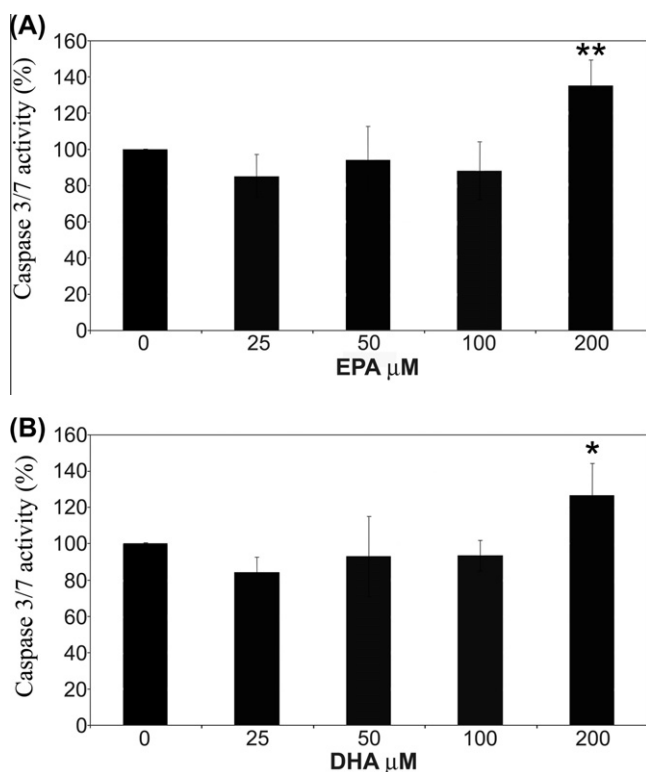
Fig. 1. Effect of EPA and DHA on caprine monocyte apoptosis rate. Positively selected blood monocytes were cultured for 24 h with different concentration of EPA (panel A) or DHA (panel B). Apoptosis was determined by measuring the modification of the caspase-3/7 enzymatic activities. Each assay was carried out in triplicate. The data are expressed as percentage of enzymatic activity and are means ± SEM of six independent experiments compared with control (100%). Asterisks indicate statistically significant difference from control ($^*P < 0.05$; $^{**}P < 0.01$).

EPA and DHA differentially modulated the PLIN2 and PLIN3 mRNA expression. EPA at 200 μM significantly ($P < 0.05$) upregulated PLIN3 mRNA expression, when compared with lower fatty acid concentration. On the contrary, DHA at 200 μM upregulated ($P < 0.05$) PLIN2 mRNA expression, when compared with lower concentrations, while PLIN3 abundance remained unchanged.

4. Discussion

The present study explored the possible relationship between EPA and DHA, two ω-3 PUFA commonly utilized as lipid diet integrators, and *in vitro* lipid droplet formation in caprine monocytes. The number of lipid droplets per cell was found to be dependent on both type and concentration of fatty acid. There is growing evidence that lipid droplets are not simply storage depots of neutral lipids, but play a precise, yet still partially undisclosed, role as inflammatory organelles (Bozza et al., 2009), probably behaving as intracellular site for eicosanoid generation during inflammation and storage of other important signaling molecules (Bozza et al., 2007).

Both EPA and DHA modulate immune activities of goat granulocytes and monocytes, including phagocytosis and oxidative burst (Pisani et al., 2009; Lecchi et al., 2011). In general, the immunomodulatory effect on leukocytes is attributed to the incorporation of dietary PUFA in phospholipids of leukocyte cellular membranes, where they are supposed to affect cellular eicosanoid synthesis and lipid peroxidation, thus altering the fluidity of cell membranes (Calder, 2006).

The relationship between EPA and DHA co-incubation and the formation of lipid droplets suggests a new possible innate immunity regulatory function of PUFA, at least in this species.

The major structural proteins that can be found at the surface of lipid droplets are those belonging to the PAT family, also known as perilipins (Bozza et al., 2009).

Since these proteins are also involved in lipid body biogenesis and assembly (Bickel et al., 2009), we hypothesized that EPA and DHA may influence the fusion process of lipid droplets by regulating perilipin mRNA expression. The second part of the study was therefore focused on the assessment of mRNA abundance of three proteins belonging to PAT family, namely PLIN1, PLIN2 and PLIN3, in order to ascertain whether EPA and DHA may influence their synthesis.

Goat monocytes did not produce detectable amounts of PLIN1 mRNA. On the contrary, both EPA and DHA upregulated mRNA expression of PLIN2 and PLIN3 when cells were treated with high concentrations of ω-3 PUFA. The function of PLIN2 is to stimulate lipid accumulation, lipid-droplet formation and lipolysis (Fukushima et al., 2005), whereas PLIN3 participates in nascent lipid droplet formation, when cellular lipid influx increases (Wolins et al., 2006). Since the modulation of PLIN2 and PLIN3 in caprine monocytes is associated with the increasing availability of ω-3 PUFAs, it can be hypothesized that EPA and DHA may modulate lipid droplet formation by regulating PLIN2 and PLIN3 mRNA expression. This activity can have a profound impact on monocyte immune functions, since a relationship between perilipin expression and innate immunity reactions has been recently demonstrated. For example, PLIN2 expression is increased during innate immunity response against leprosy (Mattos et al., 2011), and regulates MCP1 production by human monocytes (Wurfel et al., 2005). PLIN2 was also found to be involved in dendritic cell antigen presentation (Bougnères et al., 2009).

At the highest EPA and DHA concentration (200 ωM), the formation of lipid droplets was accompanied by the initiation of apoptosis. No pro-apoptotic effects were detected at ω-3 PUFA concentrations of 100 ωM. Therefore, neither EPA nor DHA substantially alter the lifespan of goat monocytes when utilized at doses that have been found to exert an immunomodulatory activity (Pisani et al., 2009; Lecchi et al., 2011). Polyunsaturated fatty acids' concentration utilized in the present investigation ranged from 50 to 200 μM and was consistent with physiological concentration of EPA and DHA in goat serum (180 and 115 μM, respectively) (Yeom et al., 2005).

This information may be useful in formulating goat diets, where ω-3 PUFA are commonly utilized as lipid source to ameliorate the percentage of unsaturated fatty acids in meat and dairy products (Moghadasian, 2008). Even when not protected, dietary administered ω-3 PUFAs are hydrogenated only to a small extent by rumen microorganisms (Ashes et al., 1992; Gulati et al., 1999). Therefore, EPA and DHA can be found in both blood and milk (Cattaneo et al., 2006), where they may contribute to modulate immune defences (Thanasak et al., 2004; Agazzi et al., 2004).

The capability of dietary ω-3-PUFA to skip the ruminal hydrogenation represents a double edge sword. On one hand it allows the delivery through diet of these important, and mostly beneficial, molecules, to the organism. On the other hand, poorly balanced diets and/or excessive intake by single goats may increase their blood concentration, eventually altering the processing stability and organoleptic quality of milk. In the present finding we provided the evidence that high concentrations of EPA and DHA promote monocyte apoptosis, which in turn might downregulate goat immune defences.

Therefore, the potential toxicity of high concentrations of ω-3 PUFA that have been shown in this study should be taken into account in formulating diet including these highly biologically active molecules.
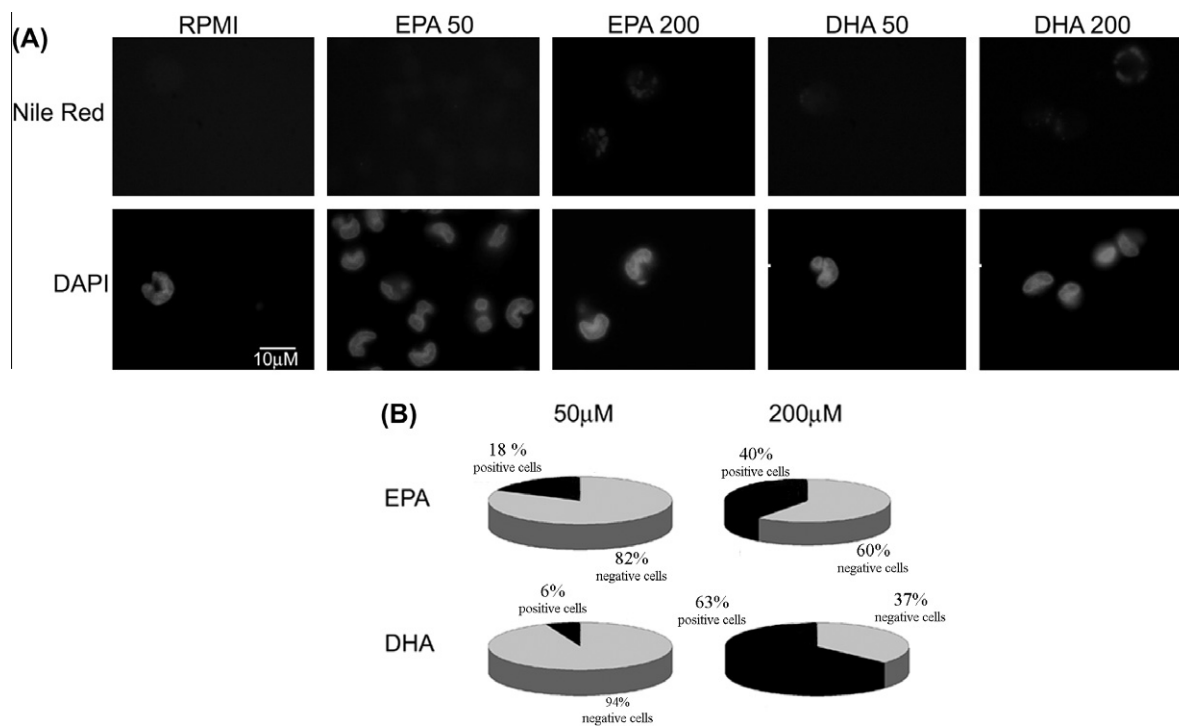
Fig. 2. Effects of EPA and DHA treatment on lipid droplet formation. Lipid droplets content in caprine monocytes incubated without fatty acids or with EPA or DHA. Isolated monocytes were cultured for 24 h in presence of 50 μM and 200 μM EPA or DHA and stained with Nile Red and DAPI nuclear staining (A). One representative set of photographs from three separate experiments is shown. RPMI cultured cells were 100% negative. Magnification: 100×. (B) Ten fields were counted and monocytes were divided in negative and positive cells.
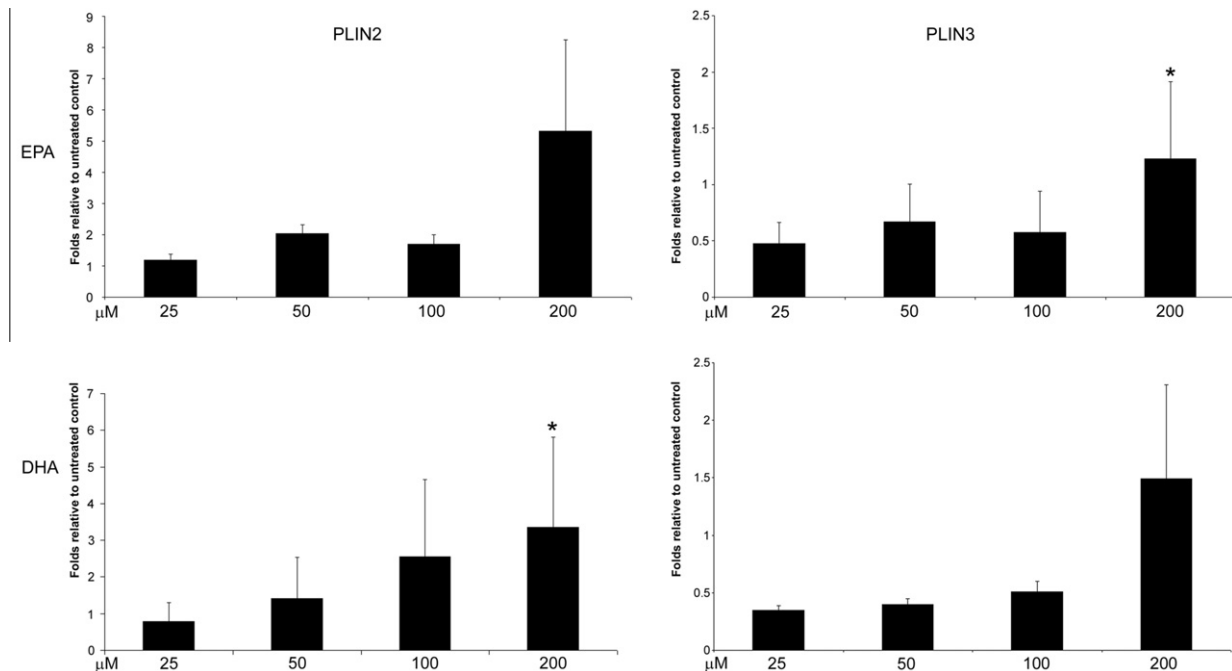

Fig. 3. Effects of EPA and DHA on PLIN2 and PLIN3 mRNA abundance. Relative PLIN2 and PLIN3 mRNA abundance in monocytes incubated with EPA (A and B) or DHA (C and D) for 24 h. The results were normalized using SDHA and YWHAZ as housekeeping genes. mRNA levels of PLIN2 and PLIN3 are expressed relative to untreated cells. Data are expressed as fold ± SEM calculated from three independent experiments. Asterisks indicate significant difference from control ($^*P < 0.05$).

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.rvsc.2012.09.019.

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