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Docosahexaenoic acid and TUG-891 activate free fatty acid-4 receptor in bovine neutrophils

Ivan Olmo, Stefanie Teuber, Camilo Larrazabal, Pablo Alarcon, Fernanda Raipane, Rafael A. Burgos*, Maria A. Hidalgo*

Laboratory of Molecular Pharmacology, Institute of Pharmacology, Faculty of Veterinary Science, Universidad Austral de Chile, Valdivia, Chile

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ABSTRACT

Fatty acids are well known metabolic intermediaries but also have a role in the immune response. Long-chain fatty acids such as omega-6 and -9 activate neutrophil function through free fatty acid (FFA)-1 receptor in bovines. Although omega-3 has also been suggested to influence neutrophil function, the details remain unclear. The goal of this study was to determine the presence of the bovine FFA4 receptor and its effect on neutrophil responses. We treated bovine neutrophils with the natural and synthetic agonists of FFA4 receptor docosahexaenoic acid (DHA) and TUG-891, respectively, and assessed oxidative and no oxidative response. We detected protein and mRNA FFA4 receptor expression through immunofluorescence, immunoblot, and RT-PCR analysis. DHA and TUG-891 both increased intracellular calcium mobilisation in bovine neutrophils, with 50% effective concentrations of 99 μM and 73 μM, respectively, which was partially reduced after treatment with the FFA4 antagonist AH7614. Furthermore, DHA and TUG-891 increased matrix metalloproteinase (MMP)-9 granules release and superoxide production. AH7614 and the intracellular calcium chelator BAPTA-AM decreased the superoxide production induced by TUG-891 and by both DHA and TUG-891, respectively, suggesting a key role of intracellular calcium in FFA4 agonists-induced superoxide production. These results highlight an important mechanism of bovine neutrophil responses mediated via FFA4 receptor, which can further inform the development of new formulations for DHA-enriched feed supplements to enhance innate immunity in dairy cattle.

1. Introduction

Neutrophils represent the first line of host defense against invasive microorganisms and are among the first cells to migrate from the bloodstream to the injured or infected tissues (Smith, 1994). Neutrophils exert several types of defensive responses in invaded or injured tissues, such as superoxide production and the release of degradative enzymes stored in cytoplasmic granules, which contribute to the inflammatory process; however, a sustained and prolonged inflammatory response can also induce potential tissue damage (Nathan, 2006; Paape et al., 2003). The release of matrix metalloproteinase (MMP)-9 granules, a no oxidative response, is triggered by several stimuli that, upon coupling to exposed extracellular receptors, produce signals to increase the intracellular generation of secondary messengers such as intracellular calcium (Faurschou and Borregaard, 2003; Sengelov et al., 1993), which is largely achieved through the endoplasmic reticulum and via calcium influx through the plasma membrane (Burgos et al., 2011; Yin and Heit, 2017).

Fatty acids are well-recognized metabolic intermediaries, but recent evidence indicates that they also function as signaling molecules with a clear role in the immune response, thus establishing a link between metabolism and immunity (Lackey and Olefsky, 2016). Indeed, fatty acids have been shown to modulate the functions of macrophages and neutrophils (Rodrigues et al., 2016; Walls et al., 2016). Short-chain fatty acids act as neutrophil chemoattractants that induce granules releases and oxidative response such as reactive oxygen species (ROS) production (Carretta et al., 2013; Maslowski et al., 2009; Vinolo et al., 2009), whereas long-chain fatty acids (LCFAs) induce different responses depending on whether omega-3, omega-6, or omega-9 fatty acids are used. For example, the omega-3 docosahexaenoic acid (DHA) reduces tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, monocyte chemoattractant protein-1, and IL-1β production along with inflammasome activation in murine macrophages (Oh et al., 2010; Williams-Bey et al., 2014), but increases TNF-α and ROS production in

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Abbreviations: FFA4, Free fatty acids-4 receptor; DHA, docosahexaenoic acid; MMP-9, matrix metalloproteinase-9; ROS, reactive oxygen species; LCFAs, long-chain fatty acids; COX-2, cyclooxygenase-2; FFA, free fatty acid; GPCR, G-protein coupled receptors; NETs, neutrophil extracellular traps

* Corresponding authors.
E-mail addresses: rburgos1@uach.cl (R.A. Burgos), mahidalgo@uach.cl (M.A. Hidalgo).

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rat neutrophils (Paschoal et al., 2013). Linoleic and oleic acids (omega-6 and omega-9, respectively) induce intracellular calcium release, mitogen-activated protein kinase phosphorylation, cyclooxygenase-2 (COX-2) expression, IL-8 secretion, ROS production, and MMP-9 release in bovine neutrophils (Hidalgo et al., 2011; Mena et al., 2013, 2016), and oleic acids further increase cell the surface expression of CD11b and ROS production in human and rat neutrophils (Hatanaka et al., 2006; Mastrangelo et al., 1998).

Fatty acids are also known to activate G-protein coupled receptors (GPCRs). LCFAs activate the GPCRs for free fatty acid (FFA)1 and FFA4 (Briscoe et al., 2003; Hirasawa et al., 2005), whereas SCFAs activate FFA2 and FFA3 receptors, which are expressed in bovine neutrophils (Alarcon et al., 2018; Carretta et al., 2013). Omega-3 fatty acids are ligands for FFA4, also known as GPR120, which has the ability to regulate gut incretin glucagon-like peptide-1 secretion (Hirasawa et al., 2005) and reduce inflammasome activation in macrophages (Oh et al., 2010). FFA4 has been described in the intestine enteroendocrine cells, lung, adipose tissue, hypothalamus, and macrophages (Cintra et al., 2012; Hirasawa et al., 2005; Miyauchi et al., 2009; Oh et al., 2010); however, its expression in bovine neutrophils has not yet been established. Omega-3 fatty acids have been proposed as a strategy to improve immune function in dairy cows. Diets containing fatty acids rich in omega-3 increased phagocytic capacity of neutrophils isolated from transition dairy cows and decreased expression of pro-inflammatory markers in milk cells and peripheral blood mononuclear cells (Gandra et al., 2016; Rezamand et al., 2016). Also, DHA attenuated inflammatory response in primary bovine mammary epithelial cells (He et al., 2017). Since neutrophils play a key role in innate immunity, and omega-3 fatty acids have potential beneficial effects in dairy cattle, the goal of our study was to determine the presence of FFA4 receptor in bovine neutrophils, and the effects of both natural and synthetic FFA4 ligands, DHA (Hirasawa et al., 2005; Oh et al., 2010) and TUG-891 (Hudson et al., 2013; Shimpukade et al., 2012), respectively, on intracellular calcium mobilisation. We further investigated the effect of DHA and TUG-891 on the release of MMP-9 granules and superoxide production given the well known role of calcium in these processes (Sengelov et al., 1993; Yin and Heit, 2017).

2. Materials and methods

2.1. Neutrophils isolation

Blood was collected from five healthy Holstein heifers of an Universidad Austral de Chile herd by jugular venipuncture, and placed in acid citrate dextrose tubes (Becton Dickinson, Franklin Lake, NJ, USA). All experiments were conducted in strict accordance with the recommendations of “Comisión Nacional de Investigación Científica y Tecnológica,” and according to the current Chilean Animal Protect Laws, by the ethical committee of the Universidad Austral de Chile. The protocol was approved by the ethical committee of the Universidad Austral de Chile (permit number: 217/2015). Neutrophils were isolated according to a previously described method (Hidalgo et al., 2004). Viability was determined by trypan blue exclusion assays and was at least 97% for all experiments. Neutrophil purity was at least 95%, as assessed by flow cytometry (BD FACSCanto II; Becton Dickinson) using a forward-scatter vs. side-scatter dot plot to determine the relative size and granularity of the cells (Quinn et al., 2007).

2.2. Intracellular calcium measurement

Neutrophils (1×10^7 cells/ml) were suspended in Hanks' balanced salt solution (HBSS) and incubated with 2.5 μM Fura-2 AM fluorescent indicator dye (Molecular Probes Inc., Eugene, OR, USA) for 30 min at 37 °C. The cells were washed three times, and then Fura-2 AM-loaded cells (2×10^6 cells/ml) were suspended in HBSS supplemented with 0.9 mM $CaCl_2$ and incubated for 5 min at 37 °C. The basal fluorescent signal was measured at 509 nm emission with 340/380 nm dual-wavelength excitation using an LS55 spectrofluorimeter (PerkinElmer Life Science), in cuvette maintained with temperature (37 °C) and stirring constant. Then the cells were treated with vehicle [0.1% dimethyl sulfoxide (DMSO)], DHA (1–200 μM), or TUG-891 (1–200 μM). The range of concentrations of DHA is according to that reported in previous studies (Abdelmagid et al., 2015; Lund-Blix et al., 2016; Mattos et al., 2003; Plewes et al., 2017). In experiments with FFA4 antagonist and inhibitors, Fura-2 AM-loaded neutrophils were incubated with AH7614 (0.5, 1, or 5 μM; Tocris, Bristol, UK), a non-competitive negative allosteric modulator of FFA4 agonists (Sparks et al., 2014; Watterson et al., 2017), for 15 min or the intracellular calcium chelator BAPTA-AM (50 μM) for 10 min at 37 °C and then stimulated with DHA or TUG-891 (50 μM). The 50% effective concentration (EC50) was calculated as the intracellular calcium mobilisation of the area under the curve (AUC) at 300 s after addition of the stimulus.

2.3. Cell viability

Neutrophils (2×10^4) were incubated with DHA (1–100 μM), TUG (1–100 μM), or vehicle (0.1% DMSO) for 5 min, and cell viability was assessed with the Celltiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WA, USA) in 96-well clear plates (SPL Life Science).

2.4. Zymography

Neutrophils (1×10^6) were incubated with DHA (1–200 μM), TUG-891 (1–200 μM), or vehicle (0.1% DMSO) for 5 min at 37 °C. In assays with the FFA4 antagonist, neutrophils were incubated with AH7614 (0.5, 1, or 5 μM) for 15 min at 37 °C and then stimulated with DHA or TUG-891 (50 μM) for 5 min at 37 °C. To block intracellular or extracellular calcium, neutrophils were incubated with BAPTA-AM (50 μM) for 10 min or EGTA (0.3 mM), respectively, for 15 s at 37 °C, and then treated with DHA or TUG-891 (50 μM) for 5 min at 37 °C. After incubation, the neutrophils were centrifuged at 600 $\times g$ for 6 min, and equal amounts of supernatants were assayed for gelatinase activity by zymography, as described previously (Manosalva et al., 2015). In brief, 10 μl of the supernatant was loaded onto 10% polyacrylamide gels (0.75-mm thick) containing 0.2% gelatin. The gels were run at 200 V for 1 h in a Mini Protean II apparatus (Bio-Rad Laboratories, Richmond, CA, USA) and then soaked twice in 2.5% Triton X-100 in distilled water on a shaker at room temperature for 30 min. The gels were then soaked in reaction buffer consisting of 100 mM Tris (pH 7.5) and 10 mM $CaCl_2$ at 37 °C overnight, followed by staining in 0.5% Coomassie Brilliant Blue R-250 (Winkler, Santiago, Chile) in acetic acid:methanol:water (1:3:6). Evidence of enzymatic activity was determined by measuring the non-staining areas in which the gelatin was degraded. To measure the gelatinolytic activity of MMP-9, the gel images were digitized, and the intensity of the bands was determined using ImageJ 1.35 s software (National Institutes of Health, Bethesda, MD, USA). MMP-9 was analysed at 5 min of incubation because shorter time periods are indicative of granules release in neutrophils (Carretta et al., 2013; Hidalgo et al., 2011; Opdenakker et al., 2001).

2.5. Superoxide production

Neutrophils (1×10^6 cells) were loaded with 10 μM hydroethidine (Molecular Probes, Eugene, OR, USA) for 5 min at 37 °C, and then 2.5×10^5 neutrophils/250 μl (final volume) were incubated with DHA (1–200 μM), TUG-891 (1–200 μM), or vehicle (0.1% DMSO) for 5 min at 37 °C. In assays with the FFA4 antagonist, the neutrophils were incubated with AH7614 (1 μM) for 15 min at 37 °C and then stimulated with DHA or TUG-891 (50 μM) for 5 min at 37 °C. In experiments with BAPTA-AM or EGTA, the neutrophils were incubated in the same manner as described above, and then with DHA or TUG-891 for 5 min at

37 °C. Fluorescence was analysed using a flow cytometer and FlowJo 7.6 software (Tree Star, Inc. USA).

2.6. Immunocytochemistry

Neutrophils (2.5×10^5) were incubated in 3% paraformaldehyde/ 0.19 M sucrose (100 µl final volume) for 15 min and then transferred to slides by cytospinning. The slides were washed with phosphate-buffered saline (PBS) before the neutrophils were permeabilized with 100 µM digitonin for 10 min. The slides were then washed, incubated with buffer blocking (PBS, 0.1% Tween, 1% bovine serum albumin) for 1 h, and then incubated with the rabbit polyclonal anti-FFA4 antibody (1:200 dilution) (97272 Abcam, USA) overnight. The slides were then incubated with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (1:200 dilution) (Molecular Probes, Eugene, OR, USA). To visualize nuclei, the slides were stained with Hoechst (1:4000 dilution) (H3570 Invitrogen, USA) and then analysed using a confocal microscope (Fluoview FV1000, Olympus).

2.7. Immunoblotting

Neutrophils (5×10^6 cells) were lysed and analysed according to a previously described method (Hidalgo et al., 2011). FFA4 was detected with rabbit polyclonal anti-FFA4 antibody (1:1000 dilution) (97272 Abcam, USA) and the secondary antibody IRDye® 800-conjugated goat anti-rabbit (1:15,000 dilution) (Licor, Lincoln, NE, USA) according to the manufacturer's instructions. Detection was performed on an Odyssey® Fc Imaging System (LI-COR). Bovine endometrial cells were used as control.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the EZNA™ Total RNA Isolation Kit (Omega Bio-Tek, USA). For cDNA synthesis, 250 ng of total RNA was subjected to reverse transcription with Affinity Script RT (Stratagene, USA), and then real-time PCR was performed using the Brilliant II SYBRGreen qPCR kit (Stratagene) with primers specific for bovine FFA4 (forward 5′–ACCAGCTCCATCCCGCTCGT-3′; reverse, 5′-CTGACTGCCG CCAGCGTGAA-3′) and GAPDH (Hidalgo et al., 2011). The following conditions were used: 95 °C for 10 min and 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. This was followed by three additional steps (dissociation curve): 95 °C for 1 min, 60 °C for 30 s and 95 °C for 30 s. Products were separated on 2% agarose gels and stained with ethidium bromide for analysis.

2.9. Statistical analysis

All experimental protocols were performed in quintuplicate (independent samples). The results are presented in bar graphs as the mean ± SEM. For statistical analysis, analysis of variance and Dunnett's multiple comparison or t-tests were performed. All analyses were performed using Graph Pad Prism v6.01 software (GraphPad Inc., La Jolla, CA, USA) and judged at a significance level of 5% ($p < 0.05$).

3. Results

3.1. DHA and TUG-891 induce intracellular calcium mobilisation

We used the known natural FFA4 ligand DHA (Hirasawa et al., 2005; Oh et al., 2010) and the potent and selective synthetic FFA4 ligand TUG-891 (Hudson et al., 2013; Shimpukade et al., 2012). Both DHA and TUG-891 induced early and sustained intracellular calcium mobilisation in neutrophils. The calcium response was concentration-dependent, with an EC50 value of 99.33 µM and 73.74 µM for DHA and TUG-891, respectively (Fig. 1A and B).

The effect of DHA and TUG-891 on intracellular calcium was assessed for 5 min, therefore also we determined if these agonists affect cell viability. Neutrophils were incubated with different concentrations (1–100 µM) DHA or TUG-891 for 5 min, and we observed that the highest concentration (100 µM) of DHA and TUG-891 tested significantly reduced cell viability to 34.58 ± 14.99% and 41.8 ± 13.8%, respectively, compared to the vehicle-only control; therefore, the following experiments were performed with 50 µM of each stimulus. To explore possible mechanisms of death induced by DHA, we assessed whether 50 µM DHA could induce apoptosis. Neutrophils were incubated with 50 µM DHA for 5 min in presence or absence of diphenyleneiodonium (DPI, a NOX2 inhibitor) and apoptosis was assessed by Annexin V-PI staining and FACS analysis. We observed that DHA did not induce apoptosis (Supplementary figure). Superoxide production was also assessed and we observed that DPI reduced DHA-induced superoxide production. Next, we intended to evaluate whether DHA could induce another mechanism of death, such as netosis, because it has been described that neutrophil extracellular traps (NETs) formation is dependent of NADPH oxidase-derived superoxide (Kirchner et al., 2012). Neutrophils were incubated for 5 min with DHA in absence or presence of DPI or AH7614 (a FFA4 antagonist) and the NETs formation was evaluated by staining with PicoGreen. DHA induced NETs formation, and the treatment with DPI reduced DHA-induced NET formation, suggesting that superoxide participates in the DHA-induced NETs formation (Supplementary figure). Interesting, we observed a reduction in DHA-induced NETs formation in neutrophils treated with AH7614. All these results suggest that DHA, acting through FFA4 receptor could reduce viability in neutrophils by a mechanism alternative to apoptosis. These data suggest NETs formation a potential mechanism, which should be further studied.

To assess a possible role of FFA4 receptor in intracellular calcium increase induced by DHA and TUG-891, we used the FFA4 antagonist AH7614 (Sparks et al., 2014; Watterson et al., 2017), which has been used to demonstrate the role of FFA4 in various previous studies (Houthuijzen et al., 2017; Quesada-Lopez et al., 2016; Tran et al., 2016; Villegas-Comonfort et al., 2017). AH7614 partially reduced the intracellular calcium mobilisation (based on the AUC value) at all concentration tested (0.5, 1, and 5 µM) compared to the DMSO vehicle control (Fig. 1C and D).

3.2. FFA4 is expressed in neutrophils

The results above suggested the presence of FFA4 in neutrophils; therefore, we next evaluated whether FFA4 mRNA and protein are expressed in these cells. Fig. 2A shows the results of confocal microscopy of FFA4 immunofluorescence in neutrophils, demonstrated positive staining mainly toward the cell membrane, which is the typical localization of GPCRs such as FFA4. Immunoblotting further demonstrated a main band near 38 kDa (and two nearby minor bands), which is in accordance with the predicted size of FFA4 (42 kDa) (Fig. 2B). In addition, the RT-PCR analysis from neutrophil RNA showed amplification of a single product of 134 bp, which corresponds with the size expected from the primers used (Fig. 2C).

3.3. DHA and TUG-891 induce the release of MMP-9 granules and superoxide production

Since is well known the role of calcium in MMP-9 granules release and superoxide production in neutrophils (Sengelov et al., 1993; Yin and Heit, 2017), and the present data about FFA4 agonist-induced calcium, we studied the effect of DHA and TUG-891 in these responses. DHA and TUG-891 (50–200 µM) significantly induced the release of MMP-9 granules (Fig. 3A) and increased superoxide production (Fig. 3B) after 5 min. The estimated EC50 values for MMP-9 granules release were 53 µM and 47 µM, for DHA and TUG-891, respectively, and for superoxide production were 58 µM and 48 µM, for DHA and TUG-891, respectively.

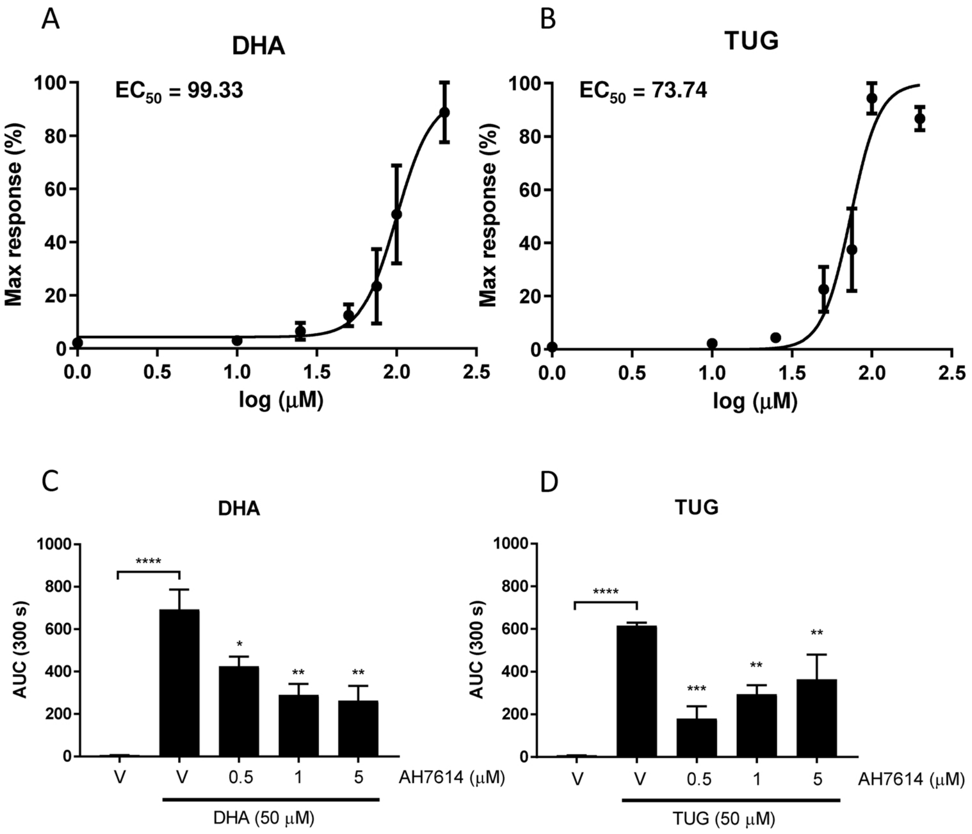

Fig. 1. DHA and TUG-891 induce intracellular calcium mobilisation via FFA4 receptor. (A and B) Dose-response curve of DHA and TUG-891 for intracellular calcium mobilisation in Fura-2 AM-loaded neutrophils. (C and D) Effect of the FFA4 antagonist AH7614 on intracellular calcium mobilisation induced by DHA or TUG-891 (50 μM). V: vehicle (0.1% DMSO). (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

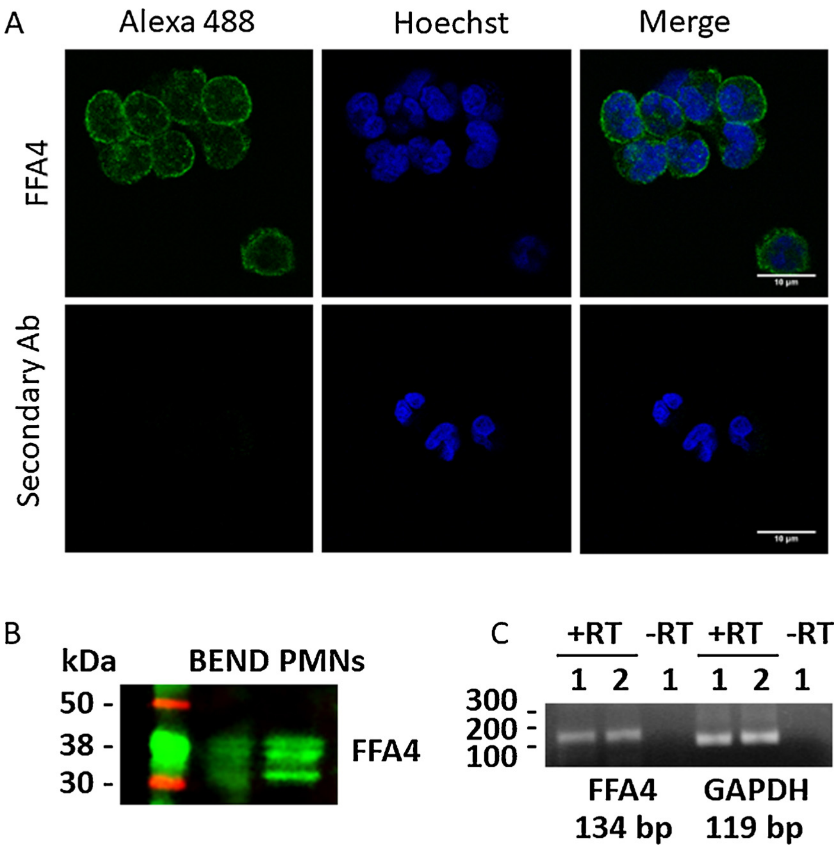

Fig. 2. Neutrophils express the FFA4 receptor. (A) Immunofluorescence and confocal microscopy analysis of FFA4 in neutrophils. Nuclei were stained with Hoescht. Control without primary antibody (Secondary Ab) is shown. (B) Immunoblot of FFA4 from total proteins of neutrophils; bovine endometrial cells (BEND) were used as a control. (C) RT-PCR of *FFA4* expression from total RNA of neutrophils. *GAPDH* was used as housekeeping gene for normalization. 1 and 2 are samples from two different animals. + RT: reaction in presence of reverse transcriptase; - RT: reaction in absence of reverse transcriptase.

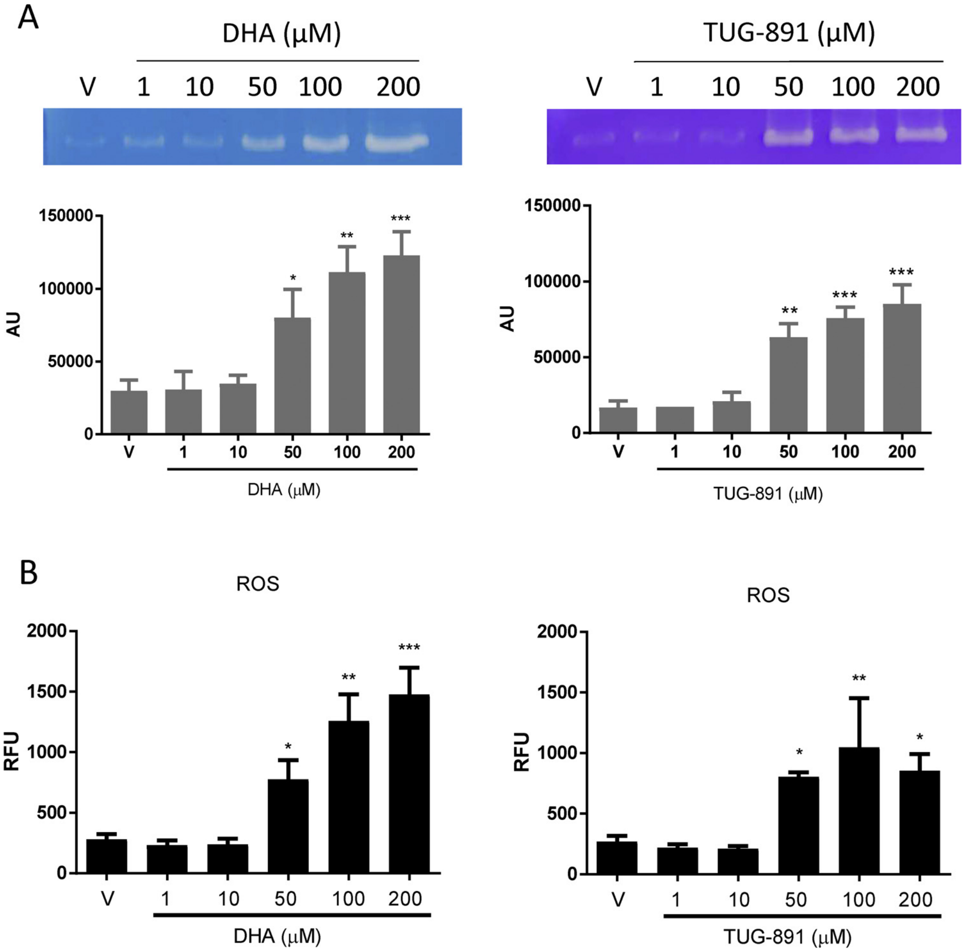

Fig. 3. FFA4 agonists increase MMP-9 granules release and superoxide production. Neutrophils were stimulated for 5 min with different concentrations of DHA or TUG-891, and the MMP-9 release in the supernatant (A) and superoxide production (B) were analysed by zymography and flow cytometry, respectively. V: vehicle. AU: arbitrary units. RFU: relative fluorescence units. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the vehicle.

The specific role of FFA4 in these responses was then assessed by incubation with the antagonist AH7614. As shown in Fig. 4A, AH7614 did not reduce the DHA- or TUG-891-induced MMP-9 release. Although the FFA4 antagonist suppressed the superoxide production induced by TUG-891 it did not affect that induced by DHA (Fig. 4B).

We next evaluated whether the calcium levels remaining inside the cells in the presence of AH7614 and DHA or TUG-891 would be sufficient for MMP-9 granules release or superoxide production. Blocking intracellular calcium with BAPTA-AM significantly reduced the superoxide production induced by DHA and TUG-891, but did not affect MMP-9 release (Fig. 5A and B). A representative experiment of the effect of BAPTA-AM on intracellular calcium mobilisation induced by DHA or TUG-891 is shown in Fig. 5C. Blocking extracellular calcium with EGTA did not reduced MMP-9 release and superoxide production induced by DHA and TUG-891. These results suggest that the calcium levels inside neutrophils maintained after treatment with AH7614 and FFA4 agonists would be sufficient to induce superoxide production.

4. Discussion

Several studies have described the mechanisms by which omega-3 fatty acids have beneficial effects on the immune response, such as their effects on the inflammasome and autophagy in human or murine cells, highlighting a role of FFA4 as the main omega-3 receptor (Oh et al., 2010; Williams-Bey et al., 2014). In this study, we further demonstrate that bovine neutrophils express FFA4 receptor, which induces intracellular calcium release. In addition, FFA4 agonists could induce the release of MMP-9 granules and superoxide production in neutrophils.

A previous study showed that DHA and eicosapentaenoic acid could differentially modulate certain responses in rat neutrophils such as superoxide release, phagocytic capacity, and cytokine release (Paschoal et al., 2013); however, the role of FFA4 was not assessed. The detection of mRNA and protein levels of FFA4 receptor in bovine neutrophils, activated by natural and synthetic FFA4 agonists, further suggest a role of FFA4 in the immune response. Both DHA and TUG-891 increased intracellular calcium mobilisation, a well known response of FFA4 activation. Similarly, intracellular calcium release has been observed in different cell types with the use of other FFA4 agonists such as grifolic acid, FFA4 agonist III, α-linolenic acid, along with TUG-891 (Briscoe et al., 2006; Hudson et al., 2013; Paillamanque et al., 2017). To assess the participation of FFA4 in the intracellular calcium mobilisation induced by DHA and TUG-891, we used the pharmacological antagonist AH7614, and observed a statistically significant reduction in this response; however, AH7614 did not completely block intracellular calcium mobilisation, even at higher concentrations. The -log half-maximal inhibitory concentration of AH7614 was reported to be 7.1; however, several concentrations have been tested in various studies (1–100 µM) to inhibit responses stimulated by FFA4 agonists (Houthuijzen et al., 2017; Quesada-Lopez et al., 2016; Sparks et al., 2014; Tran et al., 2016; Villegas-Comonfort et al., 2017). By contrast, one study described that AH7614 alone induced intracellular calcium in rat spermatids (Paillamanque et al., 2017). These differences in the effect of AH7614 could be due to its role as a non-competitive negative allosteric modulator of FFA4 agonists (Watterson et al., 2017).

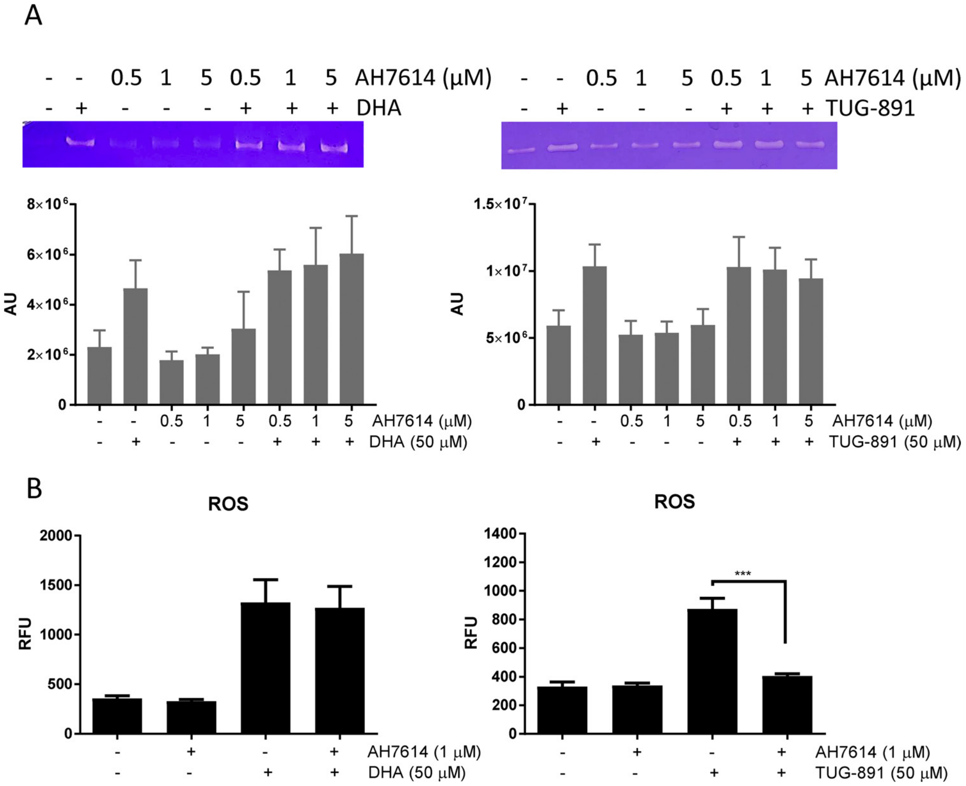

Fig. 4. Effect of the FFA4 antagonist AH7614 on MMP-9 release and superoxide production. Neutrophils were treated with AH7614 for 15 min and then DHA or TUG-891 was added for 5 min. MMP-9 release (A) and superoxide production (B) were analysed. AU: arbitrary units. RFU: relative fluorescence units. ***p $<$ 0.001.

Currently, AH7614 is the only available tool to assess the role of FFA4 in neutrophils, because a knockdown assay is difficult owing to the short lifespan and high level of activation in neutrophils after typical transfection protocols (Tamassia et al., 2012).

In addition to the robust intracellular calcium mobilisation, DHA and TUG-891 also induced MMP-9 granules release and superoxide production, supporting a key role of calcium in bovine neutrophil defense responses (Burgos et al., 2011). Pisani et al. (Pisani et al., 2009) described a reduction of basal extracellular ROS production in DHA-treated goat neutrophils, which would appear to conflict with our results at first glance; however, this discrepancy might be explained by differences in the study protocols such as the use of an assay of extracellular ROS measurement and time of the assay (30–120 min), because DHA at 30–40 min can be converted to 17S series resolvins by lipoxygenation, which are important mediators in resolution (Hong et al., 2003). In addition, this result could suggest the basal activation of neutrophils. Surprisingly, co-treatment of neutrophils with AH7614 and DHA or TUG-891 did not result in inhibition of MMP-9, and superoxide production was reduced by AH7614 only in the TUG-891-stimulated neutrophils. This result suggests that DHA could independently affect FFA4, although the mechanism remains to be elucidated. He (He et al., 2017) suggested that DHA reduced the lipopolysaccharide-stimulated inflammatory response in mammary epithelial cells through a mechanism partly dependent on peroxisome proliferator-activated receptor gamma activation, and other studies have suggested that DHA produces changes in the physical-chemical properties of the membrane owing to the incorporation of DHA in lipid rafts (Corsetto et al., 2017; Schaefer et al., 2016).

Since AH7614 did not completely block the intracellular calcium mobilisation induced by DHA or TUG-891, it is possible that the remaining intracellular calcium could be sufficient to induce MMP-9 release, superoxide production, and other calcium-dependent responses in neutrophils. We confirmed that intracellular calcium is crucial for the superoxide production induced by FFA4 agonists because only BAPTA-AM (the intracellular calcium chelator) reduced this response, whereas MMP-9 release was not inhibited by either BAPTA-AM or EGTA (an extracellular calcium inhibitor), suggesting that other mechanisms could be more important in this response induced by FFA4 agonists. Moreover, different calcium pools are necessary to induce specific neutrophil responses. For example, oleic acid, an FFA1 agonist, was found to induce CD11b surface expression in an intracellular calcium-dependent manner, whereas oleic acid-induced MMP-9 release required extracellular calcium (Hidalgo et al., 2011).

5. Conclusion

We have provided the first demonstration of the presence of FFA4 receptor in neutrophils, and that FFA4 agonists could increase intracellular calcium through the FFA4 receptor. We also demonstrated that DHA and TUG-891 induced an oxidative and no oxidative response, and we suggested that DHA could partially increase oxidative response through FFA4; however, other mechanisms should be investigated. The presence of FFA4 receptor in bovine neutrophils and its role in neutrophil function contributes important insight for further uncovering the mechanism by which DHA exerts its well-known beneficial biological effects in cows toward improving the innate immunity of cows through food supplements rich in DHA.

Author contributions

IO, ST, CL, and FR performed the experiments. PA performed the immunofluorescence experiment and data analysis. RB contributed to the data analysis and writing of the manuscript. MH conceived and designed the experiments, and wrote the manuscript.

Additional information

The authors declared no conflicts of interest with respect to the

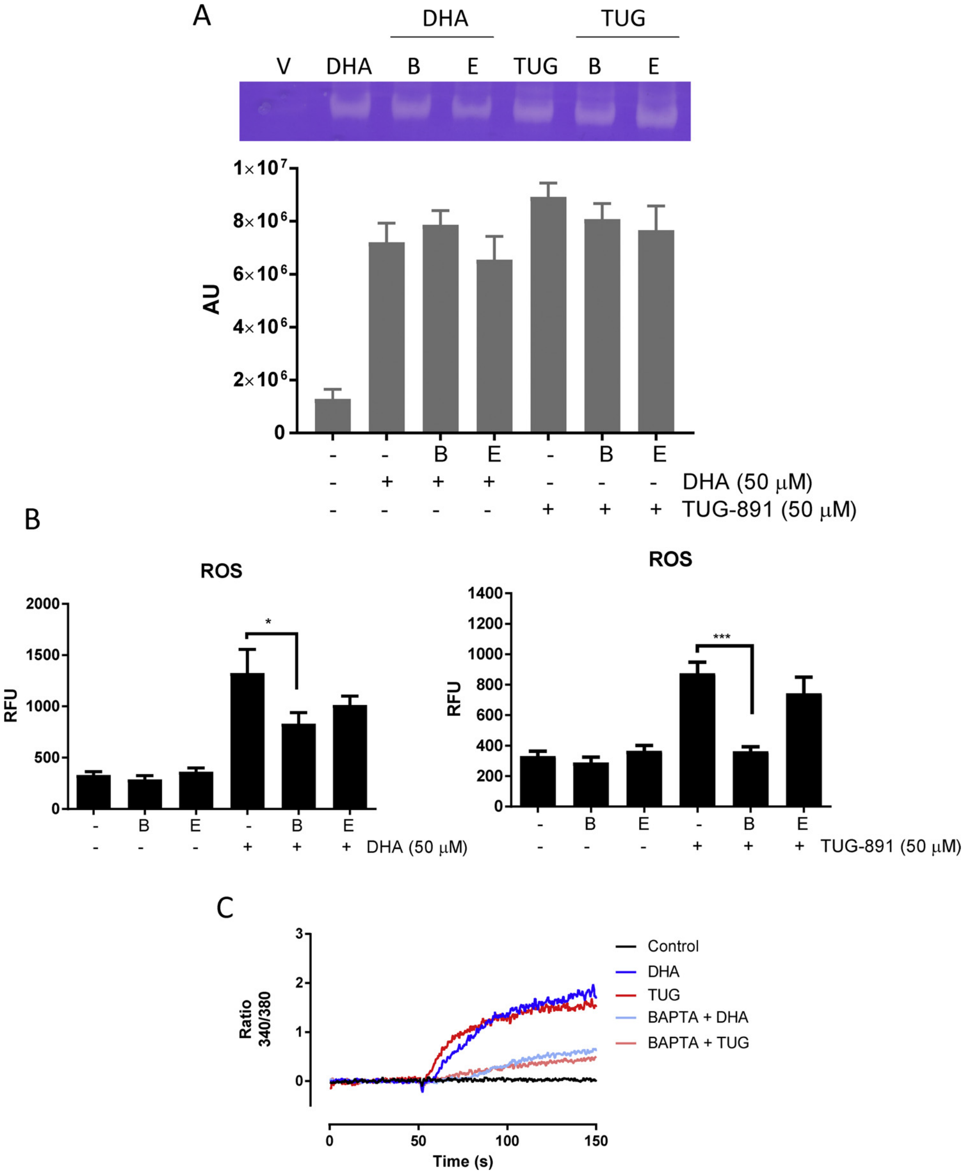

Fig. 5. Intracellular calcium is required for superoxide production induced by DHA or TUG-891. Neutrophils were incubated with BAPTA-AM (50 μM) or EGTA (0.3 mM), and DHA, TUG-891 or vehicle (V) (0.1% DMSO) was added for 5 min. MMP-9 release (A), superoxide production (B), and intracellular calcium mobilisation (C) were analysed. B: BAPTA-AM. E: EGTA. $^{*}p < 0.05$, $^{***}p < 0.001$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetimm.2019.02.008.

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