Oral Administration of Oleic or Linoleic Acid Accelerates the Inflammatory Phase of Wound Healing

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The effects of oral ingestion of oleic (OLA) and linoleic (LNA) acids on wound healing in rats were investigated. LNA increased the influx of inflammatory cells, the concentration of hydrogen peroxide (H_2O_2) and cytokine-induced neutrophil chemoattractant-2αβ (CINC-2αβ), and the activation of the transcription factor activator protein-1 (AP-1) in the wound at 1 hour post wounding. LNA decreased the number of inflammatory cells and IL-1, IL-6, and macrophage inflammatory protein-3 (MIP-3) concentrations, as well as NF-κB activation in the wound at 24 hours post wounding. LNA accelerated wound closure over a period of 7 days. OLA increased TNF-α concentration and NF-κB activation at 1 hour post wounding. A reduction of IL-1, IL-6, and MIP-3α concentrations, as well as NF-κB activation, was observed 24 hours post wounding in the OLA group. These data suggest that OLA and LNA accelerate the inflammatory phase of wound healing, but that they achieve this through different mechanisms.

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INTRODUCTION

Wound healing has been recognized as important to health at least since the time of Hippocrates. This process involves an intrinsic and coordinated cascade of events that requires the communication among different cell types (neutrophils, macrophages, endothelial cells, fibroblasts, and keratinocytes) and the generation of mediators such as cytokines (IL-1β, tumor necrosis factor-α (TNF-α), and cytokine-induced neutrophil chemoattractant-2αβ (CINC-2αβ)), reactive oxygen species (ROS), and vascular endothelial growth factors (VEGFs). Most therapeutic approaches to wound healing are focused on the maintenance of the water barrier and infection control (Broughton, 2006). Therefore, the dressings used in patients with hard-to-heal wounds differ mainly in the compounds they are impregnated with, such as silver, growth factors, or fatty acids. Silver has been used because of its antibacterial effects. However, some studies demonstrate that silver has cytotoxic activity toward different cell types and can delay wound healing (Atiyeh *et al.*, 2007). The use of topical growth factors is based upon the roles of these proteins in the repair process and some studies have demonstrated that these can be effective in wound treatment (Berlanga-Acosta *et al.*, 2009). The topical administration of oils or fatty acids on open wounds is a low-cost alternative in the treatment of wounds. However, literature on this approach is relatively scarce. Because of their physicochemical characteristics, oils prevent water loss and the invasion of microorganisms, thus avoiding the development of wound infections (Declair, 1997).

Early studies identifying essential fatty acids (EFAs) showed impaired wound healing as a sign of EFA deficiency (Burr, 1930). The EFA linoleic acid (LNA) is the main fatty acid in the epidermis (McCusker and Grant-Kels, 2010), in which it has an important functional role such as maintenance of the stratum corneum permeability barrier, maturation and differentiation of stratum corneum, formation and secretion of lamellar bodies, and promotion of wound healing. However, when the ingestion of EFA and other polyunsaturated fatty acids is deficient, an imbalance among the fatty acids in the epidermis is seen, with increased accumulation of the nonessential oleic acid (OLA), resulting in abnormal stratum corneum appearance and permeability (Hansen and Jensen, 1985).

OLA and LNA are the main unsaturated fatty acids present in Western diets (U.S. Department of Agriculture, 2008). Both

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Abbreviations: AP-1, activator protein-1; CINC-2αβ, cytokine-induced neutrophil chemoattractant-2αβ; EFA, essential fatty acid; H_2O_2, hydrogen peroxide; LNA, linoleic acid; MIP-3, macrophage inflammatory protein-3; OLA, oleic acid; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor

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OLA and LNA have an impact on the risk of various chronic diseases, and both are able to modify a range of immune cell functions (Pompeia *et al.*, 2003; Cury-Boaventura and Curi, 2005; Gorjao *et al.*, 2007; Martins de Lima *et al.*, 2007; Cury-Boaventura *et al.*, 2008; Rodrigues *et al.* 2010), as well as have an effect on epidermis constitution (Hansen and Jensen, 1985; Declair, 1997; McCusker and Grant-Kels, 2010). However, their roles in wound healing have been little explored. Considering the fact that immune cells are involved in all the phases of the wound healing process and that OLA and LNA can affect immune cell functions, we investigated the effects of these two fatty acids on the wound healing process in rats. To our knowledge, this is previously unreported.

RESULTS

Rats were subjected to full-thickness 10 mm^2 punch biopsies on the left dorsal shaved flank and monitored for up to 16 days. Pure OLA and LNA were administered orally each day from 5 days before wounding until wound closure. Improvement in wound closure was noticed 7 days after wounding, based on wound area (%) in animals supplemented with LNA in comparison with the control and OLA groups (Figure 1a and b).

The morphological analysis of the wounds showed that a layer of connective tissue (mostly loose) from the deep dermis still remained. At 1 hour after wounding, some degree of hemorrhage was present at the wound surface, and very few inflammatory cells were observed in the control and OLA groups (Figure 2). The LNA group, however, showed a visible infiltration of inflammatory cells, mainly neutrophils (~40%), especially surrounding the microvasculature (Figure 2c and f). After 24 hours of wounding, the connective tissue was highly infiltrated with inflammatory cells (mostly neutrophils and lymphocytes), and a thick superficial mass constituting clot, dead tissue, and inflammatory cells was observed in all groups. The inflammatory infiltrate under this mass was visibly lower in the LNA group than in the other two groups (Figure 2).

To identify the phenotype of the inflammatory cells present at the wound site, cell suspensions were prepared and analyzed by flow cytometry. The population (R1) was characterized by high forward scatter and low side scatter

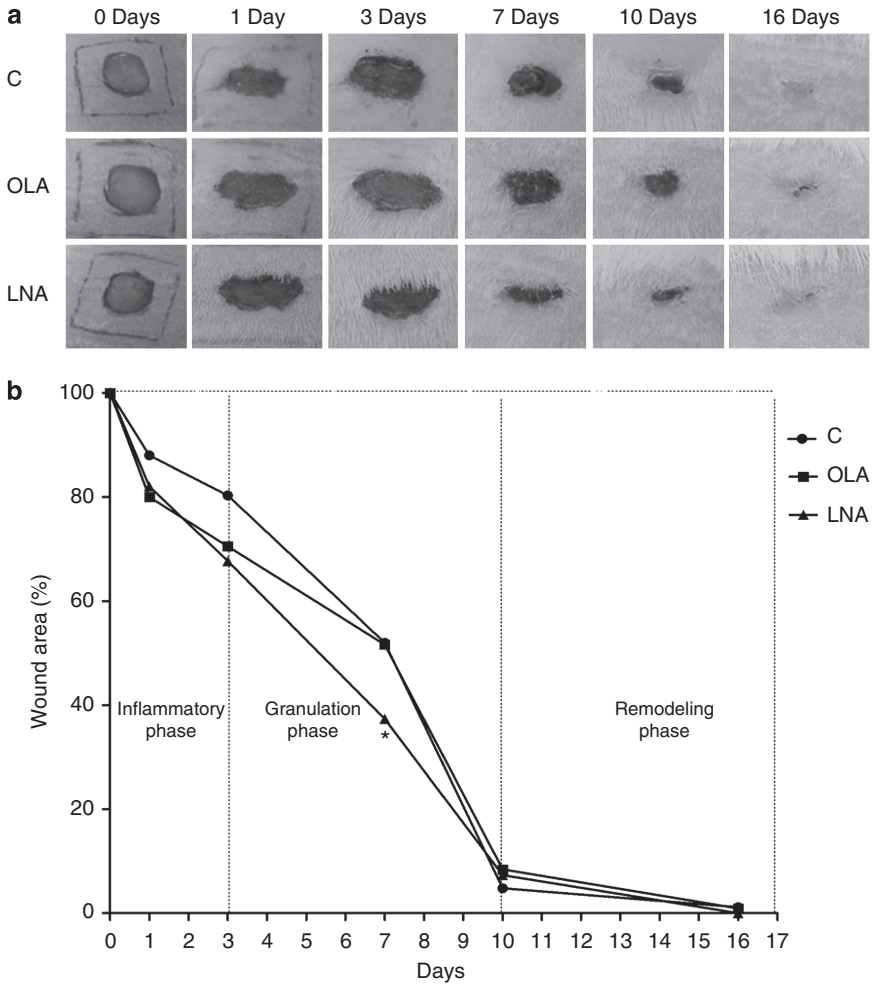

Figure 1. Macroscopic wound closure of control rats (C) or rats supplemented daily with oleic acid (OLA) or linoleic acid (LNA). (**a**, **b**) Representative photos and wound area values during the time course of 16 days. Values are expressed as mean ± SEM of at least 10 animals per group. *$P<0.05$ versus control as indicated by analysis of variance (ANOVA) and *post hoc* Tukey's test.

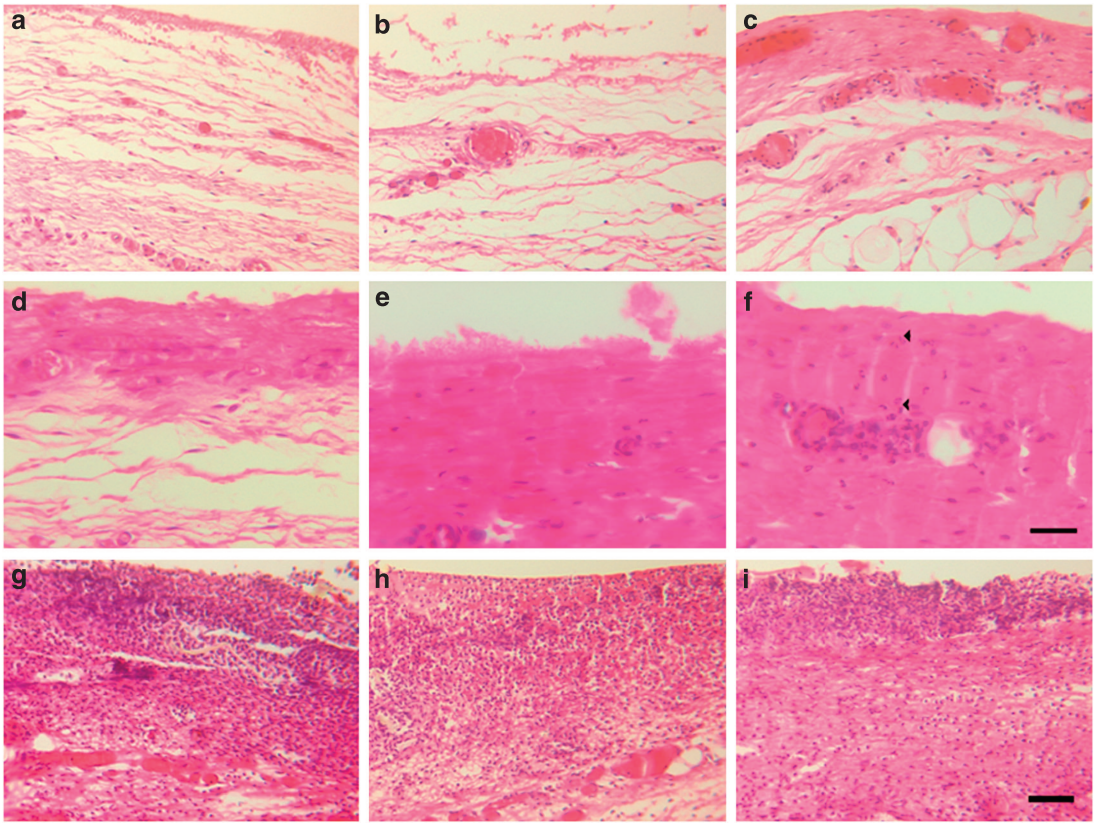

Figure 2. Morphology of the wounds. 1 Hour (**a–f**) and 24 hours (**g–i**) in control rats (left column, **a, d, g**) or rats supplemented daily with oleic acid (center column, **b, e, h**) or linoleic acid (right column, **c, f, i**). Arrowheads in **f** show neutrophils in dermal connective tissue 1 hour after wounding in the linoleic acid group. Bar in **f** = 50 μm (for **d–f**); bar in **i** = 100 μm (for **a–c** and **g–i**).

(Supplementary Figure S1 online). At 1 hour after wounding, almost 50% of the cells were positive for CD45, a leukocyte marker, and this percentage reached >90% at 24 hours after wounding in both groups, confirming a massive migration of inflammatory cells as observed in the histological analysis. The other two main cell types present were MHC-II$^+$CD11c$^-$ and CD4$^+$ (~20% each). CD8$^+$ and MHC-II$^+$CD11c$^+$ cells comprised <20% at both time points. There were no differences among the groups (Supplementary Table S2 online).

The importance of ROS for appropriate healing has been demonstrated (Roy *et al.*, 2006). At 1 hour after wounding, the content of hydrogen peroxide (H_2O_2) was elevated in the LNA group (Figure 3). OLA decreased the H_2O_2 content of wound tissue 24 hours after wound induction in comparison with the control group (Figure 3).

The administration of OLA resulted in greater activation of NF-κB 1 hour after wound induction compared with the control group (Figure 4). However, after 24 hours, both the OLA and LNA groups showed lower NF-κB activation in relation to the control group (Figure 4). At this time point, the decrease in NF-κB activation was more pronounced in the LNA group (Figure 4). The response of AP-1 activation was different. LNA resulted in greater activation after 1 hour in relation to the control and OLA groups. OLA had no effect in comparison with the control group. No differences in AP-1

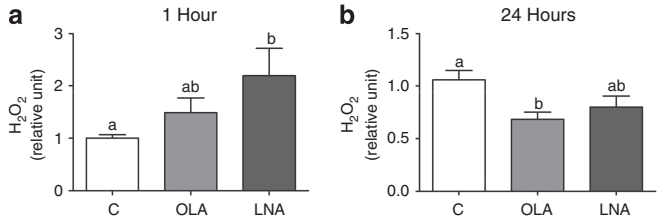

Figure 3. Concentration of hydrogen peroxide (H_2O_2) in homogenates of wounds. 1 Hour (**a**) and 24 hours (**b**) after induction of wounds in control rats (C) or rats supplemented daily with oleic acid (OLA) or linoleic (LNA) acids. Values are expressed as mean ± SEM of at least six animals per group. Different letters indicate significant differences among the groups, as indicated by analysis of variance (ANOVA) and *post hoc* Tukey's test.

activation between groups were observed 24 hours after wounding (Figure 4).

At 24 hours after wound induction, CINC-2αβ and IL-1β reached their maximal concentrations in the control group (Figure 5). After 120 hours, CINC-2αβ remained elevated in relation to time 0, but IL-1β and IL-6 returned to their initial levels. IL-6 and macrophage inflammatory protein-3α (MIP-3α) were not detected before 24 hours. TNF-α and MIP-3α concentrations were not modified during the wound healing process. In the OLA group, the concentrations of TNF-α were maximal 1 hour after wound induction and returned to control levels at 24 hours. Concentrations of

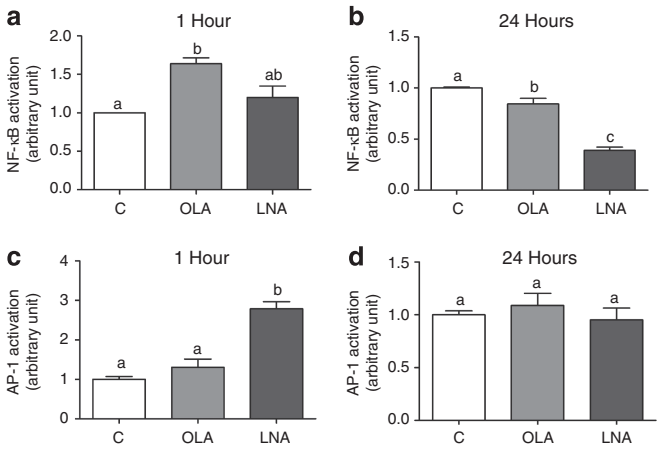

Figure 4. Transcrption factors activation. NF-κB (**a**, **b**) and activator protein-1 (AP-1; **c**, **d**) in scar tissue removed 1 and 24 hours after induction of wounds in control rats (C) and rats supplemented daily with oleic (OLA) or linoleic (LNA) acids. Values are expressed as mean ± SEM of five animals per group. Different letters indicate significant differences among the groups, as indicated by analysis of variance (ANOVA) and *post hoc* Tukey's test.

IL-1β, IL-6, and MIP-3α were significantly lower after 24 hours in comparison with the control group. Treatment with LNA increased the concentration of CINC-2αβ at 1 hour compared with the control and OLA groups, but there were no differences between groups at the later time points. However, as occurred in the OLA group, the levels of IL-1β, IL-6, and MIP-3α were diminished 24 hours after wounding in comparison with control animals (Figure 5). No differences in cytokine concentrations were observed between the OLA and LNA groups at any time point in the ELISA analysis. The real-time PCR analysis demonstrated generally similar patterns of response to the different treatments as seen for the proteins measured by ELISA. However, administration of OLA increased cytokine expression of CINC-2, IL-1β, IL-6, and TNF-α in relation to LNA group (Figure 5).

DISCUSSION

Our findings suggest that oral administration of both OLA and LNA accelerates the inflammatory phase of wound healing with early production/liberation of cytokines and H_2O_2 and activation of transcription factors, followed by inhibition of these factors in the resolution phase of the inflammatory response. These alterations resulted in faster wound closure in the LNA group (Table 1 and Figure 6).

Previous studies demonstrated that topical application of oils improves some parameters of wound healing such as stimulation of connective tissue fiber (Cardoso *et al.*, 2004), reduction in the thickness of the necrotic cell layer edge around the wound (Pereira *et al.*, 2008), and acceleration of wound closure (Park *et al.*, 2010; Otranto *et al.*, 2010). Oils contain many different constituents, and it is not clear whether the effects seen are due to the fatty acid components or other minor components such as antioxidants and vitamins. To our knowledge, the use of oral unesterified fatty acids in the treatment of wounds was previously unreported. The effects seen should encourage trials in humans to

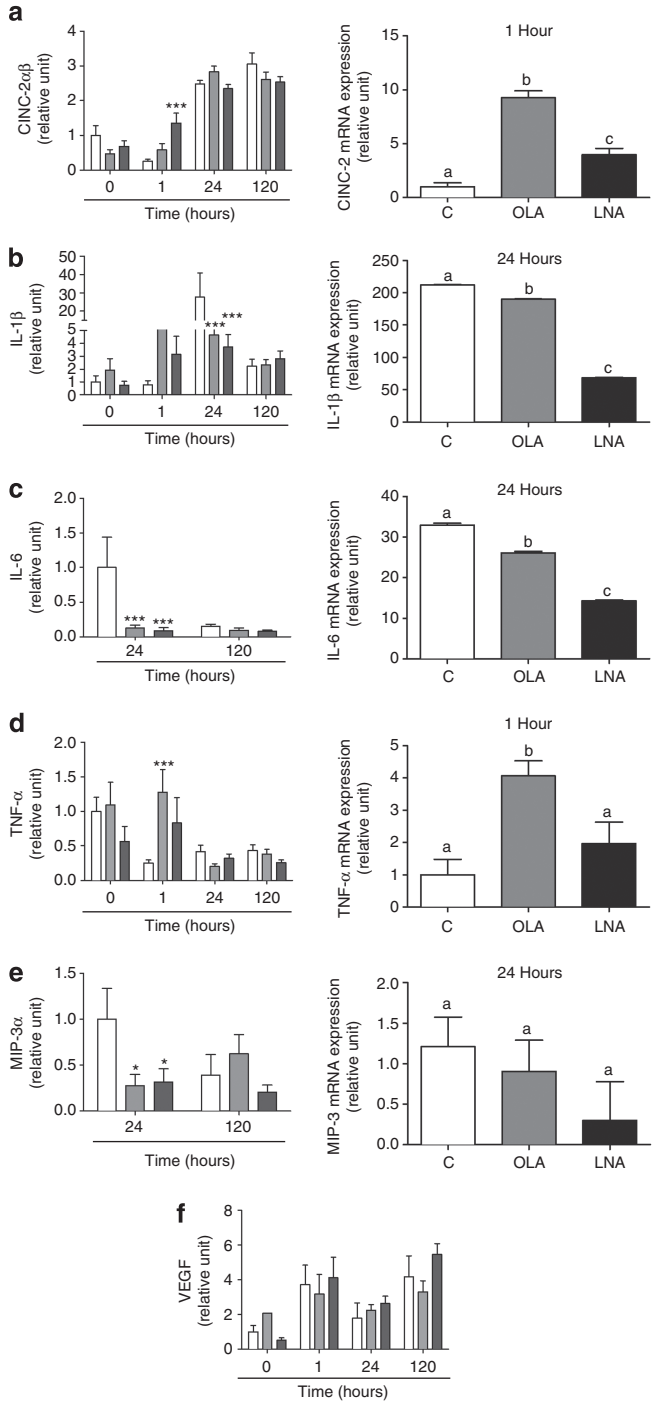

Figure 5. Concentrations and mRNA expression of cytokines. (**a**) Cytokine-induced neutrophil chemoattractant-2αβ (CINC-2αβ), (**b**) IL-1β, (**c**) IL-6, (**d**) tumor necrosis factor-α (TNF-α), (**e**) macrophage inflammatory protein-3α (MIP-3α), and (**f**) vascular endothelial growth factor (VEGF) in wounds of control rats (C, white bar) and rats supplemented daily with oleic acid (OLA, gray bar) or linoleic acid (LNA, black bar). The samples were analyzed at different periods (0, 1, 24, and 120 hours). Values are expressed as mean ± SEM of at least seven animals per group. *$P<0.05$ versus control as indicated by analysis of variance (ANOVA) and *post hoc* Tukey's test. ***$P<0.001$ versus control as indicated by ANOVA and *post hoc* Tukey's test. Different letters indicate significant differences among the groups in the mRNA expression as indicated by ANOVA and *post hoc* Tukey's test.

Table 1. Main findings of the effects of administration of OLA and LNA acids on the wound healing process

Parameter analyzed	Effect of OLA	Effect of LNA
Wound closure	Not changed	Better closure at day 7
Inflammatory cell influx	Not changed	Increased at 1 h Decreased at 24 h
H_2O_2	Not changed at 1 h Decreased at 24 h	Increased at 1 h Not changed at 24 h
AP-1	Not changed	Activated at 1 h
NF-κB	Activated at 1 h Inhibited at 24 h	Inhibited at 24 h
TNF-α	Increased at 1 h	Not changed
IL-1β	Not changed at 1 h Decreased at 24 h	Not changed at 1 h Decreased at 24 h
CINC-2αβ	Not changed	Increased at 1 h Not changed at 24 h
IL-6	Decreased at 24 h	Decreased at 24 h
MIP-3	Decreased at 24 h	Decreased at 24 h
VEGF	Not changed	Not changed

Abbreviations: AP-1, activator protein-1; CINC-2αβ, cytokine-induced neutrophil chemoattractant-2αβ; H_2O_2, hydrogen peroxide; LNA, linoleic acid; MIP-3, macrophage inflammatory protein-3; OLA, oleic acid; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

evaluate these fatty acids with the aim of developing them as therapeutic strategies in wound care. In this study, oral supplementation ($0.22\,g\,kg^{-1}\,day^{-1}$) with unesterified LNA demonstrated faster wound closure, this being statistically significant at the seventh day post wounding in comparison with the control and OLA groups. We previously showed that this supplementation protocol results in incorporation of the fatty acids into neutrophils and modification of some neutrophil functions such as the migratory response and cytokine production (Rodrigues *et al.*, 2010). Because of the alterations in these functions, and the relevance of neutrophils in the inflammatory phase of healing process, OLA and LNA may accelerate the beginning of the next phase (granulation tissue formation) of wound healing and, as a cascade, accelerate the entire process.

Wound healing can be didactically divided into four phases: blood clotting, inflammation, granulation tissue formation, and tissue remodeling. The acute inflammatory response is one of the main events that occurs after tissue damage, and 1 day after the injury neutrophils comprise at least 50% of the cells in the wound site (Engelhardt *et al.*, 1998). Other cell types are also involved with wound healing, such as macrophages (important source of cytokines and ROS), mast cells, and T lymphocytes (mainly in the remodeling phase; Eming *et al.*, 2007). Neutrophils function to destroy

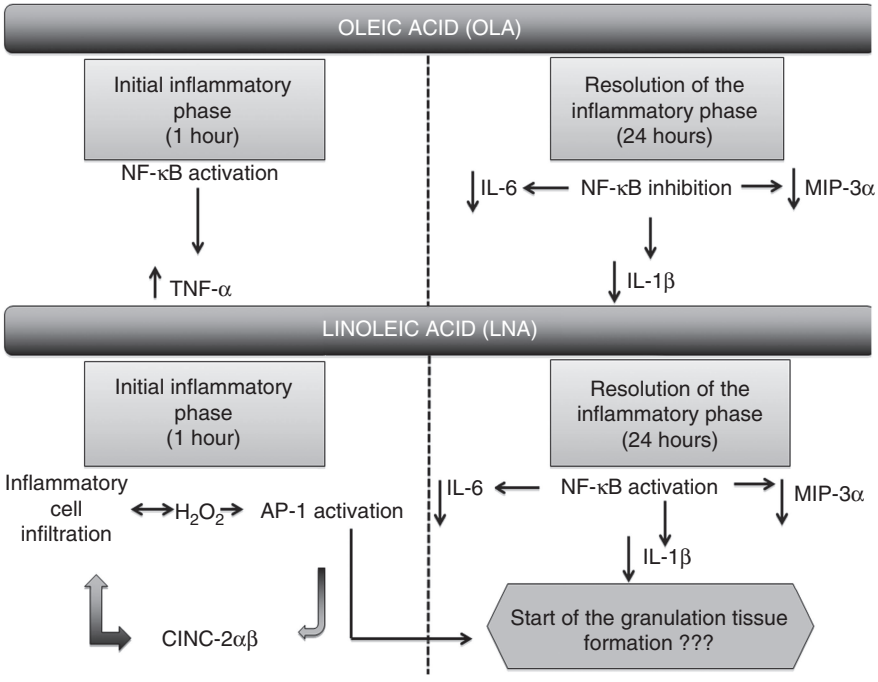

Figure 6. Scheme of the effects of administration of oleic (OLA) and linoleic (LNA) acids on the wound healing process. One of the first events to occur after skin lesion is hypoxia. The compromised tissue oxygenation, due to the disruption of vasculature, limits the healing process. The reactive oxygen species (ROS) generated are important not only to disinfect the wound site but also may serve as signaling messengers and regulate the activation of transcription factors, the migratory response of inflammatory cells, and the production of cytokines. As shown, the production of hydrogen peroxide (H_2O_2) is modulated by LNA in the first hour after injury. The increase in H_2O_2 content could activate activator protein-1 (AP-1), triggering the expression of inflammatory cytokines and growth factors. AP-1 activation is also associated with proliferation of cells such as keratinocytes and fibroblasts, which are important for the closure of the wound. On the other hand, in the OLA group, the H_2O_2 content was not changed in the first hour after wounding, the migratory response of inflammatory cells seems to be similar to the control group, and there was no modification in AP-1 activation. Put together, these results suggest a direct effect of OLA on NF-κB activation and then the increase in the production of proinflammatory cytokines, without the participation of H_2O_2.

invading pathogens through an array of microbicidal mechanisms such as phagocytosis, as well as production of ROS, cytokines, and proteolytic enzymes (Ley *et al.*, 2007).

One of the first events to occur after skin lesion is hypoxia. The compromised tissue oxygenation, due to the disruption of vasculature, limits the healing process. The wound site contains micromolar concentrations of H_2O_2 and low concentrations of this molecule support wound healing (Roy *et al.*, 2006). ROS generated are important not only to disinfect the wound site but also may serve as signaling messengers and regulate the activation of transcription factors (Morgan and Liu, 2011), the migratory response of inflammatory cells (Tobar *et al.*, 2008), and the production of cytokines (Han *et al.*, 2009). LNA increased the concentration of H_2O_2 1 hour after wound induction. Nakamura *et al.* (2003) suggested that the increase in ROS production in inflamed skin is related to the elevation in neutrophil infiltration and priming of these cells.

The leukocyte migratory response is a highly regulated process that involves several mechanisms such as the presence or absence of specific chemoattractants, the ability of cells to migrate along a chemotactic gradient, the interaction of receptor–ligand, and the expression of adhesion molecules on both the inflammatory cells and vessel wall (Rollins, 1991). Apart from the ligand–receptor interaction, cell membrane fluidity also mediates chemotactic activity (Wolach *et al.*, 1992). Locati *et al.* (1994) described that the release of arachidonic acid from phospholipids is a prerequisite to chemotaxis of monocytes. The results of this study demonstrate that LNA induces a strong initial migratory response and that later this action is less intense. This fatty acid has both pro- and anti-inflammatory actions, as seen in the production of cytokines, which are temporal in nature. The present result could be explained partially by the elevation in the chemokine CINC-2αβ (Figure 5) or in the H_2O_2 content (Figure 3).

The production of ROS is also intrinsically related to the NF-κB and AP-1 signaling cascades. Considering the fact that activation of NF-κB and AP-1 after a skin injury remains little explored (Yates and Rayner, 2002), we studied the effects of fatty acid supplementation on activation of these transcription factors in wound healing. The NF-κB family consists of homo- or hetero-dimeric proteins that remain in an inactive state in the cytoplasm. The activation of proinflammatory receptors triggers a signal transduction cascade resulting in NF-κB translocation to the nucleus where it binds to DNA and induces expression of inflammatory genes such as IL-1β, TNF-α, and IL-6 (Vallabhapurapu and Karin, 2009). AP-1 is an important transcription factor involved in tissue repair, and consists of subunits that belong to the Jun and Fos families (Neub *et al.*, 2007). Neub *et al.* (2007) observed that the regulation of AP-1 subunits expression, in healthy skin, is biphasic, which means that initially there is low expression of c-Jun, Jun B, Jun D, c-Fos, and Fos B, and 24 hours after injury all the subunits are expressed in higher amounts in the wound tissue. This behavior is associated with wound healing as the prolonged activation of this transcription factor is observed in chronic wounds (Neub *et al.*, 2007). The increase in H_2O_2 content 1 hour after wounding in the LNA group could activate the transcription factor AP-1, triggering the expression of inflammatory cytokines and growth factors. AP-1 activation is also related to the proliferation and differentiation of endothelial cells, keratinocytes, and fibroblasts. At 24 hours after wound induction, there was no reduction in AP-1 activation, suggesting the beginning of the next phase.

After a physical trauma, cytokines are produced/released at the site of tissue damage, and their systemic elevation occurs a few hours after the injury. Although much is known about these peptides, there is more limited information about their role in skin homeostasis (Grellner *et al.*, 2000, 2005; Roupe *et al.*, 2010). Cytokines function at low concentrations and, directly or indirectly, modulate wound healing due to stimulation of neutrophil, macrophage, and fibroblast chemotaxis, the growth of fibroblasts and keratinocytes, as well as the extracellular matrix reorganization (Hubner *et al.*, 1996).

Cytokine (CINC-2αβ, IL-1β, TNF-α, and VEGF) concentrations were determined 1 hour after wound induction, and the peak was reached at 24 hours. Supplementation with OLA and LNA increased the wound tissue concentration of TNF-α and CINC-2αβ, respectively. IL-1 is an important cytokine involved in various skin diseases such as bacterial infections, bullous disease, and UV damage (Yano *et al.*, 2008). Considering the fact that IL-1β and TNF-α release precedes the production of growth factors (Brown *et al.*, 1992), the enhanced release of these cytokines can accelerate the repair process.

CINC-2αβ is a potent chemoattractant to neutrophils and has many other proinflammatory effects such as neutrophil degranulation, induction of oxidative burst (Walz *et al.*, 1987), and stimulation of the interaction between leukocytes and endothelial cells (Detmers *et al.*, 1990). However, to avoid the overproduction and the deleterious effects of cytokines, their production is transient and time dependent (Thomson, 2003). Thus, the reduction in IL-1β, IL-6, and MIP-3α concentrations demonstrates that the inflammatory response was in decline at 24 hours in the treated groups.

In summary, the data presented herein suggest that administration of LNA initially increases the production of H_2O_2, which could be related to the influx of inflammatory cells that produce cytokines and growth factors. At the same time, the elevation in ROS content also signals to activation of AP-1. AP-1 activation is related to proliferation/differentiation of keratinocytes and fibroblasts, important cells that participate in the next phase of wound repair. Later, the proinflammatory scenario appears to be switched off, particularly the activation of NF-κB and the production of proinflammatory cytokines, although AP-1 activation remains. This early resolution of the inflammatory phase and the beginning of the next phase (granulation tissue formation) could explain the accelerated healing observed in this group. On the other hand, oral administration of OLA did not modify the H_2O_2 content, but induced NF-κB activation and increased the production of cytokines such as TNF-α. The lack of modification in AP-1 activation state after OLA treatment could partially explain the difference in healing when compared with LNA.

MATERIALS AND METHODS

Reagents

OLA, LNA, phenylmethanesulfonyl fluoride (PMSF), and aprotinin were obtained from Sigma Chemical (St Louis, MO). Amplex Ultrared was obtained from Invitrogen (Carlsbad, CA). Duo Set ELISA kits were obtained from R&D Systems (Minneapolis, MN).

Animals

Male Wistar rats weighing 180 ± 20 g (from the Department of Physiology and Biophysics, Institute of Biomedical Sciences, São Paulo University, Brazil) were maintained at 23 °C under a light/dark cycle of 12:12 hours. Animals received chow (Nuvital, Curitiba, Brazil) and water *ad libitum*. The Animal Care Committee of the Institute of Biomedical Sciences approved the experimental procedure of this study (Protocol number: 86).

Administration of OLA and LNA

Unesterified OLA or LNA at a dose of 0.22 g per kg body weight was administered daily by single gavage. Control animals received 0.22 g per kg body weight of water by gavage. As published previously, this mode of administration does not alter nutritional parameters (food ingestion, caloric intake, weight gain) or the general health status of the animals (Rodrigues *et al.*, 2010). Considering that the caloric content of the fatty acids was small (1.98 Kcal day^{-1}), we choose to use water as an inert liquid control.

Wound induction

After 5 days of fatty acid supplementation, the animals were anesthetized and an area of 10 mm^2 of dorsal region skin was removed by surgery. Animals were killed 1, 24, 120 hours, and 16 days after the surgery.

Wound measurement

To evaluate wound closure, the wounds were photographed daily with a Sony cyber shot (model DSC-S950S 10MP 4 × Optical zoom) by the same examiner. After digitization, the wound area was measured using ImageJ software (National Institutes of Health, Bethesda, MD). Wound closure was defined as a reduction of wound area and results were expressed as percentage (%) of the original wound area.

Histological examination of wound tissue

Wound lesions with adjacent normal skin were removed, fixed with Bouin's fixative for 24 hours at room temperature, and processed and embedded in Paraplast (Sigma Chemical). Sections (7 μm) were stained with hematoxylin/eosin in order to evaluate the general aspects of the wound.

Determination of cytokine concentrations in wound tissue

Wound lesions removed at 1, 24, and 120 hours after induction were immediately packed in dry ice and kept frozen (−80 °C) until they were homogenized. For homogenization, phosphate-buffered saline was supplemented with protease inhibitors (0.5 M PMSF and 25 IU ml^{-1} aprotinin). Tissue (100 mg) was homogenized with a Polytron PT 3100 (Kinematica, Lucerne, Switzerland). Samples were then sonicated for 1 minute and centrifuged at 1,000 × *g* at 4 °C for 10 minutes. The concentrations of cytokines (CINC-2αβ, IL-1β, TNF-α, MIP-3, IL-6, and VEGF) in the supernatants were assessed by ELISA using the Duo Set kit (R&D System). The concentrations were normalized by the amount of protein in the samples, determined by the method described by Bradford (1976).

Real-time PCR

Total RNA was extracted (RNeasy Mini Kit, Qiagen, Venlo, The Netherlands) from wound tissue and reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The primers used for real-time PCR are in the Supplementary Table S1 online. Reactions were performed using SYBR-Green PCR master mix (Invitrogen) in a Rotor Gene Q (Qiagen, Duesseldorf, Germany). As an internal control, levels of glyceraldehyde-3-phosphate-dehydrogenase were quantified in parallel with target genes. Normalization and fold changes were calculated using the ΔΔCt method (Miller and Chen, 2006).

Determination of H_2O_2 concentrations in wound tissue

Wound tissue was incubated for 1 hour in 1.5 ml of Krebs-Ringer (containing in mM: NaCl 118.5, $NaHCO_3$ 24.7, KCl 4.7, $MgSO_4$ 1.18, KH_2PO_4 1.18, and $CaCl_2$ 2.5, and equilibrated with 95% O_2/5% CO_2), pH 7.4, at 37 °C with 5.3 mM glucose, 1% BSA in the presence of Amplex Ultrared reagent (50 mM) and horseradish peroxidase (0.1 U ml^{-1}). After this period of incubation, 20 μl of the medium was collected and the fluorescence intensity was measured using the excitation/emission wavelengths of 530 nm and 590 nm, respectively.

Measurement of NF-κB and AP-1 activation in wound tissue

Wound tissue removed at 1 and 24 hours after wounding was homogenized in buffer (HEPES (10 mM), $MgCl_2$ (1.5 mM), KCl (10 mM), dithiothreitol (0.5 mM) and PMSF (0.2 mM)). The homogenate was centrifuged at 900 × *g* for 10 minutes and the remaining pellet containing nuclear proteins was resuspended in extraction buffer (HEPES (20 mM), pH 7.6, NaCl (0.45 M), EDTA (2.5 mM), glycerol (25%), dithiothreitol (2.5 mM), and PMSF (1 mM)). After incubation for 30 minutes at 4 °C under agitation, the suspension was centrifuged at 10,000 × g for 2 minutes at 4 °C. Double-stranded oligonucleotides containing the NF-κB and AP-1 consensus binding sites (NF-κB: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and AP-1: 5′-CGCTTGATG AGTCAGCCGGAA-3′) were end labeled using T4 polynucleotide kinase and [γ-32P]ATP (Amersham Biosciences, Little Chalfont, UK). The reactions were made as described in the previous study conducted by our group (Vinolo *et al.*, 2011). The blots were analyzed by scanner densitometry (Image Master 1D, Amersham Biosciences) and results were expressed as arbitrary unit in relation to control.

Statistical analysis

Comparisons between groups were made using one-way analysis of variance and Tukey's multiple comparison post-test. The significance was set at $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Atiyeh BS, Costagliola M, Hayek SH *et al.* (2007) Effect of silver on burn wound infection control and healing: review of the literature. *Burns* 33:139–48

Berlanga-Acosta J, Gawilongo-Cowley J, Lopez-Saura P *et al.* (2009) Epidermal growth factor in clinical practice–a review of its biological actions, clinical indications and safety implications. *Int Wound J* 6:331–46

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–54

Broughton G (2006) A brief history of wound care. Plast Reconstr Surg 117:6s

Brown LF, Yeo KT, Berse B *et al.* (1992) Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med* 176:1375–9

Burr GO BM (1930) On the nature and role of the fatty acids essential in nutrition. *J Biol Chem*, 587–621

Cardoso CR, Souza MA, Ferro EA *et al.* (2004) Influence of topical administration of n-3 and n-6 essential and n-9 nonessential fatty acids on the healing of cutaneous wounds. *Wound Repair Regen* 12:235–43

Cury-Boaventura MF, Curi R (2005) Regulation of reactive oxygen species (ROS) production by C18 fatty acids in Jurkat and Raji cells. *Clin Sci (Lond)* 108:245–53

Cury-Boaventura MF, Gorjao R, de Lima TM *et al.* (2008) Effect of olive oil-based emulsion on human lymphocyte and neutrophil death. *JPEN* 32:81–7

Declair V (1997) The usefulness of topical application of essential fatty acids (EFA) to prevent pressure ulcers. *Ostomy Wound Manage* 43:48–52, 54

Detmers PA, Lo SK, Olsen-Egbert E *et al.* (1990) Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. *J Exp Med* 171:1155–62

Eming SA, Krieg T, Davidson JM (2007) Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol* 127:514–25

Engelhardt E, Toksoy A, Goebeler M *et al.* (1998) Chemokines IL-8, GROalpha, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *Am J Pathol* 153:1849–60

Gorjao R, Cury-Boaventura MF, de Lima TM *et al.* (2007) Regulation of human lymphocyte proliferation by fatty acids. *Cell Biochem Funct* 25:305–15

Grellner W, Georg T, Wilske J (2000) Quantitative analysis of proinflammatory cytokines (IL-1beta, IL-6, TNF-alpha) in human skin wounds. *Forensic Sci Int* 113:251–64

Grellner W, Vieler S, Madea B (2005) Transforming growth factors (TGF-alpha and TGF-beta1) in the determination of vitality and wound age: immunohistochemical study on human skin wounds. *Forensic Sci Int* 153:174–80

Han D, Ybanez MD, Ahmad S *et al.* (2009) Redox regulation of tumor necrosis factor signaling. *Antioxid Redox Signal* 11:2245–63

Hansen HS, Jensen B (1985) Essential function of linoleic acid esterified in acylglucosylceramide and acylceramide in maintaining the epidermal water permeability barrier. Evidence from feeding studies with oleate, linoleate, arachidonate, columbinate and alpha-linolenate. *Biochim et Biophys Acta* 834:357–63

Hubner G, Brauchle M, Smola H *et al.* (1996) Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice. *Cytokine* 8:548–56

Ley K, Laudanna C, Cybulsky MI *et al.* (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev* 7:678–89

Locati M, Zhou D, Luini W *et al.* (1994) Rapid induction of arachidonic acid release by monocyte chemotactic protein-1 and related chemokines. Role of Ca2+ influx, synergism with platelet-activating factor and significance for chemotaxis. *J Biol Chem* 269:4746–53

Martins de Lima T, Gorjao R, Hatanaka E *et al.* (2007) Mechanisms by which fatty acids regulate leucocyte function. *Clin Sci (Lond)* 113:65–77

McCusker MM, Grant-Kels JM (2010) Healing fats of the skin: the structural and immunologic roles of the omega-6 and omega-3 fatty acids. *Clin Dermatol* 28:440–51

Miller GE, Chen E (2006) Life stress and diminished expression of genes encoding glucocorticoid receptor and beta2-adrenergic receptor in children with asthma. *Proc Natl Acad Sci USA* 103:5496–501

Morgan MJ, Liu Z-G (2011) Crosstalk of reactive oxygen species and NF-κB signaling. *Cell Res* 21:103–15

Nakamura Y, Kozuka M, Naniwa K *et al.* (2003) Arachidonic acid cascade inhibitors modulate phorbol ester-induced oxidative stress in female ICR mouse skin: differential roles of 5-lipoxygenase and cyclooxygenase-2 in leukocyte infiltration and activation. *Free Radic Biol Med* 35:997–1007

Neub A, Houdek P, Ohnemus U *et al.* (2007) Biphasic regulation of AP-1 subunits during human epidermal wound healing. *J Invest Dermatol* 127:2453–62

Otranto M, do Nascimento AP, Monte-Alto-Costa A (2010) Effects of supplementation with diferente edible oils on cutaneous wound healing. *Wound Rep Reg* 18:629–36

Park NY, Valacchi G, Lim Y (2010) Effect of dietary conjugated linoleic acid supplementation on early inflammatory responses during wound healing. *Mediators of Inflamm* 2010:1–8

Pereira LM, Hatanaka E, Martins EF *et al.* (2008) Effect of oleic and linoleic acids on the inflammatory phase of wound healing in rats. *Cell Biochem Funct* 26:197–204

Pompeia C, Cury-Boaventura MF, Curi R (2003) Arachidonic acid triggers an oxidative burst in leukocytes. *Braz J Med Biol Res* 36:1549–60

Rodrigues HG, Vinolo MA, Magdalon J *et al.* (2010) Dietary free oleic and linoleic acid enhances neutrophil function and modulates the inflammatory response in rats. *Lipids* 45:809–19

Rollins BJ (1991) JE/MCP-1: an early-response gene encodes a monocyte-specific cytokine. *Cancer Cells* 3:517–24

Roupe KM, Nybo M, Sjobring U *et al.* (2010) Injury is a major inducer of epidermal innate immune responses during wound healing. *J Invest Dermatol* 130:1167–77

Roy S, Khanna S, Nallu K *et al.* (2006) Dermal wound healing is subject to redox control. *Mol Ther* 13:211–20

Thomson AW, Lotze MT (eds) (2003) *The Cytokine Handbook*, 4th edn. Academic Press: New York

Tobar N, Caceres M, Santibanez JP *et al.* (2008) RAC1 activity and intracellular ROS modulate the migratory potential of MCF-7 cells through a NADPH oxidase and NFkappaB-dependent mechanism. *Cancer Lett* 267:125–32

U.S. Department of Agriculture ARS (2008) Nutrient intakes from food: mean amounts consumed per individual, one day, 2005-2006

Vallabhapurapu S, Karin M (2009) Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* 27:693–733

Vinolo MAR, Rodrigues HG, Hatanaka E *et al.* (2011) Suppressive effect of short-chain fatty acids on production of pro-inflammatory mediators by neutrophils. *J Nutr Biochem* 22:849–55

Walz A, Peveri P, Aschauer H *et al.* (1987) Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. *Biochem Biophys Res Commun* 149:755–61

Wolach B, Ben Dor M, Chomsky O *et al.* (1992) Improved chemotactic ability of neonatal polymorphonuclear cells induced by mild membrane rigidification. *J Leukoc Biol* 51:324–8

Yano S, Banno T, Walsh R *et al.* (2008) Transcriptional responses of human epidermal keratinocytes to cytokine interleukin-1. *J Cell Physiol* 214:1–13

Yates S, Rayner TE (2002) Transcription factor activation in response to cutaneous injury: role of AP-1 in reepithelialization. *Wound Repair Regen* 10:5–15