The Effect of Gamma Linolenic Acid on the Expression of c-Fos and Inflammatory Factors on the Formalin Induced Pain

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Research Article

Keywords: Gamma Linolenic Acid, formalin, c-Fos, TNF-α, IL-1β

Posted Date: May 25th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2969782/v1

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Abstract

Background

The sensation of pain as a pathological entity has always been discussed. The current study was conducted to determine the analgesic effect of gamma-linolenic acid (GLA) in the formalin test.

Methods

The formalin was injected into the right hind paw in rats. The pain behaviors were determined as a numerical score for 60 minutes after the injection of formalin. The spinal cord was removed to evaluate the protein expression of c-Fos. Also, the levels of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were measured in the skin.

Results

The attenuation of nociceptive response persisted after GLA injection in formalin treated rats. Formalin injection also enhanced the levels of cytokines, including TNF-α and IL-1β in the hind paw, which could be repressed by GLA. Also, western blot analysis showed that formalin increase c-Fos protein in the spinal cord, which could be suppressed by GLA. The molecular analysis targeting c-Fos and inflammatory cytokines such as TNF-α and IL-1b also showed an effect of GLA, which matched the results of the behavioral data analysis.

Conclusion

Our results demonstrated that pretreatment, with GLA, produced antinociceptive effects in the formalin test and may be effective for pain relief.

Introduction

Pain may be caused by tissue damage, chemical stimuli, or abnormal activation of the immune system that stimulates the pain receptors. Peripheral stimulation of pain receptors causes the release of local inflammatory mediators, resulting in peripheral and central sensitization [1–3].

It is well established that a biphasic pain response occurs after the injection of formalin into the hind paw or tails of rodents. The primary mechanism involved in the pain relief process in the acute phase of the formalin test is the stimulation of the peripheral nervous system. In addition, after the acute phase, secondary pain caused by formalin injection is observed. It has been shown that primary acute pain response and secondary hyperalgesia are independent [4, 5]. Some studies have reported that formalin-induced pain is maintained by the spinal dorsal horn (SDH) or descending facilitation from the rostral ventral medulla (RVM) [5, 6].

The c-Fos gene is rapidly and transiently expressed in neurons in response to a pain stimulus and encodes the nuclear protein Fos [7]. FOS may contribute to the long-term modulation of spinal pain processes by altering spinal pain circuits leading to allodynia (non-noxious stimuli) or hyperalgesia (increased sensitivity to noxious stimuli) [8–10].

It has been shown that in acute and chronic pain, the production of interleukin (IL)-1β in the dorsal horn of the spinal cord is increased by peripheral macrophages and activated microglia [11–14]. Also, injection of formalin in the hind paw increases inflammatory cytokines such as IL-1β and TNF-α in the cortex and blood, after 24 hours [15]. Furthermore, the
concentration of these inflammatory cytokines increases within the skin of the hind paw 1 h after injection of formalin [16].

Inflammation plays a role in most chronic diseases. Currently, studies of the relationship between diet and disease are expanding. Among dietary components, fat plays the most significant role in influencing health. Polyunsaturated fatty acids (PUFAs) generally have a positive effect on health. Omega-3 and Omega-6 PUFAs play a role in health and disease by producing modulating molecules such as eicosanoids and cytokines, as well as influencing the expression of various genes. Linoleic acid is converted into gamma-linolenic acid (GLA, all cis 6, 9, 12-octadecatrienoic acid, C18:3, n-6), which is an essential fatty acid [17]. GLA is beneficial in the management of breast pain. Also, GLA may reduce symptoms of nerve pain in people with diabetic neuropathy [18]. GLA inhibits inflammation by inactivating NF-κB and AP-1. The mechanism of this effect is due to the reduction of oxidative stress and the inhibition of the ERK and JNK signal transmission pathways [19]. The goal of the current study was to investigate the effect of GLA on the expression of c-Fos and inflammatory factors on the formalin-induced pain.

Materials and methods

Animals and Drugs

Male rats (250 ± 20gr) were housed on a 12/12h light/dark cycle with access to water and food ad libitum. The experimental protocols of this study were by the instructions approved by the Ethics Committee of Shahid Chamran University of Ahwaz, Ahwaz, Iran (Ethic code: EE/1401.2.24.14812/scu.ac.ir). Formalin solution was bought from Merck KGaA, Darmstadt, Germany Company (Merck, Germany). GLA (Merck, Germany) was purchased.

Study design

Forty-eight rats were used in this research. The first group (Sham): rats receiving normal saline intraperitoneally (i.p.) + saline injection in the right hind paw. The second group (Control): rats receiving normal saline (i.p.) + formalin 2.5% injection in the right hind paw. The third group (Diclofenac): rats receiving diclofenac 10 mg/kg (i.p.) + formalin test. The fourth group (GLA 50): rats receiving GLA 50 mg/kg (i.p.) + formalin test. The fifth group (GLA 100): rats receiving GLA 100 mg/kg (i.p.) + formalin test. The sixth group (GLA 150): rats receiving GLA 150 mg/kg (i.p.) + formalin test.

Formalin test

The Formalin test was developed to investigate the pain response. The rats were placed in the test chamber for 20 minutes before the experiment. After accustoming the animals to the test chamber, 50 microliters of 2.5% formalin solution (dissolved in saline) injected subcutaneously into the hind paw using a syringe. After the administration of formalin, the data was recorded for 60 minutes.

All the following behavioral recordings were performed by an experimenter who was blind to the experimental condition. The test chamber was made of Plexiglas (25 x 25 x 40 cm). Pain behaviors were recorded manually. Behavioral rating criteria were as follows: (0) No pain: normal weight bearing on the injected paw (1). Favoring: the injected paw is gently placed on the ground and slightly retracted. (2) Painful: the animal retracts the leg into the abdomen or taps the ground. (3) Severe pain: biting or licking the injected paw [20, 21].

Tissue sampling

To investigate the expression of c-Fos protein, the animals were euthanized. The spinal cord of the animals was removed immediately and kept at -70 °C until the experiment. Also, for the expression of inflammatory genes, the tissue of the right paw was removed and placed at -70 °C.

Measurement of cytokines levels
To determine TNF-α and IL-1β levels, the skin of hid paw in each group was homogenate in PBS, then centrifuged at 10,000 × g for 20 min at 4°C, and the supernatant was collected and stored at −70°C. The levels of TNF-α and IL-1β were measured with the ELISA method according to the manufacturer’s instructions (BT-Lab, England).

**Western blot**

The L4-6 spinal cord was isolated on the 1h day after formalin injection. The tissues were lysed in a buffer that contained 50 mM Tris (pH 7.4), 150 mM sodium chloride, 0.25% sodium deoxycholate, 0.1% Triton X-100%, EDTA 5.84 g, and 0.1% sodium dodecyl sulfate. One protease inhibitor tablet was used for every 10 ml of solution. The samples were homogenized for 2 minutes at 4°C using a homogenizer (analytikjena, Speed Mill plus, Germany) at 25,000 rpm. The homogenized samples were centrifuged for 10 minutes at 4°C with 14,000 rpm, and then the protein concentration was measured using the Bradford method. The samples after boiling at 100°C for 5 min were transferred to a gel with 10% sodium deoxycholate-polyacrylamide (Genshare Biology, CN) for electrophoretic separation and immunoblotting. The samples were transferred on a PVDF lm with a current of 0.25 A onto (Millipore, Burlington, MA, United States) for 90 min. The films were blocked with 5% skim milk for 1h and then incubated at 4°C overnight with the following antibodies: rabbit anti-GAPDH (1:1,000, Cell Signaling Technology, Inc.), rabbit anti-ASC antibody (1:1,000, CST), rabbit anti-c-Fos antibody (1:1,000, Cell Signaling Technology, Inc.), mouse anti-rabbit IgG-HRP: sc-2357 (1:200, Santa Cruz Biotechnology, Inc.). Photosensitive papers were scanned using a JS 2000 scanner (BonninTech, China) and the band density was calculated. To quantify the bands, the density of each protein compared to the calibrator protein in the studied groups was analyzed by JS 2000 software.

**Statistical analyses**

Results are expressed as mean ± SEM. Statistical analysis was performed with the SPSS program (version 16). Differences among multiple groups were determined in one way analysis of variance (ANOVA) followed by post hoc Tukey test. Statistical significance was set at P < 0.05.

**Results**

**The Analgesic Effects of GLA on the Formalin-Induced Pain**

The results of the formalin test are shown in Table 1 (mean ± ESM). In the first five minutes of the formalin test: the average pain intensity in the control group was higher than in the sham group. The average pain intensity in the diclofenac 10 mg/kg group was significantly different from the control group (P < 0.05). The average pain intensity in GLA 100 and 150 groups was lower than the control group (P < 0.05). The average pain intensity in GLA 50 group was not significantly different from the control group. From the beginning of the sixth minute to the end of the fifteenth minute, there was no significant difference between any of the studied groups. From the beginning of the 20th minute to the end of the 60th minute, the pain intensity in the control group was higher than in the sham group (P < 0.05). Also, from the beginning of the 20th minute to the end of the 60th minute, the pain intensity in the GLA 50, 100, and 150 groups was lower than the control group, but this difference was not significant. In other cases, significant differences were not observed (Fig. 1).
### Table 1
Formalin test.

<table>
<thead>
<tr>
<th>min</th>
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<tr>
<td>Group 1</td>
<td>0.049 ± 0.09a</td>
<td>0.06 ± 0.12a</td>
<td>0.15 ± 0.13a</td>
<td>0.08 ± 0.14a</td>
<td>0.01 ± 0.03a</td>
<td>0.0 ± 0.00a</td>
<td>0.0 ± 0.00a</td>
<td>0.0 ± 0.00a</td>
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<tr>
<td>Group 2</td>
<td>2.0 ± 0.33d</td>
<td>1.04 ± 1.02a</td>
<td>0.475 ± 0.95a</td>
<td>1.825 ± 0.39a</td>
<td>2.0 ± 0.14b</td>
<td>2.0 ± 0.37b</td>
<td>2.22 ± 0.10b</td>
<td>2.13 ± 0.19b</td>
<td>2.05 ± 0.11b</td>
<td>1.88 ± 0.23b</td>
<td>1.55 ± 0.53b</td>
<td>1.51 ± 0.57b</td>
</tr>
<tr>
<td>Group 3</td>
<td>1.8 ± 0.16cd</td>
<td>0.97 ± 0.74a</td>
<td>0.44 ± 0.55a</td>
<td>1.26 ± 0.90a</td>
<td>1.8 ± 0.85b</td>
<td>2.17 ± 0.67b</td>
<td>2.17 ± 0.16b</td>
<td>2.23 ± 0.08b</td>
<td>2.05 ± 0.09b</td>
<td>1.81 ± 0.45b</td>
<td>1.67 ± 0.42b</td>
<td>1.21 ± 0.61b</td>
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<tr>
<td>Group 4</td>
<td>0.66 ± 0.35ab</td>
<td>1.0 ± 0.74a</td>
<td>0.74 ± 0.72a</td>
<td>1.58 ± 1.07a</td>
<td>1.81 ± 0.86b</td>
<td>2.0 ± 0.73b</td>
<td>1.94 ± 0.56b</td>
<td>1.78 ± 0.46b</td>
<td>1.7 ± 0.45b</td>
<td>1.25 ± 0.37b</td>
<td>1.25 ± 0.50b</td>
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<tr>
<td>Group 5</td>
<td>1.63 ± 0.99bcd</td>
<td>0.72 ± 0.47a</td>
<td>1.13 ± 0.60a</td>
<td>1.56 ± 0.74a</td>
<td>1.85 ± 0.65b</td>
<td>1.96 ± 0.47b</td>
<td>1.85 ± 0.38b</td>
<td>1.78 ± 0.60b</td>
<td>1.81 ± 0.39b</td>
<td>1.57 ± 0.40b</td>
<td>1.1 ± 0.69b</td>
<td>1.17 ± 0.63b</td>
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<tr>
<td>Group 6</td>
<td>0.78 ± 0.39abc</td>
<td>1.35 ± 0.44a</td>
<td>0.37 ± 0.40a</td>
<td>1.41 ± 1.02a</td>
<td>2.02 ± 0.43b</td>
<td>2.1 ± 0.13b</td>
<td>2.02 ± 0.42b</td>
<td>1.97 ± 0.10b</td>
<td>1.7 ± 0.37b</td>
<td>1.38 ± 0.45b</td>
<td>1.08 ± 0.58b</td>
<td>0.99 ± 0.77b</td>
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**Formalin Injection increased the levels of IL-1β and TNF-α in the hind paw**

The levels of IL-1β were initiated by formalin injection in the control group compared with the sham group (one-way ANOVA, $F(12, 5) = 17.60, P < 0.001$). The levels of IL-1β in the diclofenac (10 mg/kg) group did show a significant difference in compared with the control group. GLA 100 and 150 mg/kg decrease of IL-1β compared with a control group, but such an effect was not seen in the case of the 50 mg dose. (GLA 50 mg: one way ANOVA, $F(12, 5) = 17.60, P > 0.05$. GLA 100 mg: one-way ANOVA, $F(12, 5) = 17.60, P < 0.001$. GLA 150 mg: one-way ANOVA, $F(12, 5) = 17.60, P < 0.001$ respectively).

Also, the levels of TNF-α were initiated by formalin injection in the control group compared with the sham group (one-way ANOVA, $F(12, 5) = 12.99, P < 0.001$). The levels of TNF-α in the diclofenac (10 mg/kg) group did show a significant difference with the control group. GLA 100 and 150 mg/kg reduced of TNF-α with the control group, but such an effect was not seen in the case of the 50 mg dose. (GLA 50 mg: one way ANOVA, $F(12, 5) = 12.99, P > 0.05$. GLA 100 mg: one-way ANOVA, $F(12, 5) = 12.99, P < 0.001$. GLA 150 mg: one-way ANOVA, $F(12, 5) = 12.99, P < 0.001$ respectively).

**Formalin Injection increased the c-Fos expression in the spinal cord**

There was elevated c-Fos protein observed in the spinal cord after formalin injection in the control group compared with the sham group (one-way ANOVA, $F(4, 85) = 20.11, P < 0.001$). Also, we analyzed the effects of GLA on the c-Fos expressions in the spinal cord in Formalin-Induced Pain. There was a significant difference between the control group and GLA 100 and 150 mg/kg groups, but such an effect was not seen in the case of the 50 mg dose. (GLA 50 mg: one way ANOVA, $F(4, 85) = 20.11, P < 0.001$. GLA 100 mg: one-way ANOVA, $F(4, 85) = 20.11, P < 0.001$. GLA 150 mg: one-way ANOVA, $F(4, 85) = 20.11, P < 0.001$ respectively). In addition, the expression of the c-Fos in the diclofenac (10 mg/kg) group did show a significant difference from the control group.

**Discussion**
GLA (18:3n-6) is an omega-6 (n-6), 18 carbon (18C) polyunsaturated fatty acid that is found in botanical seed oils (blackcurrant ~ 17%GLA, borage ~ 21% GLA, evening primrose ~ 9%GLA), and milk [22]. In the search we did, there were few studies describing the application of GLA in pain. However, GLA has been reported in previous studies to exert significant anti-inflammatory effects [17, 19]. GLA reduces diabetic neuropathy complications [23]. In addition, GLA has been shown to reduce breast pain [18]. In the current study, GLA 100 and 150 mg/kg reduced the pain intensity in the acute phase of the formalin test.

Activation of macrophages peripheral tissue and the DRG and microglia in the spinal cord develop inflammatory pain [24]. Macrophages play a key role in the initiation of inflammation [25]. Macrophages are important enhanced pro-inflammatory mediators which trigger inflammatory responses [26]. In our study, 1h after formalin injection in the hind paw, the levels of proinflammatory cytokines in the injection area (IL-1β and TNF-α) were increased. Such molecules are released as a host response due to inflammasome activation. Therapeutic strategies targeting proinflammatory cytokines such as IL-1β and TNF-α have proved ineffective in attenuating of pain. In addition to increasing pro-inflammatory cytokines, local inflammatory stimulation also decreases anti-inflammatory cytokines. Cytokines may be transported retrogradely from the environment, via axonal or non-axonal mechanisms, to the DRG and dorsal horn, where they can affect neuronal activity and thus contribute to nociception [27]. GLA has anti-inflammatory effects [17]. GLA, through the inactivation of NF-kB and AP-1 inhibits inflammatory responses [19]. Inactivation of NF-kB suppresses the production of proinflammatory cytokines such as IL-6 and TNF-α [28]. Some studies support the anti-inflammatory effects of essential fatty acids [29]; however further studies could fill the research gaps.

c-Fos expression increased after formalin injection in the lumbar spinal cord in rats [30]. The c-Fos oncogene is an immediate-early gene. The information encoded in the c-Fos gene quickly converts into messenger RNA. Fos protein product is expressed for up to 60 minutes [31]. The expression of c-Fos in the spinal cord enhanced after stimulation of primary sensory neurons. c-Fos has an important role in developing pain, as part of the adaptive response of nociceptive input [32]. Therefore, the identification of c-fos as a marker for pain enables the search for drugs capable of preventing the induction of pain [33]. In the current study GLA 100 and 150 mg/kg attenuated c-Fos expression in the spinal cord after formalin injection. Essential fatty acids are an attractive adjunctive treatment for pain associated with inflammatory bowel disease, rheumatoid arthritis, and dysmenorrhea [34].

In conclusion, the analgesic effects of GLA were significantly observed in the early (or acute) phase of the formalin test. Acute pain or nociception is one of the main characteristics of inflammation [3]. Inflammatory mediators cause pain by binding to their receptors on primary pain sensory neurons in the peripheral nervous system, including the skin [35]. The results of this study showed that 1h after formalin injection, the level of IL-1β and TNF-α increased at the injection site. Previous studies have also shown that peripheral inflammation increases the expression of inflammatory mediators in the spinal cord [36]. High levels of inflammatory cytokines in the spinal cord can lead to increased expression of c-Fos [37]. Therefore, our results suggest that GLA can reduce pain by reducing pro-inflammatory mediators and thus reducing neuronal sensitivity due to decreased c-Fos expression.

**Declarations**

**Funding declaration**

We thank Shahid Chamran University of Ahvaz, Ahvaz, Iran, for funding the study (No: 1401.2.24.14812).

**Data Availability**

Not applicable.

**Conflict of interest**
The authors declare that there is no conflict of interest in this study.

References


Figures
Figure 1

Levels of IL-1β in study groups.

(Sham): normal saline intraperitoneally (i.p.) + saline injection in the right hind paw. (Control): normal saline (i.p.) + formalin 2.5%. (Diclofenac): rats receiving diclofenac 10 mg/kg (i.p.) + formalin test. (GLA 50): rats receiving GLA 50 mg/kg (i.p.) + formalin test. The fifth group (GLA 100): rats receiving GLA 100 mg/kg (i.p.) + formalin test. The sixth group (GLA 150): rats receiving GLA 150 mg/kg (i.p.) + formalin test. *Significant difference between the control and sham groups. ** Significant difference between the Diclofenac 10, GLA 50, GLA 100 and GLA 150 mg/kg and control groups.
Figure 2

Levels of TNF-α in study groups.

(Sham): normal saline intraperitoneally (i.p.) + saline injection in the right hind paw. (Control): normal saline (i.p.) + formalin 2.5%. (Diclofenac): rats receiving diclofenac 10 mg/kg (i.p.) + formalin test. (GLA 50): rats receiving GLA 50 mg/kg (i.p.) + formalin test. The fifth group (GLA 100): rats receiving GLA 100 mg/kg (i.p.) + formalin test. The sixth group (GLA 150): rats receiving GLA 150 mg/kg (i.p.) + formalin test. *Significant difference between the control and sham groups. ** Significant difference between the Diclofenac 10, GLA 50, GLA 100 and GLA 150 mg/kg and control groups.
Figure 3

Expression of c-Fos protein in study groups.

(Sham): normal saline intraperitoneally (i.p.) + saline injection in the right hind paw. (Control): normal saline (i.p.) + formalin 2.5%. (Diclofenac): rats receiving diclofenac 10 mg/kg (i.p.) + formalin test. (GLA 50): rats receiving GLA 50 mg/kg (i.p.) + formalin test. The fifth group (GLA 100): rats receiving GLA 100 mg/kg (i.p.) + formalin test. The sixth group (GLA 150): rats receiving GLA 150 mg/kg (i.p.) + formalin test. *Significant difference between the control and sham groups. ** Significant difference between the Diclofenac 10, GLA 50, GLA 100 and GLA 150 mg/kg and control groups.