A study of the mechanisms underlying the anti-inflammatory effect of ellagic acid in carrageenan-induced paw edema in rats

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ABSTRACT

Objectives: Ellagic acid (EA) has shown antinociceptive and anti-inflammatory effects. Inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2) enzymes and also cytokines play a key role in many inflammatory conditions. This study was aimed to investigate the mechanisms involved in the anti-inflammatory effect of EA.

Materials and Methods: Carrageenan-induced mouse paw edema model was used for induction of inflammation.

Results: The results showed that intraplantar injection of carrageenan led to time-dependent development of peripheral inflammation, which resulted in a significant increase in the levels of tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1) β, nitric oxide (NO) and prostaglandin E2 (PGE2), and also iNOS and COX-2 protein expression in inflamed paw. However, systemic administration of EA (1–30 mg/kg, intraperitoneal [i.p.]) could reduce edema in a dose-dependent fashion in inflamed rat paws with ED50 value 8.41 (5.26–14.76) mg/kg. It decreased the serum concentration of NO, PGE2, aspartate aminotransferase and alanine aminotransferase, and suppressed the protein expression of iNOS, COX-2 enzymes, and attenuated the formation of PGE2, TNF-α and IL-1 β in inflamed paw tissue. We also demonstrated that EA significantly decreased the malondialdehyde (MDA) level in liver at 5 h after carrageenan injection. Moreover, histopathological studies indicated that EA significantly diminished migration of polymorphonuclear leukocytes into site of inflammation, as did indomethacin.

Conclusions: Collectively, the anti-inflammatory mechanisms of EA might be related to the decrease in the level of MDA, iNOS, and COX-2 in the edema paw via the suppression of pro-inflammatory cytokines (TNFα, IL1 β), NO and PGE2 overproduction.

KEY WORDS: Carrageenan, ellagic acid, inducible nitric oxide synthase, prostaglandin E2, Rat

Introduction

Inflammation is the body’s response against invading pathogens, which is typically characterized by redness, swelling, pain, and heat. Several reports have provided evidence that inflammation is involved in the pathogenesis of many diseases including aging,[1] cancer,[2] cardiovascular dysfunction,[3] and other life-threatening and debilitating disorders.

Acute inflammation is a process that involved the overproduction of free radicals, activation of a complex enzymes, and release of several inflammatory and pro-inflammatory mediators. The carrageenan-induced paw edema is a well-known
acute model of inflammation that is widely used for screening novel anti-inflammatory compounds. Carrageenan injection into the subplantar surface of rat paw induced a biphasic edema. The early phase observed around 1 h is related to the release of histamine, serotonin, bradykinin, and to a less extent prostaglandins produced by cyclooxygenase enzymes (COX), whereas the delayed phase (after 1 h) is attributed to neutrophil infiltration, and the continuing of the prostaglandin generation. Release of the neutrophil-derived free radicals, nitric oxide (NO) and pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), and interleukin-1 β (IL-1 β) also involved in the delayed phase of carrageenan-induced acute inflammation. Different observations suggest that drugs targeting COX enzyme, free radical formation and pro-inflammatory protein expression (e.g. inducible nitric oxide synthase; iNOS) might provide a better control over inflammatory states than the therapeutic agents currently available.

Plant polyphenols play an important role in human nutrition. There is increasing evidence that dietary phenolic compounds have biological properties including antioxidant, anti-inflammatory, anticancer and antiatherosclerotic effects. Among these phytochemicals, ellagic acid (EA; 2,3,7,8 tetrahydroxy benzopyranol [5,4,3-cde] benzopyran-5,10-dione) is a naturally-synthesized polyphenolic compound that present in fruits and nuts such as raspberries, strawberries, pomegranates, cranberries, walnuts, pecans, and other plant foods as EA-glycosides, or bound as ellagitannins. Extracts from red raspberry leaves or seeds, pomegranates, or various other sources containing high levels of EA are commercially available as dietary supplements. This compound exhibits anti-carcinogenic, anti-oxidative, antinoceiceptive and anti-inflammatory activities. Furthermore, it has been shown that EA suppress the release of NO and prostaglandine E₂ (PGE₂) by down-regulating inducible NOS and COX, respectively in the endothelial cells and rat model of colon cancer.

Based on the mentioned above, this study was aimed to assess the mechanisms involved in the anti-inflammatory effect of EA in carrageenan-induced paw edema in rats.

**Materials and Methods**

**Animals**

Adult male Wistar rats (160–200 g) were housed at controlled temperature (22 ± 2°C) and allowed free access to food and drinking water. Testing took place in the middle of the light period of a 12 h/12 h light/dark cycle. All animal experiments were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The Institutional Animal Ethical Committee of Jundishapur University, formed under Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Reg. No. PRC150) approved the pharmacological protocols. The animals were used only once and then euthanized.

**Drugs and Chemicals**

Ellagic acid, 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), phenylmethylsulfonyl fluoride (PMSF), λ-carrageenan, thiobarbituric acid (TBA), aprotinin, pepstatin, and leupeptin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Indomethacin was donated by Iran Daru Pharmaceutical Co. (Tehran, Iran). TNF-α and IL1 β kits were purchased from eBioscience, USA. Anti-iNOS, anti-COX-2, and anti-β-actin antibody were obtained from Abcam Biochemical Co. (Cambridge, UK).

**Carrageenan-induced Rat Paw Edema**

Paw edema was induced in a right hind paw of each rat by intraplantar injection of 100 μl of 1% (suspension in saline) lambda carrageenan. EA (1, 3, 10 and 30 mg/kg) and indomethacin (5 mg/kg) were intraperitoneally administered 30 min before the carrageenan injection. The paw volume of rats was measured by plethysmometer (Ugo Basile, Varese, Italy), before and after injection of carrageenan at different time intervals (1, 2, 3, 4, and 5 h). The degree of edema induced was assessed by a = b, where a and b were the volume of the right hind paw after and before carrageenan treatment, respectively. After 5 h, the animals were sacrificed and the carrageenan-induced edema feet were dissected and stored at −80°C. The whole liver tissue was rinsed in ice-cold normal saline, and immediately were homogenized in cold KCl solution (1.5%) to give a 10% homogenate suspension used for measuring malondialdehyde (MDA). Furthermore, blood was withdrawn and serum expressed from the clotted blood was kept at −80°C for measuring PGE₂ and NO levels.

**Measurement of the Tumor Necrosis Factor α and Interleukin 1 β Level in Paw Tissue**

Paw tissue were homogenized in 500 μl of buffer containing protease inhibitors, and IL-1 β and TNF-α levels were determined using a commercially available ELISA kit (eBioscience, USA) according to the instructions of the manufacturer.

**Western Blot Analysis of Inducible Nitric Oxide Synthase and Cyclooxygenase 2**

Soft tissues were removed from rats paw and homogenized in a lysis solution containing 10 mM CHAPS, 1 mM PMSE, 5 μg/ml aprotinin, 1 mM pepstatin, and 10 μM leupeptin. The homogenates were centrifuged at 12,000 g for 20 min, and 30 μg of protein from the supernatants was then separated on 10% SDS-PAGE using a standard method and transferred to polyvinylidene difluoride membranes. The membranes were then incubated with anti-iNOS, anti-COX-2 or anti-β-actin antibodies in 5% skim milk in TBST for 2 h at room temperature. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody. The immunoreactive protein bands were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent.

**Measurement of Prostaglandine E₂ and Nitric Oxide Level in Paw Tissue and Serum**

Tissue levels of PGE₂ were determined using a commercially available ELISA kit, following the manufacturer’s guidelines (Abcam Biochemical, UK). Furthermore, nitrate reductase enzyme was used to measure NO products following the manufacturer’s guidelines (Cayman, USA). Values obtained by this procedure represented the sums of nitrite and nitrate. Similarly, PGE₂ and NO levels in serum were measured.

**Hepatic Malondialdehyde Assay**

Malondialdehyde levels, an index of lipid peroxidation, produced by free radicals were measured to check
the hepatoprotective and antioxidant activity of EA in carrageenan-induced liver injury. MDA reacts with TBA to produce a red colored complex that has peak absorbance at 532 nm. 1,1,3,3-tetramethoxypropane was used as a standard.[14]

**Hepatic Assessment of the Aspartate Aminotransferase and Alanine Aminotransferase Levels**

Plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured as indicators of liver function parameters using commercial diagnostic assay kits from Bionik (Tehran, Iran) following the instructions of the manufacturer.

**Histopathological Examination**

After 5 h of carrageenan treatment, rats were sacrificed, and the tissue of the paws was removed and fixed in 10% neutral-buffered formaldehyde solution. Each sample was embedded in paraffin wax, sectioned at 5 μm and stained with H and E. A representative area was selected for qualitative light microscopic analysis of the inflammatory cellular response with a ×40 objective.

**Statistical Analysis**

All experimental results are given as means ± standard error of the mean for 6–8 animals per group. The statistical analyses were performed using one-way ANOVA, followed by Tukey’s post-hoc test. The ED50 value for the anti-inflammatory effect of EA accompanied by its respective 95% confidence limits were determined by linear regression from individual experiments using GraphPad Prism 5.0 software (San Diego, CA, USA).

**Results**

**Effects of Ellagic Acid on Carrageenan-induced Rat Paw Edema**

Intraplantar injection of carrageenan in rats resulted in a time-dependent increase in paw volume [Figure 1]. However, treatment with EA reduced the paw edema in a dose-dependent manner at 1, 2, 3, 4 and 5 h after an injection of carrageenan. ED50 values with 95% confidence intervals for the anti-inflammatory effect of EA were 8.41 (5.26–14.76) mg/kg. As expected, the reference drug, indomethacin (5 mg/kg), caused a significant inhibition of postcarrageenan edema [Figure 1].

**Effects of Ellagic Acid on Cytokine Secretion**

As shown in Figure 2a and b, carrageenan increased the level of TNF-α and IL-1β in paw edema, respectively. Meanwhile, in the range dose 1–30 mg/kg, EA could inhibit the level of TNF-α and IL-1β to 40–17% and 68–36% of that observed in carrageenan group, respectively. Moreover, indomethacin (5 mg/kg) significantly decreased TNF-α level in the rat edema paw.

**Effects of Ellagic Acid on Inducible Nitric Oxide Synthase and Cyclooxygenase 2 Expression**

To determine whether the inhibition of NO and PGE2 production was due to a decreased iNOS and COX-2 protein level, the effect of EA on iNOS and COX-2 protein expression was studied using Western blotting analysis. The results showed that treatment with EA at 30 mg/kg for 5 h after carrageenan injection reduced iNOS and COX-2 proteins expression in the rat paw edema [Figure 3]. In addition, the protein expression showed a reduction of iNOS and COX-2 protein expression after treatment with indomethacin at 5 mg/kg compared with the carrageenan-induced alone [Figure 3].

**Effects of Ellagic Acid on the Nitric Oxide Level**

As shown in Figure 4a and b, injection of carrageenan into the rat hind paw induced a marked increase in the hind paw and also serum NO level 5 h after injection. The i.p. treatment of rats with EA (10 and 30 mg/kg) and indomethacin (5 mg/kg, i.p.) caused a significant reduction of increased NO generation by carrageenan in serum ($P < 0.05$). However, EA had no effect on NO level in edema paw tissue (Figure 4a, $P > 0.05$).

**Effects of Ellagic Acid on the Prostaglandine $E_2$ Level**

Figure 5a and b show the PGE2 level increased significantly in the paw tissue and serum 5 h after carrageenan injection ($P < 0.05$). The i.p. treatment of rats with EA (10 and 30 mg/kg) and indomethacin (5 mg/kg, i.p.) caused a significant reduction of increased PGE2 level by carrageenan in serum ($P < 0.05$). However, the effect of EA in paw tissue was more potent than that of serum.

**Effects of Ellagic Acid on the Malondialdehyde, Alanine Aminotransferase and Aspartate Aminotransferase Level**

To determine whether the anti-inflammatory effect of EA was partly due to antioxidant activity, the hepatoprotective properties of EA were assessed by measurement the MDA levels as an index of lipid peroxidation and plasma contents of AST and ALT enzymes. As shown in Figure 6a, the MDA level increased significantly in liver tissue of rats at 5 h after carrageenan injection ($P < 0.05$). However, the MDA level was decreased significantly by treatment with EA. Furthermore, injection of carrageenan into the rat hind paw induced an increase in the serum levels of AST and ALT enzymes 5 h after injection compared with control group ($P < 0.05$). EA (1-30 mg/kg, i.p.) resulted in a significant reduction in AST and ALT levels [Figure 6b and c].

**Histopathological Examination**

As shown in Figure 7a, there was no sign of inflammation in the paw tissue of normal rats. Paw tissue of vehicle-treated rats showed a reduction of iNOS and COX-2 proteins expression after treatment with indomethacin at 5 mg/kg compared with the carrageenan-induced alone [Figure 3].
Indo: Indomethacin (5 mg/kg, i.p.)
an internal control. EA: Ellagic acid (30 mg/kg, intraperitoneal [i.p.]),
group, respectively (one-way ANOVA followed by Tukey's test). VEH: Vehicle, EA: Ellagic acid, Indo: Indomethacin (5 mg/kg, intraperitoneal)

Discussion

in the number of cellular infiltrates. indomethacin (5 mg/kg, i.p.) exhibited a significant decrease
in Figure 7c and d, treatment of rats with EA at 30 mg/kg and
mainly polymorphonuclear leukocytes [Figure 7b]. As observed
showed an acute inflammation with extensive extravasations,
mainly polymorphonuclear leukocytes [Figure 7b]. As observed
in Figure 7c and d, treatment of rats with EA at 30 mg/kg and
indomethacin (5 mg/kg, i.p.) exhibited a significant decrease
in the number of cellular infiltrates.

The results of this study showed that EA significantly
suppressed the rat hind paw edema induced by intraplantar
injection of carrageenan, indicating marked anti-acute
inflammatory efficacy. In the current study, the anti-inflammatory
effect of EA was further evaluated to elucidate the underlying
mechanisms involved in this animal model. We demonstrated
that the effect may be due to inhibition of the pro-inflammatory
cytokines release, inflammatory enzymes (iNOS and COX-2)
expression and also their products (NO and PGE2). Furthermore,
we showed that EA could prevent liver damage induced by
carrageenan through the anti-oxidative mechanisms.
The carrageenan-induced paw edema is a well-defined model
of acute inflammation that a variety of inflammatory mediators
involves in its development and has wildly been used to evaluate
the anti-edematous effect of natural products. In the present
study, we showed that EA produced anti-inflammatory effects
in carrageenan-induced rat paw edema dose-dependently. Our
results confirmed previous findings that EA exhibit a noticeable
anti-inflammatory effect in experimental models. On the other
hand, it is well characterized that neutrophil infiltration plays
a key role in the inflammation induced by carrageenan in hind
paw.[19] Our results indicated that EA elicited a marked reduction
in the infiltration of neutrophils into the carrageenan-treated
paws, according to a histopathological examination.

A growing lines of evidence have demonstrated that
flavonoids, phenolic acids, and triterpenoid possessed
antinociceptive and anti-inflammatory effects in animal
models.[14] Studies have also reported that flavonoids such as
rutin, quercetin, luteolin produced significant antinociceptive
and anti-inflammatory activities.[17] Hence, it was suggested that
the antioxidant and anti-inflammatory activities of EA may be
related to its phenolic content.

The expression of the inducible isoform of NO synthase (iNOS)
has been proposed as an important mediator of inflammation.
Moreover, a large amount of NO is produced from L-arginine
by iNOS, thereby causing detrimental effects. Although
physiological NO production has beneficial anti-microbial
and anti-tumor effects, excessive NO produced by iNOS is a
mediator of the inflammatory diseases and causes cell injury
by generating reactive radicals, such as peroxynitrite.[18] Our
finding revealed that EA suppressed carrageenan-induced
production of iNOS and its product, NO. So, we suggest the
anti-inflammatory mechanism of EA may be through the
L-arginine-NO pathway because EA significantly inhibits the NO
production by iNOS. In accordance with this data, we previously
showed the involvement of L-arginine-NO pathway in the
anti-nociceptive effect of EA in acetic acid-induced visceral
pain.[11] Furthermore, Dhingra and Chhillar suggested the role
of iNOS in antidepressant-like activity of EA.[19] Moreover,
it has been indicated that EA inhibit the release of NO by
down-regulating iNOS.[13]

In vitro and in vivo studies have been indicated an existing
cross talk between the release of NO and PGs in the modulation
of inflammation.[20] Data indicate that NO has a profound effect
on COX-2 catalytic activity.[21] However, the mechanisms of
this effect have not been elucidated. One possibility is that
NO increases COX-2 half-life by generating free radicals and
inhibiting COX-2 autoinactivation.[26] Another possibility is
that lipid peroxidation initiated by peroxynitrite, the product
of the reaction of NO with superoxide, liberates arachidonic
acid from the cell membrane, which in turn activates COX-2.[22]
PGE2, a product of COX-2, is considered as one of the strongest
inflammatory mediators in the inflammatory response. Our
study showed that EA could inhibit PGE_2 production and reduce the COX-2 expression significantly in carrageenan-induced rat paw edema suggested that the anti-inflammatory effect of EA might be associated to its inhibitive effect on PGE_2 production through blocking COX-2 protein expression. On the other hand, carrageenan injection also provokes the release of some important pro-inflammatory cytokines such as TNF-α and IL-1β. TNF-α triggers the release of IL-1β and cytokine-induced neutrophil chemo-attractant 1. These mediators are responsible for stimulation of the PGs synthesis by COX-2. Moreover, TNF-α induces NO synthesis by activating iNOS and augments the responses of neutrophils to inflammatory stimuli. Our findings demonstrated that EA considerably reduced the TNF-α and IL-1β levels in the carrageenan-injected paw tissues. Inhibition of cytokine production by EA might account for the inhibition of COX-2 expression because both cytokines induce it. Also, EA has been found to inhibit the activation of transcription factor NF-xB and, therefore, reduced expression of COX-2 protein. Karin and Ben-Neriah showed that transcription of pro-inflammatory mediators such as iNOS, COX-2, TNF-α, and IL-1β is regulated by activation of NF-xB.

Free radical has been proposed to play an important role in the carrageenan-induced acute inflammatory response. In a number of pathophysiological conditions associated with inflammation or oxidative stress, free radical have been proposed to mediate cell damage in the liver. As a marker of oxidative damage, lipid peroxidation indicates changes in membrane fluidity and permeability and thus increases in rates of protein degradation, which will eventually lead to cell lysis. One of the consequences of lipid peroxidation can also result in enzyme activity changes. MDA is a metabolic product of lipid peroxidation, the level of which is raised in oxidative stress. The present results showed that EA reduce the severity of liver damage by lowering MDA level. Furthermore, there were significant decreases in AST and ALT level with EA treatment. We assume that the suppression of AST and ALT level is probably due to the inhibition of lipid peroxidation. A growing body of evidence suggests the anti-oxidative effect of EA, which is consistent with our results.

In conclusion, the results of the current study suggested that EA possessed anti-inflammatory effects in carrageenan-induced rat paw edema, which is comparable to indomethacin. The anti-inflammatory mechanism of EA may be related to the reduction of TNF-α and IL-1β that could result in inhibition of iNOS and COX-2 expression and its product (NO and PGE_2). Furthermore, the protective effect of EA on liver damage may result from anti-oxidative effects. Our findings provide new perspectives on the therapeutic use EA in the management of inflammatory diseases.
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Figure 6: Effects of ellagic acid (EA) on carrageenan (Carr)-induced (a) malondialdehyde (MDA), (b) aspartate aminotransferase (AST) and (c) alanine aminotransferase (ALT) concentrations at 5 h in rats. MDA was measured in liver homogenate. AST and ALT were measured in serum. Each value represents the mean ± standard error of the mean. *P < 0.05 and #P < 0.05 as compared to the VEH and Carr group, respectively (one-way ANOVA followed by Tukey's test). VEH: Vehicle, EA: Ellagic acid, Indo: Indomethacin (5 mg/kg, intraperitoneal).

Figure 7: Histopathologic examination of paw tissue of rats treated with EA, 5 h after injection of carrageenan (Carr). (a) Normal rats show the normal appearance of epidermis and dermis without any lesion. (b) Carrageenan-injected paw tissue in control group. Vasodilatation with edema, and migration of neutrophil were observed. (c and d) Carrageenan-injected paw of rats treated with EA (30 mg/kg, intraperitoneal [i.p.]) and indomethacin (5 mg/kg, i.p.), respectively. The migration of neutrophil and edema reduced. Sections were stained with H and E, ×40. 1: Neutrophil, 2: Edema, 3: Vessel.
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