International Immunopharmacology 19 (2014) 290–299

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Ellagic acid protects against carrageenan-induced acute inflammation through inhibition of nuclear factor kappa B, inducible cyclooxygenase and proinflammatory cytokines and enhancement of interleukin-10 via an antioxidant mechanism

Nagla A. El-Shitany [a,b,*], Eman A. El-Bastawissy [c], Karema El-desoky [d]

[a] Department of Pharmacology and Toxicology, College of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia
[b] Department of Pharmacology and Toxicology, College of Pharmacy, Tanta University, Tanta, Egypt
[c] Department of Pharmaceutical Chemistry, College of Pharmacy, Tanta University, Tanta, Egypt
[d] Department of Pathology, College of Medicine, Tanta University, Tanta, Egypt

ARTICLE INFO

Article history:
Received 25 April 2013
Received in revised form 3 February 2014
Accepted 3 February 2014
Available online 15 February 2014

Keywords:
Ellagic acid
Carrageenan
Inducible cyclooxygenase
Nuclear factor kappa beta
Interleukin
Antioxidant

ABSTRACT

There are several hypotheses that explain the process of acute inflammation, including free radical overproduction, pro-inflammatory enzyme activation, and release of pro-inflammatory cytokines. In this study, the protective role of ellagic acid against carrageenan-induced acute inflammation was assessed. In addition, the immunomodulatory action, the antioxidant effects, and the role of COX-2 and NF-κB were also investigated. Inflammation was induced by the injection of 100 μl of 1.5% carrageenan solution. Ellagic acid (10, 25, 50, 100 and 200 mg/kg), indomethacin (10 mg/kg), meloxicam (4 mg/kg), and saline, were injected 2 h before carrageenan injection. The percentage inhibition in the paw weight was calculated. Paws, MDA, NO, GSH, IL-1β, TNF-α, IL-10 and NF-κB mRNA expression were estimated. Formalin fixed hind paws were used for histopathological examination and immunohistochemical staining for COX-2 expression. Ellagic acid, meloxicam and indomethacin reduced paws, edema, MDA and NO formation. In addition, all of them restored the depleted GSH contents in the paws. Ellagic acid, meloxicam and indomethacin reduced NF-κB mRNA expression. Ellagic acid ameliorated COX-2 expression; meloxicam inhibited while indomethacin failed. Both ellagic acid and meloxicam increased IL-10 while indomethacin did not. The docking study revealed a high affinity of ellagic acid towards COX-2. Ellagic acid exhibited a potent anti-inflammatory effect against carrageenan-induced inflammation. The mechanisms of ellagic acid induced protection were proved to be due to reduction of NO, MDA, IL-1β, TNF-α, COX-2 and NF-κB expression and induction of GSH and IL-10 production.

1. Introduction

There are several hypotheses that explain the process of acute inflammation including, overproduction of oxygen and nitrogen-derived free radicals, activation of a complex array of enzymes, and release of several inflammatory mediators and pro-inflammatory cytokines [1,2]. There are now real evidences that much of the cytotoxicity occurs during the acute phase of inflammation is attributed to the deleterious action of reactive oxygen species (ROS) and nitric oxide (NO) [3–5]. Nuclear factor kappa B (NF-κB) plays an important role in the promotion of acute inflammation. It regulates the expression of many genes involved in inflammation such as inducible enzymes, chemokines and cytokines [6]. Furthermore, the inflammatory response is mediated by various signaling molecules and enzymatic pathways, among which COX-2 (inducible cyclooxygenase isoform) is a key enzyme playing an important role in regulating the formation of prostaglandins (PGs) from arachidonic acid during inflammation [7,8]. In addition, Holtmann et al. [9] reported that, tumor necrosis factor-α (TNF-α) and interleukin-1 β (IL-1β) are important primary mediators, which play a critical role in both acute and chronic inflammation. Interleukin-10 (IL-10) is known as the most important anti-inflammatory cytokine found within the immune response. IL-10 inhibits monocyte/macrophage-derived TNF-α, IL-1, IL-6, IL-8, and IL-12 [10,11].

Ellagic acid (2,3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3-cde][1] benzopyran-5,10-dione) is a phenolic constituent in certain fruits and nuts, such as raspberries, strawberries, walnuts, longan seed, mango kernel and pomegranate [12,13]. It was documented that ellagic acid possesses radical scavenging, metal chelating, antibacterial, antiviral, antifibrotic, antiatherogenic, antimutagenic and anticancer activities [14]. Although very little information exists on the mechanism of action of ellagic acid, its potent scavenging action on both OH and O_2^- might

* Corresponding author at: Department of Pharmacology and Toxicology, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia. Tel.: +966 545923440, +966 26323428.
E-mail address: Nagla_fouad@yahoo.com (N.A. El-Shitany).

http://dx.doi.org/10.1016/j.intimp.2014.02.004
1567-5769/

be responsible for these effects [15,16]. Recently, researchers have shown the possible involvement of COX protein expression in the mechanism of action of ellagic acid in the animal models. If ellagic acid is a COX inhibitor, then it may be a potent anti-inflammatory agent. Few studies have investigated the anti-inflammatory effect of ellagic acid in vivo [17].

Therefore, this study aimed to investigate the anti-inflammatory, the antioxidative and the immunomodulating effects of ellagic acid in a model of carrageenan-induced acute local inflammation in rats. Furthermore, ellagic acid probable therapeutic effects were compared with both indomethacin (non selective COX inhibitor) and meloxicam (selective COX-2 inhibitor). In addition, the effect of ellagic acid on NF-κB and COX-2 expression as well as the interaction of ellagic acid with COX-2 receptor were also investigated.

2. Materials and methods

2.1. Chemicals

Ellagic acid, indomethacin, carrageenan sodium (Sigma, USA), and meloxicam injection (15 mg meloxicam/2 ml; Amoun Pharma, Cairo, Egypt) were used in this study. All other chemicals were of high grade.

2.2. Animals and treatments

Adult male albino rats (150–180 g) (Egyptian offspring) were used in this study. Rats were maintained under controlled conditions and had free access to food and water. Ellagic acid (10, 25, 50, 100, 200 mg/kg) (24 rats for each dose), meloxicam (4 mg/kg) (24 rats) [18], indomethacin (10 mg/kg) (24 rats) [19] and 0.9% saline (carrageenan group) (24 rats) were injected i.p. at 2 h before carrageenan injection. A set of non-carrageenan injected rats served as a control group (6 rats). All experiments were performed according to the rules and regulations of Tanta University Animal Ethics Committee, Egypt.

2.3. Induction and assessment of carrageenan-induced paw edema

100 μl of 1.5% carrageenan sodium dissolved in 0.9% saline was s.c. injected into the plantar side of the right hind paw of rats [20]. The weight of both the left paw (non-carrageenan injected) and the right paw (carrageenan injected) was determined at 1, 2, 3, and 4 h after intraplantar carrageenan injection [21]. Edema weight was assessed by calculating the difference between the right paw weight and the left paw weight at all the indicated time points. The percentage increase in paw weight was calculated by dividing the edema weight at each time point by the left paw weight of the same animal multiplied by 100.

$$\text{The percentage increase in paw weight} = \frac{\text{Right Paw Weight} - \text{Left Paw Weight}}{\text{Left Paw Weight}} \times 100.$$

2.4. Sample collection

Four hours after carrageenan injection, the rat hind paws were collected, frozen (−80 °C) and then used for malondialdehyde (MDA), reduced glutathione (GSH), NO metabolites (nitrate plus nitrite), IL-1β, TNF-α and IL-10 determination. Formalin fixed entire hind paw was used for histopathologic examination (hematoxylin and eosin) (H&E) and immunohistochemical staining for COX-2 expression.

2.5. Sample preparation for MDA and GSH determination

The soft tissues of the rat paws were dissected, washed with ice-cold normal saline, and then, homogenized in four times their volume cold normal saline at 4 °C. The homogenate was then centrifuged at 12,074 *g* for 5 min. The supernatant obtained was stored at −20 °C for GSH and MDA detection [22].

2.6. Determination of hind paw lipid peroxides measured as MDA

Lipid peroxidation product, MDA was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS) according to the method of Uchiyama and Mihara [23]. The formed color resulting from reaction of tissue homogenate with thiobarbituric acid was extracted with n-butanol. The intense pink color was measured spectrophotometrically at 535 nm. The paw MDA level was expressed as nmol/g tissue.

2.7. Determination of hind paws GSH

Glutathione was determined according to the method of Son et al. [24]. The method is based on the reduction of bis-(3-carboxy-4-nitrophenyl) disulfide reagent by the thiol group of GSH forming 2-nitro-5-mercaptobenzoic acid. The intense yellow color of 2-nitro-5-mercaptobenzoic acid was measured spectrophotometrically at 412 nm.

2.8. Determination of hind paws NO

Nitric oxide was determined by measuring NO metabolites (nitrate + nitrite), according to Miranda et al. [25]. According to Posadas et al. [26], soft tissues were dissected from the rat's paw and homogenized in deionized water at 12,074 *g* for 5 min. The proteins were precipitated by ethanol. Vanadium chloride was added to reduce nitrate to nitrite. The intense pink color resulted after the addition of Griess reagent was measured spectrophotometrically at 540 nm.

2.9. Determination of hind paws IL-1β, TNFα and IL-10

The soft tissues of the rat hind paws were homogenized in ice cold phosphate buffered saline containing protease inhibitor cocktail and 0.05% Tween 20. Samples were centrifuged at 12,074 *g* for 10 min. The resultant supernatant was used for analyzing IL-1β, TNFα and IL-10 levels in an ELISA assay as part of an Assaymax IL-1β, TNFα and IL-10 kits (Gentaur, Dublin, Ireland) using monoclonal antibodies specific for TNF-α and IL-10, respectively. Cytokine concentrations were calculated using a standard purified recombinant cytokines.

2.10. Immunohistochemical determination of COX-2 expression in the hind paw sections

COX-2 expression was detected by the immunostaining of tissue sections prepared from formalin-fixed, paraffin-embedded hind paws using a kit obtained from Lab Vision (Fremont, CA). An immunoperoxidase (PAP, peroxidase/anti-peroxidase) technique was adopted. In this way, the cytoplasm of each COX-2 (+) cell was stained brown. The brown staining was graded as follows: no brown color (−) (negative staining), mild brown staining (+) (mild positive) and moderate brown staining (++) (moderate positive).

2.11. Preparation of paw tissues for PCR analysis

The soft tissues were dissected from the rat's paw and total RNA was extracted using an RNA extraction kit (Bio Basic Inc., Markham, ON, Canada) according to the manufacturer's instructions. RNA was then transcribed using revert aidTM first strand cDNA synthesis kit (Ferments life science, Fort Collins, CO, USA).

2.12. Determination of NF-κB mRNA expression by semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR)

A fragment of 256 bp of rat NF-κB or 154 bp of β-actin (Table 1) was amplified using the PCR master mix (Ferments life science). In all experiments, control reactions were performed by substituting sterile