In vivo anti-inflammatory action of eugenol on lipopolysaccharide-induced lung injury

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Magalhães CB, Riva DR, DePaula LJ, Brando-Lima A, Koatz VL, Leal-Cardoso JH, Zin WA, Faffe DS. In vivo anti-inflammatory action of eugenol on lipopolysaccharide-induced lung injury. J Appl Physiol 108: 845–851, 2010. First published January 14, 2010; doi:10.1152/japplphysiol.00560.2009.—Eugenol, a methoxyphenol component of clove oil, suppresses cyclooxygenase-2 expression, while eugenol dimers prevent nuclear factor-kB (NF-kB) activation and inflammatory cytokine expression in lipopolysaccharide-stimulated macrophages. Our aim was to examine the in vivo anti-inflammatory effects of eugenol. BALB/c mice were divided into four groups. Mice received saline [0.05 ml intratracheally (it), control (Ctrl) and eugenol (Eug) groups] or Escherichia coli LPS (10 µg it, LPS and LPSEug groups). After 6 h, mice received saline (0.2 ml ip, Ctrl and LPS groups) or eugenol (160 mg/kg ip, Eug and LPSEug groups). Twenty-four hours after LPS injection, pulmonary resistive (ΔP1) and viscoelastic (ΔP2) pressures, static elastance (Ea), and viscoelastic component of elastance (ΔE) were measured. Lungs were prepared for histology. In parallel mice, bronchoalveolar lavage fluid was collected 24 h after LPS injection. TNF-α was determined by ELISA. Lung tissue expression of NF-kB was determined by EMSA. ΔP1, ΔP2, Ea, and ΔE were significantly higher in the LPS group than in the other groups. LPS mice also showed significantly more alveolar collapse, collagen fibers, and neutrophil influx and higher TNF-α levels and NF-κB expression than the other groups. Eugenol treatment reduced LPS-induced lung inflammation, improving lung function. Our results suggest that eugenol exhibits in vivo anti-inflammatory action in LPS-induced lung injury.

acute lung injury; inflammation; lung mechanics

EUGENOL (4-ALLYL-2-METHOXYPHENOL), a component of clove oil, is commonly used as a flavoring agent in cosmetics and food products and, in particular, in dentistry in zinc oxide-eugenol chelating cement. Previous studies demonstrated that eugenol and other phenolic compounds show antioxidative and anti-inflammatory activities, which stem from the inhibition of prostaglandin synthesis and neutrophil chemotaxis (18, 22, 23). Also, it has been shown that phenolic antioxidants inhibit nuclear factor-kB (NF-kB) activation induced by tumor necrosis factor-α (TNF-α) (5) and block cyclooxygenase (COX)-2 expression in lipopolysaccharide (LPS)-stimulated macrophages (23). TNF-α, a monokine known to mediate inflammation and carcinogenesis in various pathophysiological processes, acts in part through activation of NF-κB (5), an important transcriptional factor that regulates inflammatory response and the expression of inflammatory cytokines (7). Additionally, TNF-α stimulates the secretory activity of airway smooth muscle cells, having a main role in orchestrating and perpetuating the inflammatory process (11). On the other hand, COX, the rate-limiting enzyme in the conversion of arachidonic acid to prostanoids, exists as two isoforms: COX-1, constitutively expressed in most cell types, and COX-2, induced by growth factors, cytokines, and LPS via activation of transcription factors such as NF-κB in a variety of cells (23).

In vitro data suggest that methoxyphenols and related compounds constitute likely candidates to interfere with TNF signaling leading to activation of NF-kB (5, 22, 23). LPS, a component of the cellular wall of gram-negative microorganisms responsible for local and systemic toxicity of gram-negative bacteria (31), strongly stimulates the expression of NF-kB in macrophages (15). In vivo administration of LPS leads to an inflammatory response network characterized by the release of different proinflammatory mediators (30). Mononuclear phagocyte activation plays a major role in this process, which expresses, among other cytokines, TNF-α, a central mediator in endotoxin-induced lung injury (13, 31). TNF-α release induces neutrophil adhesion to endothelial cells, resulting in neutrophil migration and infiltration of pulmonary spaces (13, 30). LPS-induced acute lung injury (ALI) is associated with respiratory mechanical changes, with increased pulmonary resistance secondary to tissue and small airways involvement (8). Endotoxin and LPS, its purified form, are also commonly found in the air (20, 24). In humans, acute inhalation of endotoxin can induce inflammatory reaction with neutrophil and macrophage activation and pulmonary response characterized by bronchoconstriction (4, 27). Additionally, chronic exposure to endotoxin has been associated with increased risk of nonatopic chronic obstructive pulmonary disease and severe asthma (20).

Since eugenol and related compounds exhibit anti-inflammatory properties and show inhibitory effects on LPS-stimulated NF-kB activation and inflammatory cytokine and COX-2 expressions in both mouse and human macrophages in culture, we examined the anti-inflammatory effects of eugenol in an in vivo model of LPS-induced lung injury. Our results indicate improvement of LPS-induced changes in lung inflammation, as well as in elastic, resistive, and viscoelastic components of lung mechanics after eugenol treatment.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro. All animals received humane care according to the Helsinki convention for the use and care of animals. The experimental study was carried on in a research laboratory.
Animal preparation. Twenty-eight BALB/c mice (20–25 g) were randomly divided into four groups. In control (Ctrl) and LPS groups, mice were intratracheally (i.t.) injected with 0.05 ml of sterile saline solution (0.9% NaCl) or 10 μg of LPS (Escherichia coli serotype O55:B5; Sigma Chemical, St. Louis, MO) in 0.05 ml of saline, respectively. With 10 μg of LPS we can observe well-established functional, structural, and remodeling changes in lung parenchyma (24 h, 19, 26, 28). This dose of LPS yields a 1.5-fold increase in lung static elastance (E_{st}) compared with control animals (19). Six hours later, mice received intraperitoneal (i.p.) injection of either 0.2 ml of saline [Ctrl (n = 7) and LPS (n = 9) groups] or eugenol [160 mg/kg body wt in 0.2 ml saline; eugenol (Eug, n = 4) and LPSEug (n = 8) groups]. All animals were analyzed 24 h after saline or LPS administration.

Pulmonary mechanics. Twenty-four hours after saline or LPS administration, the animals were sedated with diazepam (1 mg ip), anesthetized with pentobarbital sodium (20 mg/kg body wt ip), paralyzed with pancuronium bromide (5 mg/kg body wt iv), and mechanically ventilated (Samay VR15, Universidad de la Republica, Montevideo, Uruguay) with a frequency of 100 breaths/min, tidal volume of 0.2 ml, flow of 1 ml/s, and positive-end-expiratory pressure of 2.0 cmH_{2}O. The anterior chest wall was surgically removed. A pneumotachograph (1.5-mm ID; length = 4.2 cm, distance between side ports = 2.1 cm) was connected to the tracheal cannula for the measurements of airflow (V'), and changes in lung volume were obtained by flow signal digital integration. The pressure gradient across the pneumotachograph was determined by means of a Validyne MP-45-2 differential pressure transducer (Validyne Engineering, Northridge, CA). Equipment resistive pressure (= R_{eq} V') was subtracted from pulmonary resistive pressure so that the present results represent intrinsic values. Transpulmonary pressure was measured with a Validyne MP-45 differential pressure transducer (Validyne Engineering) (8).

Lung resistive (∆P1) and viscoelastic/inhomogeneous (∆P2) pressures, total pressure drop after flow interruption (P_{tot} = ∆P1 + ∆P2), lung static (E_{st}) and dynamic (E_{dy}) elastances, as well as ∆E (E_{dyn} − E_{st}) were computed by the end-inflation occlusion method. ∆P1 selectively reflects airway resistance, and ∆P2 reflects stress relaxation, or viscoelastic properties of the lung (2). Lung mechanics were measured 10–15 times in each animal.

Histological study. Heparin (1,000 IU) was intravenously injected immediately after the determination of respiratory mechanics. The trachea was clamped at end expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly killed the animals. Lungs were perfused with an infusion of formaldehyde 10% in Millonig’s phosphate buffer (100 ml HCHO, 900 ml H_{2}O, 18.6 g Na_{2}HPO_{4}, 4.2 g NaOH) and then removed en bloc. After fixation, the tissue was embedded in paraffin. Four-μm-thick slices were cut and stained with hematoxylin and eosin (H & E) and picrosirius staining for collagen detection.

Morphometric analysis of lung architecture, the volume fraction of collapsed alveoli, was determined in each sample by the point-counting technique (14) across 10 random nonoverlapping microscopic fields, at ×400 magnification, on a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The amounts of mononuclear (MN) and polymorphonuclear (PMN) cells in the pulmonary tissue were evaluated at ×1,000 magnification. Points falling on MN and PMN cells were counted and divided by the total number of points falling on tissue area in each microscopic field (14). The interstitial area of lung parenchyma occupied by collagen was quantified in picrosirius-stained sections by the point-counting technique on images captured in a blinded manner across 15 random noncoincident fields (×400 magnification). The quantification was done on captured high-quality images (2,048 × 1,536 pixels) with Image Pro Plus 4.5.1 software (Media Cybernetics, Silver Spring, MD). A single observer performed morphological measurement in a blinded manner. Results were expressed as percentage of surface density/tissue.
TNF-α, NF-κB activation, and neutrophil influx in the BALF (Fig. 3). These findings are in accordance with previous reports on LPS-induced lung injury in mice, showing established functional, structural, and remodeling changes in lung parenchyma 24 h after LPS challenge (8, 26).

Eugenol treatment, applied 6 h after LPS-induced lung injury, significantly improved viscous, elastic, and viscoelastic components of lung mechanics (Fig. 1). Animals injected with LPS and treated with eugenol showed less pulmonary inflammation and remodeling than the LPS group, characterized by lower alveolar collapse, PMN infiltration, and collagen fiber deposition in lung parenchyma (Fig. 2 and Table 1). Eugenol treatment also significantly reduced neutrophil infiltration and TNF-α release in the BALF and abrogated NF-κB activation (Fig. 4) compared with the LPS-challenged group without treatment.

Spearman correlation test showed that LPS-induced elastic and viscoelastic changes in lung mechanics were positively correlated with the fractional area of alveolar collapse, all groups included (Fig. 5).

**DISCUSSION**

Our results demonstrate that eugenol treatment 6 h after the induction of lung injury in vivo avoided LPS-induced increase in lung impedance (Fig. 1), through the modulation of lung inflammatory and remodeling processes elicited by LPS (Table 1 and Fig. 3). LPS-induced ALI is a frequently used model to understand morphological and functional changes secondary to circulating LPS, such as those observed in the acute respiratory distress syndrome. The administration of LPS causes a cascade of inflammatory responses that trigger an acute inflammatory response (8, 12), with TNF-α release and neutrophil influx into lung spaces (29, 32). More recent studies highlight the significance of LPS-induced TNF-α secretion and positive TNF-α feedback mechanisms with upregulation of NF-κB activity on
ALI pathophysiology (6, 29). In the present study we used a model of ALI induced by intratracheal instillation of LPS, which yields in vivo respiratory mechanical changes associated with lung morphometric alterations, collagen deposition in lung parenchyma, neutrophil infiltration in lung tissue and BALF, as well as TNF-α release (19, 26, 28). The present results support the physiological and morphometric changes previously reported in this LPS-induced model of ALI.

Increased influx of RMN cells into lung parenchyma and BALF, as well as of lung myeloperoxidase (MPO) activity, represent early events in LPS-induced lung injury. Indeed, they have been observed in the first 6 h after aerosol or intratracheal exposure, beginning as early as 2 h after exposure (8, 32). In this line, Asti et al. (1) also demonstrated a massive margination of PMN cells in lung parenchyma and an increase in MPO activity in the lung at 4 h (131%), reaching a peak (147%) at 6 h after endotoxin challenge with a similar dose of intratracheal LPS. Evidence of NF-κB activation was also reported 6 h after LPS challenge in a murine model of lung inflammation (3). Thus eugenol was delivered after the challenge (at the peak of the inflammatory process as evaluated by MPO) because our objective was to assess its anti-inflammatory potential after the triggering of the inflammatory cascade, i.e., as a compound that could be used to treat LPS-induced inflammation, rather than just prevent LPS-induced changes.

Eugenol given at 6 h after LPS administration was able to avoid the aforementioned influx of neutrophils into lung tissue and BALF (Fig. 3A, Table 1) and the higher amounts of inflammatory cells and macrophages in the BALF of LPS-treated mice (Fig. 3, A–C).

The precise mechanisms of the anti-inflammatory action of eugenol are still disputed. A preventive effect of eugenol and other related compounds has been shown on LPS-induced NF-κB activation, inflammatory cytokine release, and COX-2 expression in human and mouse macrophage cells stimulated in culture (22, 23). Phenolic antioxidants can also block TNF-α expression at a transcriptional level, with inhibition of NF-κB activation, the major regulator of TNF-α transcription in macrophages (18), at both gene and protein levels (22, 23). Although some eugenol dimers inhibit LPS-stimulated transcriptional activity, this effect was not observed with eugenol (22, 23). On the other hand, eugenol was able to inhibit prostaglandin production induced by LPS through inhibition of COX-2 in mouse macrophages. In contrast to these previous studies in murine macrophages, Lee and colleagues (16) recently demonstrated (2007) in vitro that eugenol downregulated protein levels of IL-1β and TNF-α induced by LPS in human macrophages; in the present study TNF-α levels were kept at control levels by the administration of eugenol (Fig. 3D). Together these findings suggest that the molecular mechanisms by which eugenol modulates cytokine and prostaglandin expression in the presence of LPS remain to be examined, indicating a specificity of the polyphenol activity likely depending on cell type, phenolic chemical structure, and concentration (23).
It is noteworthy, however, that most reports on the beneficial effects of polyphenols have been obtained from in vitro studies, and more detailed investigations are required to extrapolate these results to in vivo situations (25). Birrell et al. (3) observed in vivo reduction of inflammatory mediators after polyphenol administration similar to the ones described in vitro but, however, with no impact on the activation of the NF-κB pathway. Absorption, bioavailability, biodistribution, and metabolism of polyphenols are not entirely known. Furthermore, resulting derivatives do not necessarily possess the same biological activity (16). In relation to eugenol, it is described that the drug is rapidly absorbed and metabolized after oral administration, being almost completely excreted in the urine within 24 h (80% of its dose is excreted within 6 h after oral administration). Eugenol undergoes a pronounced first-pass effect in serum, while unconjugated eugenol was not detected after an oral dose of 150 mg (10). In this vein, it is noteworthy that the present study is the first report of in vivo anti-inflammatory effect of eugenol in LPS-induced ALI, supporting the notion that inhibition of TNF-α release (Fig. 3D) and NF-κB activation (Fig. 4) are probably involved in this beneficial action of eugenol. Furthermore, the in vivo anti-inflammatory effect of eugenol was significant enough to yield lung functional improvement.

The inhibition of TNF-α production by phenolic antioxidants is described as complete, long-lasting, and dose dependent (18). In our in vivo study, eugenol treatment with 160 mg/kg body wt improved elastic, resistive, and viscoelastic components of lung mechanics (Fig. 1), as well as reducing alveolar collapse and PMN infiltration in lung parenchyma after LPS-induced lung injury (Fig. 2, Table 1). It is well documented that pulmonary tissue mechanics is highly affected by lung parenchyma structure and composition. LPS administration induces changes in alveolar surfactant, favoring alveolar collapse leading to increased airway resistance, resulting in tissue and airway involvement correlated to viscoelastic parameters (ΔE and ΔP2) (8). In our study, elastic and viscoelastic components of pulmonary mechanics were positively correlated with the amount of alveolar collapse, suggesting that eugenol somehow may have influenced the surfactant system, usually impaired in injured lungs (Fig. 5).

On the other hand, eugenol treatment also improved the resistive component of lung mechanics (Fig. 1). This finding could be explained by a potential direct effect of eugenol on airway smooth muscle relaxation, since relaxant properties of eugenol-related compounds have been described in tracheal and vascular smooth muscle (17). A second explanation could be the suppression of inflammatory mediators and prostanoids, modulating bronchomotor tonus. Exposure to LPS increases expression of COX-2, the inducible isof orm of COX mainly produced by inflammatory cells, and is implicated in the induction of pro-inflammatory mediators, such as TNF-α and IL-1β, which is consistent with the data presented in Table 1. Eugenol treatment with 160 mg/kg body wt significantly reduced TNF-α and IL-1β levels in BALB/c mice treated with LPS (Fig. 4).

The mechanism by which eugenol modulates COX-2 expression is not clear. However, it has been shown that eugenol is capable of modulating the NF-κB activation pathway, which is an important regulatory element in the expression of COX-2. In our study, eugenol treatment with 160 mg/kg body wt significantly reduced the levels of nuclear translocation of p65 subunit of NF-κB in alveolar macrophages from BALB/c mice treated with LPS (Fig. 4).

Fig. 4. Electrophoretic mobility shift assay showing the effect of eugenol treatment on nuclear factor (NF)-κB activation in alveolar macrophages from BALB/c mice. Animals were instilled with saline (Ctrl) or 10 μg LPS followed (6 h later) by treatment with saline (LPS) or eugenol (160 mg/kg body wt, LPSEug). Values are means ± SE of 3 animals/group. Different letters indicate significantly different values (P < 0.05). au, Arbitrary units.

Fig. 5. Spearman correlation between pulmonary mechanical parameters and % of collapsed air spaces obtained in mice instilled with saline (Ctrl) or 10 μg LPS followed (6 h later) by treatment with saline (LPS) or eugenol 160 mg/kg body wt (LPSEug). Significant correlations between alveolar collapse and ΔE (A), alveolar collapse and E0 (B), and alveolar collapse and ΔP2 (C) were found. r, Spearman correlation coefficient.
responsible for prostaglandin production. COX-2’s relevance for LPS-driven lung tissue neutrophilia is controversial (3); however, a role for COX-2 inhibition cannot be ruled out as a participating mechanism in airway relaxation.

It has been demonstrated that collagen fiber deposition in LPS-induced ALI can impair tissue mechanics independently of alveolar collapse (26). Additionally, collagen deposition in lung parenchyma is significantly increased 24 h after LPS administration, indicating that the biochemical processes involved in tissue remodeling are able to react very quickly as well (28). In our work, eugenol prevented collagen deposition in lung parenchyma (Table 1), suggesting that eugenol can also interfere positively with the remodeling process present in ALI.

Our study presents limitations. 1) Recruitment maneuvers were not performed before the measurements. On one hand, the anterior chest wall and later on the lungs were removed very carefully, to avoid compression of the lungs and the concomitant generation of atelectasis; on the other hand, the reexpansion of the lungs would eliminate at least part of the atelectasis generated by the inflammatory process. 2) The Eug group was compared with the others only from the lung mechanics point of view; we cannot dismiss the possibility that it could be biochemically different. 3) Different doses of eugenol could convey diverse results, and thus dose-response curves would enrich the conclusions.

In conclusion, eugenol effectively improved functional and structural pulmonary changes induced by LPS, modulating lung inflammation and remodeling in an in vivo model of ALI, through a mechanism involving inhibition of TNF-α release and NF-κB activation. This study demonstrates that many in vitro anti-inflammatory effects of eugenol translate in an in vivo model of lung inflammation and may lead to potential new therapies for ALI as well as other chronic lung inflammatory diseases.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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