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Eugenol—The active principle from cloves inhibits 5-lipoxygenase activity and leukotriene-C4 in human PMNL cells

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Abstract

Polymorphonuclear leukocytes (PMNL) play an important role in the modulation of inflammatory conditions in humans. PMNL cells recruited at the site of inflammation, release inflammatory mediators such as leukotrienes, proteolytic enzymes and reactive oxygen species. Among these, leukotrienes are implicated in pathophysiology of allergic and inflammatory disorders like asthma, allergic rhinitis, arthritis, inflammatory bowel disease and psoriasis. 5-Lipoxygenase (5-LO) is the key enzyme in biosynthetic pathway of leukotrienes. Our earlier studies showed that spice phenolic active principles significantly inhibit 5-LO enzyme in human PMNLs. In this study we have further characterized the inhibitory mechanism of eugenol, the active principle of spice-clove on 5-LO enzyme and also its effect on leukotriene C_4 (LTC_4). Substrate dependent enzyme kinetics showed that the inhibitory effect of eugenol on 5-LO was of a non-competitive nature. Further, eugenol was found to significantly inhibit the formation of LTC_4 in calcium ionophore A23187 and arachidonic acid (AA) stimulated PMNL cells. These data clearly suggest that eugenol inhibits 5-LO by non-competitive mechanism and also inhibits formation of LTC_4 in human PMNL cells and thus may have beneficial role in modulating 5-LO pathway in human PMNL cells.

1. Introduction

Leukotrienes are biologically active 5-lipoxygenase products of arachidonic acid (AA) metabolism and are implicated in inflammation and allergic manifestations. Unlike other biologically active molecules, leukotrienes are not stored as preformed components of immune response within the secreting cells, but are synthesized de novo using AA released from membrane phospholipids upon exposure to allergic conditions, infection and/or inflammation. 5-LO, is the key enzyme in the leukotriene biosynthetic pathway and belongs to a class of dioxygenase enzymes containing a catalytic non-heme iron atom. The distribution of 5-LO is essentially restricted to polymorphonuclear leukocytes (PMNLs), monocytes, macrophages, mast cells and B-Lymphocytes. In these cells, 5-LO catalyses the incorporation of dioxygen at C-5 of AA to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and further metabolized to leukotrienes (LTs)—LTB_4, LTC_4, LTD_4 LTE_4. All these LTs exert their biological actions by interacting with receptors on target cells. LTs have been implicated as mediators in a diversity of diseases including asthma and a number of other inflammatory pathologies such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and glomerulonephritis [1]. Although leukotrienes may not be involved in the initial stages of a disease, they appear to play an important role in the propagation of the disease by exacerbating the initial, primarily local events and eventually leading to tissue damage.

The hallmark of PMNLs in allergic and inflammatory conditions lie in its increased recruitment at the site of inflammation and secretion of elevated levels of cysteinyl leukotrienes which culminate in pathophysiology of

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such conditions. Therefore, the modulation of LT production through inhibition of 5-LO pathway may have therapeutical importance to combat the effects of its products in various inflammatory conditions. Since the elucidation of the 5-LO biosynthetic pathway, an ongoing debate in drug development has been whether inhibition of the 5-LO enzyme is more efficacious than antagonization of the peptido or non-peptidoleukotriene receptors. However, evidence suggests that 5-LO inhibitors may be superior to LT-receptor antagonists, since 5-LO inhibitors block the action of the full spectrum of 5-LO products, whereas LT-antagonists produce narrower effects [2,3]. In addition, LT-receptor antagonists appear to prolong the half-lives of LTs by hindering their further metabolism [4].

Spices are considered as nutraceuticals in view of their medicinal properties including antioxidant [5–7] anti-inflammatory [8] hypocholesterolomic [9] antimicrobial [10] and anticarcinogenic [11,12] properties in addition to their flavor and aroma enhancing properties. Our earlier studies clearly demonstrated that spice aqueous extracts and active principles, either individually or in combination significantly inhibit 5-LO, the key enzyme involved in biosynthetic pathway of leukotrienes in human PMNL cells [13]. Aqueous extract spice-clove and its active principle-eugenol, significantly inhibited 5-LO of PMNL cells. In this study, we further examined the kinetics of inhibition of 5-LO by eugenol and also the inhibition of leukotriene formation in human PMNL cells.

2. Materials and methods

2.1. Materials

All chemicals and solvents used were of AR grade. ATP, DTT, calcium ionophore and spice active principles eugenol were obtained from Sigma Chemical Co., USA. HPLC grade solvents for HPLC analysis were obtained from Merck, India. LTC_4 standard was obtained from Cayman chemicals, USA and arachidonic acid from Nuchek, USA.

2.2. Isolation of 5-LO from PMNLs of human blood

Human peripheral venous blood from healthy individuals who had not received any medication was collected with anticoagulant EDTA. Polymorphonuclear leukocytes (PMNLs) were separated from blood by Ficoll-Histopaque density gradient method and hypotonic lysis of erythrocytes [14]. All procedures were performed at 4 °C. PMNL cells were suspended in phosphate buffer saline and sonicated for 20–30 s at 20 KHz to release the cytosolic 5-LO enzyme into solution. This solution was centrifuged at 10,000 rpm, for 30 min at 4 °C. The supernatant was used as the source of enzyme. Protein was estimated by Lowry's method [15] using BSA as standard.

2.3. 5-Lipoxygenase enzyme assay

Assay of 5-LO was performed according to the method of Aharony and Stein [16]. The standard reaction mixture for the of 5-LO assay contained 100 mM phosphate buffer pH 7.4, 50 μM of DTT, 200 μM of ATP, 300 μM of $CaCl_2$, 150 μM of AA and 5.0 μg protein. Enzymatic reaction was carried out at room temperature. 5-LO activity was measured as 5-HETE formed at 234 nm using Shimadzu spectrophotometer. The molar extinction co-efficient of $25\,mM^{-1}\,cm^{-1}$ was used to calculate specific activity of the enzyme. The enzyme activity was expressed as μmoles of 5-HETE formed/min/mg protein.

2.4. Inhibitory studies with eugenol on 5-LO

Eugenol was dissolved in DMSO and incubated with the enzyme at different concentrations for 2.0 min and then the reaction was initiated with the addition of AA and the 5-LO activity was measured as 5-HETE formed at 234 nm spectrophotometrically as described above.

2.5. Enzyme kinetic studies on inhibition of 5-LO with spice principles

The enzyme kinetics on inhibition of 5-LO were studied using different concentrations of substrate AA (50 and 200 μM) versus IC_{25}, IC_{50} and IC_{75} inhibitory concentrations of spice active principles- curcumin and eugenol. A double reciprocal plots of enzyme kinetics were constructed according to Lineweaver and Burk [17].

2.6. Inhibitory studies with eugenol on LTC_4

Human PMNLs were separated from blood by Ficoll gradient method (as explained earlier). PMNLs were suspended in Hank's Balanced Salt Solution (HBSS) and counted using haemocytometer. The viability of PMNL cells were checked by Trypan blue staining. Cell suspension (2.5×10^6 cells) in HBSS were incubated with spice active principle eugenol for 15 min at 37 °C. Reaction was started by stimulating the cells by adding 2.5 μM calcium ionophore A23187 and 20.0 μM of AA and incubated for 15 min at 37 °C. The reaction was stopped by adding 30 μL of 1N HCl and sonicated for 20–30 s at 20 KHz. The sample was centrifuged at 2000*g* for 20 min at 4 °C. The supernatant was loaded on to Sep-Pack C_{18} solid phase extraction cartridges (Waters Millipore Corp. Milliford MA) and leukotrienes were eluted with ethyl acetate. Peptidoleukotriene- LTC_4 was

separated and analyzed by Shimadzu LC-10A HPLC system using Hypersil reverse phase C_{18} column (150 × 4.6 mm; 5 μm) with mobile phase of methanol:ammonium acetate(70:30). LTC_4 peak separated at 4.60 min at flow rate of 0.5 ml/min on the basis of retention time at 280 nm of authentic standard from Cayman Chemicals Co., USA. Quantification was performed by using a plot of the ratio between the peak area of sample versus standard. Data are reported as percentage of leukotriene produced by human PMNL cells stimulated with A23187 and AA in presence of spice principle and vehicle DMSO.

2.7. Statistical analysis

Data are presented as specific activity of 5-LO enzyme in presence of different concentrations of spice principles. The IC_{25}, IC_{50} and IC_{75} values were calculated from dose response curves. Values are given as mean ± SEM of three individual samples.

3. Results

Cloves active principle-eugenol significantly inhibited 5-LO activity in human PMNLs in a concentration dependent manner (Fig. 1) with an IC_{50} value of 26.0 μM. Enzyme kinetics studies were carried to understand the mechanism of inhibition of human PMNLs 5-LO by eugenol. Substrate dependent inhibitory studies were carried at varying concentrations of AA (50–200 μM) and at different concentrations of eugenol (8.5, 25.5 and 53.5 μM). A double reciprocal plot of substrate versus enzyme velocity in presence of eugenol is presented in Fig. 2. Eugenol treatment (0, 8.5, 25.5 and 53.5 μM) decreased the maximum velocity of enzyme activity or V_{max} from 4.45 to 3.9, 2.85 and 1.65 μmol of 5-HETE/min/μg protein respectively without much change in K_m values of 116, 120, 116 and 113 μM (Table 1). The enzyme kinetic data demonstrate that eugenol is a non-competitive reversible inhibitors of 5-LO of human PMNL cells.

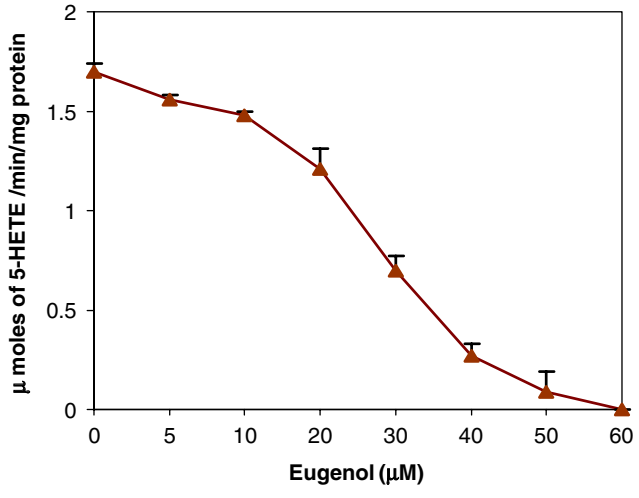

Fig. 1. Eugenol was incubated with human PMNL 5-LO enzyme for 2 min before starting the reaction with substrate AA. The enzyme activity was followed spectrophotometrically at 234 nm.Eugenol dose dependently inhibited 5-LO activity. Values are mean ± SEM of three individual experiments.

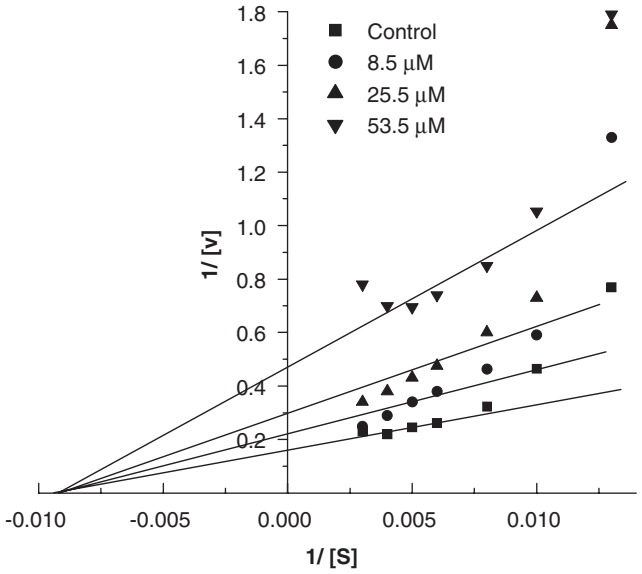

Fig. 2. A double reciprocal plot of substrate dependent enzyme kinetics on inhibition of 5-LO activity by eugenol in human PMNL cells. 5-LO enzyme was incubated with different concentrations of eugenol in presence of different concentrations of AA. No change Km values are observed in presence of different concentration of eugenol.

Table 1
Effect of eugenol on V_{max} and K_m values of 5-LO

Spice principles	Concentration (μM)	K_m (μM)	V_{max} (μmoles HETE/min/μg protein)
Eugenol	0.0	116.0	4.45
	8.5	120.0	3.40
	25.5	116.0	2.85
	53.5	112.5	1.65

Table 2
Eugenol inhibited LTC_4 in calcium ionophore and AA stimulated human PMNL cells

Spice principle	Concentration (μM)	% inhibition of LTC4
Eugenol	0	0
	13	23.2 ± 2.54
	26	40.0 ± 10.53
	39	42.5 ± 9.40
	58	55.5 ± 2.40

Values are mean ± SEM of three determinations.

The effect of eugenol on leukotriene C_4, the peptidoleukotriene implicated in inflammation, bronchocon-

striction and vasoconstriction was estimated in calcium ionophore A23187 and AA stimulated human PMNL cells by HPLC method. As shown in Table 2, eugenol inhibited LTC_4 formation in a concentration dependent manner with an IC_{50} of approximately 30.0 μM.

4. Discussion

The LTs are a potent class of biologically active lipid derivatives that play an important role in the allergic and inflammatory response. The PMNL cells are implicated in allergic and inflammatory responses due to their increased recruitment at the site of inflammation and secretion of elevated levels of cysteinyl leukotrienes, which culminate in pathophysiological conditions. Although leukotrienes may not be involved in the initial stages of a disease, they appear to play an important role in propagation of the disease, by exacerbating the initial, primarily local events and eventually leading to pathophysiological condition. LTC_4 is one of the important leukotrienes involved in immediate hypersensitivity, bronchoconstriction, smooth muscle contraction, and increased vascular permeability of epithelial mucus secretion. In view of their central role in mediating the inflammatory responses, significant efforts have been directed towards inhibition of 5-LO by spice phenolic acids and flavonoids and to modulate biosynthesis of leukotrienes [18].

Eugenol (2-methoxy-4-(2-propenyl) phenol) is naturally occurring phenolic compound in basil, cinnamon and nutmeg and the major component of clove oil. It is widely used as component of zinc oxide eugenol cement in dentistry and is applied to the oral environment [19]. In addition, eugenol is a flavoring agent in cosmetic and food products [20]. Eugenol has been shown to possess many medicinal properties such as antispasmodic [21], antipyretic [22], anti-inflammatory [23] and antibacterial activity [24]. Earlier we have reported that aqueous extract of clove and also its active principle eugenol significantly inhibits 5-LO enzyme activity in human PMNL cells. In the present study, we report that eugenol inhibits 5-LO enzyme by non-competitive mechanism and also formation of peptidoleukotriene LTC_4 in PMNL cells activated with AA and calcium ionophore A23187. The inhibitory mechanism of 5-LO studied by substrate dependent enzyme kinetics showed that eugenol at different concentrations did not change the K_m value of 5-LO (Table 1, Fig. 2), but decreased the maximum velocity (V_{max}) of the enzyme at different concentrations of the substrate AA. These results show that eugenol may not bind to the active site of the enzyme, but may combine at a different site of the enzyme or may scavenge the lipid peroxyl radicals formed during oxidation of AA and thus block the formation of 5-HPETE as evidenced by decreased V_{max} during enzyme kinetic experiments. Inhibition of 5-LO of human PMNLs by eugenol is in agreement with our earlier report of non-competitive inhibition of soybean lipoxygenase dependent lipid peroxidation by eugenol [6].

In summary, the present study shows that eugenol the active principle from spice clove modulates 5-LO activity and also inhibits formation of LTC_4 in human PMNL cells. Thus the eugenol may modulate 5-LO mediated cellular events and pathophysiological effects of leukotrienes.

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References

[1] S.W. Crooks, R.A. Stockely, Leukotriene B4, Inter. J. Biochem. Cell Biol. 30 (1998) 173–178.
[2] D.G. Redkar-Brown, D. Aharony, Inhibition of antigen-induced contraction of guinea pig trachea by ICI 198,615, Eur. J. Pharmacol. 165 (1989) 113–121.
[3] T.R. Jones, L. Charette, D. Denis, Antigen-induced contraction of guinea-pig isolated trachea: Studies with novel inhibitors and antagonists of arachidonic acid metabolites, Br. J. Pharmacol. 95 (1989) 309–321.
[4] C. Denzlinger, M. Grimberg, A. Kapp, C. Haberl, W. Wilmanns, Effect of the leukotriene receptor antagonists FPL 55712, LY 163443, and MK-571 on the elimination of cysteinyl leukotrienes in the rat, Br. J. Pharmacol. 102 (1991) 865–870.
[5] A.P. Reddy, B.R. Lokesh, Studies on spice principles as antioxidants in the inhibition of lipid peroxidation of rat liver microsomes, Mol. Cell. Biochem. 111 (1992) 117–124.
[6] K.A. Naidu, Eugenol: an inhibitor of lipoxygenase dependent lipid peroxidation, Prostaglandins Leukotr. Essent. Fatty acids 53 (1995) 381–383.
[7] S. Shobana, K.A. Naidu, Antioxidant activity of selected Indian spices, Prostaglandins Leukot. Essent. Fatty Acids 62 (2000) 107–110.
[8] A.P. Reddy, B.R. Lokesh, Studies on anti-inflammatory activity of spice principles and dietary n-3 polyunsaturated fatty acids on carrageenan induced inflammation in rats, Ann. Nutr. Metab. 38 (1994) 343–358.
[9] K. Sambaiah, M.N. Satayanarayana, Hypocholesterolemic effect to red pepper and capsaicin, Ind. J. Exp. Biol. 18 (1980) 898–899.
[10] D.S. Arora, J. Kaur, Antimicrobial activity of spices, Int. J. Antimicrob Agents 12 (1999) 257–262.
[11] H. Chen, Y.L. Zhang, D.Y. Zhou, Curcumin inhibits cell proliferation by interfering with the cell cycle and inducing apoptosis in colon carcinoma cells, Anticancer Res. 19 (1999) 3675–3680.
[12] L.G. Menon, R. Kuttan, G. Kuttun, Antimetastatic activity of curcumin and catechin, Cancer Lett. 141 (1999) 159–165.

[13] N. Satya Prasad, R. Raghavendra, B.R. Lokesh, K.A. Naidu, Spice phenolics inhibit human PMNL 5-lipoxygenase, Prostaglandins Leukot. Essent. Fatty Acids 70 (2004) 521–528.
[14] A. Boyum, Isolation of lymphocytes, granulocytes and macrophages, Scan J. Immunol. 5 (1976) 9–15.
[15] O.H. Lowry, N.J. Rosebrough, A. Farr, R.L. Randall, Protein measurement with folin phenol reagent, J. Biol. Chem. 143 (1951) 265–271.
[16] D. Aharony, R.L. Stein, Kinetic mechanism of guinea pig neutrophil 5-lipoxygenase, J. Biol. Chem. 261 (1986) 11512–11519.
[17] H. Linweaver, D. Burk, Determination of enzyme dissociation constants, J. Am. Chem. Soc. 56 (1964) 658–660.
[18] M.J. Laughton, P.J. Evans, Moroney, J.R.S. Hoult, B. Halliwell, Inhibition of mammalian 5-lipoxygenase and cyclooxygenase by flavonoids and phenolic dietary additives, Biochem. Pharmacol. 42 (1991) 1673–1681.
[19] K. Markowitz, M. Moynihan, M. Liv, S. Kim, Biological properties of eugenol and zinc oxide–eugenol, Oral Surg. Oral Pathol. 73 (1992) 729–737.
[20] IARC, Monograph, Evaluation of Carcinogenic Risk of Chemicals to Humans, vol. 36, (1985) Lyon, France, pp. 75–97.
[21] H. Wagner, K. Jurcic, R. Deininger, Antispasmodic activity of eugenol-esters and- ethers, Planta Med. 37 (1979) 9–14.
[22] J. Feng, J.M. Lipton, Eugenol: antipyretic activity in rabbits, Neuropharmacol. 26 (1987) 1775–1778.
[23] W.R. Hume, Effect of eugenol on constrictor responses in blood vessels of the rabbit ear, J. Dent. Res. 62 (1983) 1013–1015.
[24] V. Moleyar, P. Narasimham, Antibacterial activity of essential oil components, Int. J. Food Microbiol. 16 (1992) 337–342.