

Intranasal curcumin regulates chronic asthma in mice by modulating NF- κ B activation and MAPK signaling

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ABSTRACT

Background: Curcumin, a natural product found in the plant *Curcuma longa*, has been reported to have diverse range of molecular targets that influence numerous biochemical and molecular cascades including anti-inflammatory and antioxidant properties.

Purpose: The aim of the study was to investigate the therapeutic potential of intranasal curcumin on ovalbumin (OVA)-induced chronic asthma and to elucidate underlying molecular mechanisms.

Study design/method: Mice were sensitized and exposed to 2% OVA aerosol for 2 times in a week for five consecutive weeks to study effect of intranasal curcumin on various MAPK pathway enzymes involved in chronic asthma and its effect on the activation of nuclear factor κ B (NF- κ B).

Results: Curcumin treatment decreased the ROS level in BALF and nitrite level in blood serum of chronic asthmatic mice. Curcumin treatment had significantly decreased the phosphorylation of JNK, ERK1/2, and p38 and COX-2 expression thereby nuclear factor κ B (NF- κ B) activation and expression in lung tissues.

Conclusion: These results suggest that intranasal curcumin protects against asthma via action on mitogen-activated protein kinase (MAPK)/NF- κ B signaling pathways.

Introduction

Chronic asthma has been considered as a disease characterized with many airway abnormalities including airway inflammation and remodeling. Airway abnormalities particularly the increased production of various cytokines, chemokines and growth factors, leads to the activation of intracellular signaling pathways. There are many pathways which are linked to signaling molecules and their crosstalk lead to the development of asthmatic disease. Studies suggest that there is an increased expression of multiple proteins in the respiratory tract, including pro-inflammatory proteins, cytokines, chemokines, and adhesion molecules. The time course and intensity of asthmatic responses by resident and circulating cells may be regulated by various inflammatory signaling, including Src family kinases (SFKs), protein kinase C (PKC), growth factor tyrosine kinase receptors, nicotinamide adenine

dinucleotide phosphate (NADPH)/reactive oxygen species (ROS), PI3K/Akt, MAPKs, nuclear factor-kappa B (NF- κ B), activator protein-1 (AP-1), and other signaling molecules. These signaling molecules target proteins involved in airway inflammation as well as remodeling process (Lee and Yang, 2013).

The mitogen-activated protein kinase (MAPK) pathway is one of the major signal transduction pathways, which represent a converging point for many other signaling pathways, including tyrosine and serine/threonine kinases, G proteins, and calcium signaling. MAPKs regulate a variety of biological processes like cell growth and proliferation, chemotaxis, degranulation, and other secretory processes. Studies have implicated that the MAPK family of phosphoproteins are critical signaling molecules that leading to the generation of pro-inflammatory mediators. Airway remodeling is thought to be a consequence of long-term inflammation and MAPKs are key signaling molecules that drive pro-

Abbreviations: AP-1, Activator protein; BALF, Bronchoalveolar lavage fluid; Cox-2, Cyclooxygenase-2; cPLA2, Cytosolic phospholipase A2; DCFH-DA, Dichlorofluorescein diacetate; ERK 1/2, Extracellular signal-regulated kinase; GFTKR, Growth factor tyrosine kinase receptors; ICAM-1, Intercellular adhesion molecule-1; IL-1, Interleukin-1; JNK, Jun-N-terminal kinase 11.; Lox-5, Lipooxygenase-5; MMP-9, Matrix metalloproteinase-9; NADPH, Nicotinamide adenine dinucleotide phosphate; NO, Nitric oxide; NOS, Nitric oxide synthase; NF- κ B, Nuclear factor-kappa B; OVA, Ovalbumin; p 38K, p38 kinases; MAPKs, Mitogen activated protein kinase; PKC, Protein kinase C; ROS, Reactive oxygen species; SFKs, Src family kinases; TNF- α , Tumor necrosis factor- α ; VCAM-1, Vascular cell adhesion molecule-1

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inflammatory pathways. Thus, inhibition of MAPKs has emerged as an attractive strategy for reversing inflammation and remodeling in asthma. For these reasons, we examined the activation of 3 major members of the MAPK family (i.e., extracellular signal-regulated kinase [ERK] 1/2, Jun-N-terminal kinase [JNK], and p38 kinases) in lung samples from asthmatic, control mice and adapted therapeutic drug treated mice (curcumin and dexamethasone).

Transcription factors like NF- κ B regulate the expression of hundreds of genes that are involved in regulating cell growth, differentiation, inflammation, development and apoptosis. Many of the known inflammatory target proteins, such as matrix metalloproteinase-9 (MMP-9), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), cyclooxygenase-2 (COX-2), and cytosolic phospholipase A2 (cPLA2), are associated with inflammatory signaling pathways induced by various stimuli, including tumor necrosis factor (TNF- α), interleukin-1 β (IL-1 β). These signaling molecules regulate both key inflammatory signal transduction pathways and target proteins involved in airway inflammation and remodeling. Thus, inhibition of these pathways has emerged as an attractive strategy for reversing inflammation and remodeling in asthma and regulating these activated pathways may be explored as a potential strategy for management of asthma, where downregulation of these factors are relevant (Vallabhapurapu and Karin, 2009; Duan and Wong, 2006).

Curcuma longa is a perennial herb of the Zingiberaceae family (common name turmeric), 3–5 ft tall orange tuberous roots or rhizome with oblong pointed leaves and funnel-shaped yellow flowers bearing plant, widely cultivated in India, China and Indonesia (Dobelis, 1986). Its constituents include curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) and volatile constituents of the essential oil (Basnet et al., 2012). One of its constituents, curcumin has been reported to control inflammation, cell growth and apoptosis, thus useful to prevent and treat inflammatory diseases because of its antioxidant and anti-inflammatory activities. Curcumin has been shown to be a highly pleiotropic molecule interacting with numerous inflammatory molecular targets. But its bioavailability is the main concern which remains as hurdle for its long term applicability. Its major limitation is its extremely low aqueous solubility, rapidly metabolized, low gastrointestinal absorption and quick degradation at acidic pH. The chemical structure of curcumin has been shown to be diferuloylmethane. Chemically, it is a bis-a, b-unsaturated b-diketone that exhibits keto-enol tautomerism. Curcumin exists as an equilibrium mixture of two tautomeric forms in solution but its more stable form is its enol tautomer (Lin et al., 2000; Wang et al., 1997) (Fig. 1).

Intranasal drug delivery is now recognized to be a useful and reliable alternative to oral and parenteral routes (Pires et al., 2009). Being highly vascular and permeable, low enzymatic environment of nasal cavity and avoidance of hepatic first pass metabolism, it is a suitable route for drug delivery. We already have studied absorption of curcumin in blood plasma (15 min-0.90 μ g/ml, 30 min-1.55 μ g/ml) and lungs (15 min-0.27 μ g/ml, 30 min-3.6 μ g/ml) at different time points after intranasal administration (5 mg/kg, i.n.) which was found more effective than the intraperitoneal administration (Subhashini et al., 2013). This direct absorption into the blood stream avoids gastrointestinal destruction and hepatic first pass metabolism (destruction of drugs by liver enzymes) allowing drug to be effectively, rapidly, and predictably bioavailable (Alagusundaram, 2016). Due to large surface area of the epithelial and sub epithelial layer of nasal mucosa, it provides rapid drug absorption and quick onset of action. Intranasal administration of curcumin ameliorates inflammation as well as airway remodeling including fibrosis in mice model of chronic asthma (Chauhan et al., 2014). So we were interested to study underlying mechanism associated in the regulation of asthma severity in mice model of chronic asthma.

Materials and methods

Animals

Studies were performed on five to six weeks old BALB/c mice, which were purchased from CDRI and housed in a standard animal care facility. The animals were housed in polypropylene cages with rice husk as bedding material. They were given food and water *ad libitum*. Experimental protocols were approved by the Institutional Animal Ethical Committee (F.Sc./IAEC/2016-17/711 dated August 09, 2016), Banaras Hindu University (BHU), Varanasi, U.P, India.

Sensitization and challenge protocol

Sensitization and challenge protocols used in this study has summarized in Fig. 1. Briefly, mice were injected with 0.2 ml of saline containing 50 μ g Ovalbumin (OVA) in 4 mg aluminum hydroxide by intraperitoneal route on days 0, 7, and 14. Mice were then challenged for 30 min by nebulization of 2% (wt/vol) OVA in saline solution for 2 days/wk for 5 wk (11 booster exposure), $n = 6$ per group. Aerosol challenge was performed in a whole-body inhalation exposure system attached to an ultrasonic nebulizer. Control group was sensitized and challenged with saline alone (Fig. 2).

Grouping of mice

Mice were randomly divided into five groups (6 mice/group) and were named accordingly as described earlier (Table 1). Nor-Normal, Asth-Asthmatic, Veh Asth- Vehicle Asthmatic, an equal volume of vehicle was administered as used in curcumin treated group according to study design to evaluate effect of DMSO (vehicle) alone on asthma, Cur 5- Curcumin intranasal (5 mg/kg), Curcumin stock was prepared in DMSO (50 mg/ml) for intranasal (i.n.) treatment, Sd- Standard drug – Dexamethasone (1 mg/kg, i.p.).

Collection of BALF, serum and lungs

After 24 h of last aerosol challenge, mice were sacrificed by cervical dislocation, BALF, serum and lungs were collected and stored at -80°C for further analysis. BALF was collected by trachea cannulation after washing the airway lumen by chilled sterile phosphate buffered saline (PBS). The suspension was centrifuged at 3000 rpm for 10 min at 4°C . The cell pellet was used to study the ROS level. Nitrite level was measured in serum. Protein expressions of COX-2, LOX-5, phosphorylation of MAPKs (p-38, ERK and JNK) and NF- κ B, were studied in homogenized lung samples by western blotting technique.

Measurement of intracellular ROS (iROS)

Intracellular ROS were measured in BALF cells (Alaimo et al., 2011). BALF Cells were counted for total viable cells. Cells were washed with chilled PBS. iROS were labeled by incubating cells (1×10^5) in 100 μ l of 20 mM dichlorofluorescein diacetate (DCFH-DA) for 45 min at 37°C temp in the dark. After incubation, fluorescence intensity was monitored by using fluorescence spectrometer (λ_{ex} : 490 nm; λ_{em} : 515 nm). Values were normalized by the fluorescence intensity of the Normal cells.

Nitrite detection in the serum

Nitrite level, as a measure of NO was detected in serum by Griess reagent (Guzel et al., 2007). Briefly, 100 μ l of sample was mixed with 100 μ l of vanadium chloride followed by the addition of Griess reagent (100 μ l). Plate was incubated for 45 min at 37°C and absorbance was measured at 540 nm in microplate reader. Concentration of the sample was measured against sodium nitrite (NaNO_2) as standard. Values are

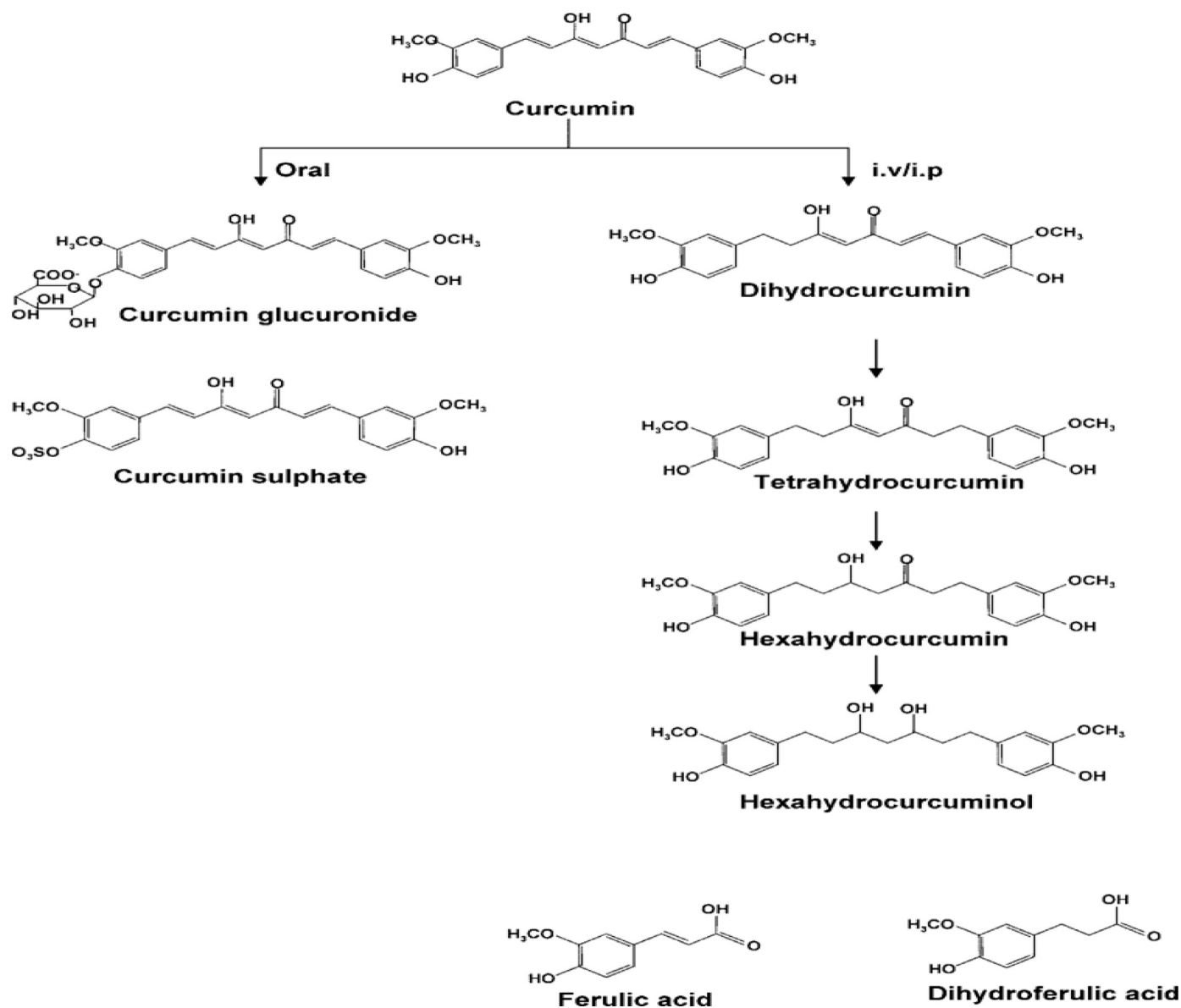


Fig. 1. Structure and its metabolites (Anand et al., 2007).

expressed in μM /ml of serum.

Analysis of COX-2, LOX-5 and activated enzymes of MAPK pathway

Lung tissue was homogenized (10% homogenate) in lysis buffer. Homogenate was centrifuged at 12000 rpm for 30 min at 4 °C and total protein concentration in the supernatant was determined (Lowry et al., 1951). Equal amount of protein (50 μg) was subjected to 12% SDS PAGE for electrophoresis at 100 V for 120 min. Separated proteins were transferred to nitro cellulose membrane. Membranes were blocked with 5% BSA dissolved in TBST buffer (10 mM Tris and 150 mM NaCl and 0.5% Tween-20) for 2 h and incubated with COX-2 antibody (1:1000 dilution, Cayman Chemical, Michigan, USA), p-p38 MAP kinase (1:2000 dilution, Cell Signaling, USA), pERK, p-Junk and β -actin (1:2000 dilution, Cell Signaling, Danver, USA), overnight at 4 °C. After the removal of primary antibody, membranes were rewashed three times with TBST at room temperature and membrane was incubated with a HRP conjugated goat-anti-mouse secondary antibody (1:10000 dilution) for 2 h. Subsequently, membrane was rewashed with TBST buffer and bands were visualized using enhanced chemiluminescence (ECL) reagent (Thermo Fischer, Massachusetts, USA) and

develop in dark room (Kim et al., 2007). β -actin has been used as standard to ensure the equal amount of protein present in sample among different lanes. The intensity of the bands was analyzed by using image J software.

Extraction of cytosolic and nuclear protein fraction for analysis of NF- κ B p65

The lungs were removed and homogenates (30 mg/500 μl) were prepared in buffer A containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM Potassium chloride (KCl), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2.0 $\mu\text{g}/\text{ml}$ aprotinin, 2.0 $\mu\text{g}/\text{ml}$ leupeptin, 0.1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT). Homogenates were incubated on ice for 30 min then 1.0% NP-40 was added, centrifuged at 12000 rpm for 15 min at 4 °C and supernatants were removed and stored at -80 °C for the analysis of NF- κ B p65 extractions. Cell pellet was incubated on ice for 10 min and re-suspended in buffer B (50 μl) containing 20 mM HEPES, 420 mM NaCl, 0.1 mM EDTA, 0.5 mM PMSF, 2.0 $\mu\text{g}/\text{ml}$ aprotinin, 2.0 $\mu\text{g}/\text{ml}$ leupeptin, 0.1 mM EGTA and

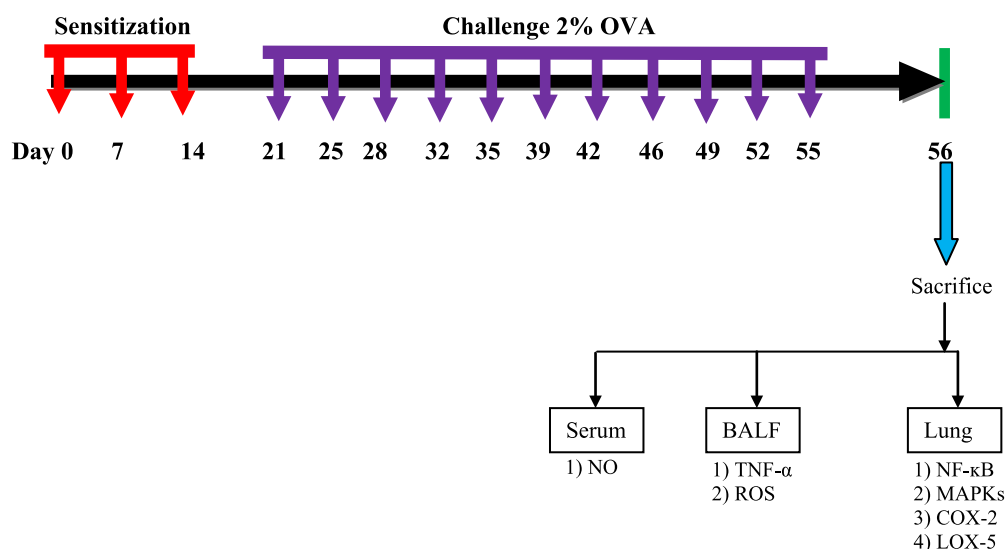


Fig. 2. Experimental protocol: BALB/c mice were sensitized by intraperitoneal injection of OVA on days 0, 7 and 14 and challenged by 2% OVA aerosol. Intranasal curcumin was administered from days 21st to 55th, an hour before every OVA aerosol challenge.

1 mM DTT to prepare soluble nuclear protein and mixture was kept on ice for half an hour with gentle stirring, vortexed for 60 s at interval of 10 min. The pellet was kept on ice for 30 min and centrifuged at 12000 rpm for 20 min. The resulting supernatants contained soluble nuclear proteins and NF-κB p65 (Kim et al., 2007). Protein expressions were analyzed using an antibody against NF-κB p65 (1:1000).

Data analyses

Values are presented as the mean $\pm$ SEM. Differences between two means were evaluated using the Student *t*-test. Statistical significance between Normal (Nor) and OVA-induced asthmatic (Asth) were estimated using the two-tailed Student's *t* test. Differences between more than two groups were assessed via ANOVA with Tukey's and post hoc test comparisons. To define statistically significant differences among different groups such as Nor vs. Asth and Vehicle Asthmatic (Veh asth), Asth vs. treated groups and Standard Drug (Sd), the data were subjected to the ANOVA followed by Tukey's and post hoc test. *P* values less than 0.05 were considered significant.

Results

Effect of curcumin on inflammation

Lower dose of curcumin (2.5 mg/kg, *i.n.*) did not inhibit the inflammatory cell count whereas higher dose (5 mg/kg, *i.n.*) has suppressed the inflammation. Therefore, lower dose was discontinued in further studies to avoid sacrifice of large number of Balb/c mice (Fig. 3(A)).

Table 1
Grouping of animals according to sensitization, challenge and treatment.

S. No	Groups	Sensitization	Challenge (aerosol)	Treatment
Group I	Nor	OVA + Alum	Saline	–
Group II	Asth	OVA + Alum	OVA	–
Group III	Veh asth (21–55 days)	OVA + Alum	OVA	DMSO (<i>i.n.</i>) (equal volumes of vehicle and curcumin were used in treated group; 2.5 μ l/nostril)
Group IV	Cur 5 (<i>i.n.</i>) (21–55 days)	OVA + Alum	OVA	Curcumin 5 mg/kg, bw (<i>i.n.</i>)
Group V	Standard drug (Sd) (21–55 days)	OVA + Alum	OVA	Dexamethasone, 1 mg/kg, bw (<i>i.p.</i>)

Effect of curcumin on nitric oxide release

Nitric oxide in the form of nitrite was measured in the serum, which was significantly higher in asthmatic group (Asth and Veh Asth) as compared to Nor. Intranasal curcumin suppressed nitrite level by 21% in both Asth and Veh Asth groups which was comparable to standard drug, Sd (dexa) (18%) (Fig. 3).

Effect of curcumin on ROS generation

Since ROS generation may serve as signaling molecule to mediate multiple cell functions, we examined whether intranasal curcumin could regulate ROS generation. As shown in Fig. 4, ROS production was enhanced after OVA sensitization and challenge (Asth and Veh Asth groups) as compared to Nor group. Results showed that pretreatment with intranasal curcumin, decreased the intracellular ROS level to 36% and 35% respectively as compared to the Asth and Veh Asth groups (Fig. 4). This result suggests that intranasal curcumin has an anti-oxidant effect similar to Standard drug, dexamethasone (42% and 41%).

Effect of curcumin on NF-κB activation in OVA-induced chronic asthma

Effect of intranasal curcumin on NF-κB activation in a mouse model of chronic asthma was investigated further and it was found that curcumin treatment at dosage of 5 mg/kg bw inhibits NF-κB activation as significant increase in expression of NF-κB p65 subunit in the nuclear fraction of lung tissues of asthmatic mice (Asth and Veh Asth), which was higher than the level in Nor mice (Fig. 4). The enhanced expression of NF-κB p65 unit was decreased significantly in intranasal curcumin group. In contrast, NF-κB p65 subunit in the cytosolic fractions was decreased in asthmatic group as compared to Nor mice, and this decrease in cytosolic NF-κB p65 subunit was also attenuated by intranasal

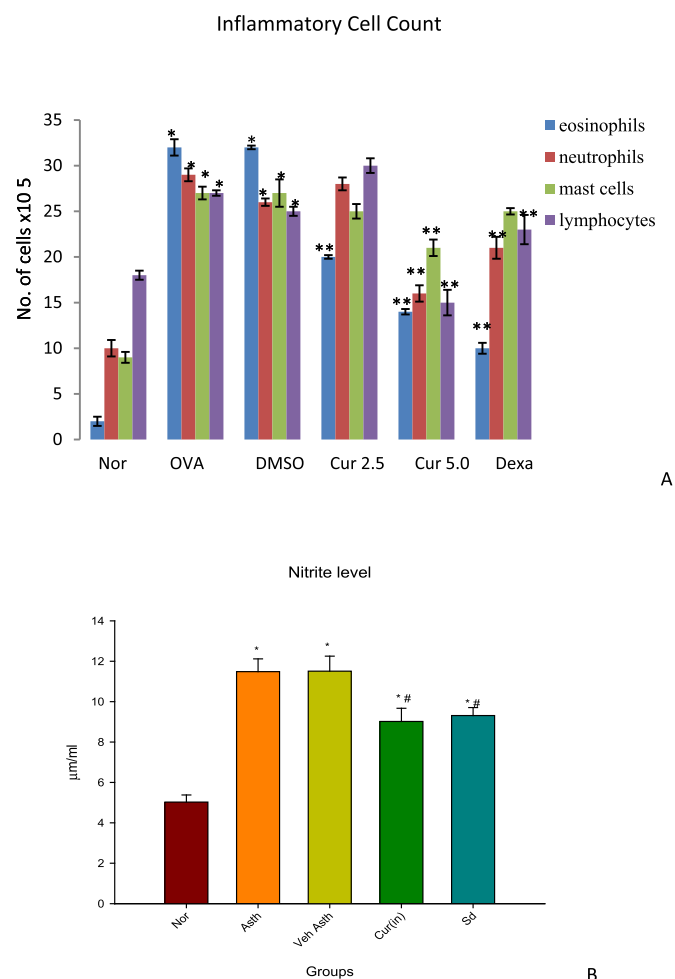


Fig. 3. A–B: Effect of intranasal curcumin on inflammatory cell count in (A) BALF cells and (B) Nitrite level in serum. Intranasal curcumin was much effective in lowering inflammatory cell count as well as nitrite level as compared to Asth groups. $p < 0.05$ ($n = 6$). Intranasal curcumin has inhibitor effects similar to Standard drug (sd) Dexamethasone. The results are shown in means $\pm$ SEMs (* $p < 0.05$ as compared to Nor, # $p < 0.05$ as compared to Asth and Veh Asth). lower dose (0.5 mg/kg) was not much effective in lowering the inflammatory cell count.

curcumin administration.

Effect of curcumin on cyclooxygenase-2 (COX-2) and lipoxygenase-5 (LOX-5) expression

Activation of COX-2 and LOX-5 enzymes is responsible for the arachidonic acid metabolism (prostaglandins and leucotrienes). In asthmatic condition, COX-2 expression was significantly enhanced in Asth and Veh Asth mice groups as compared to Nor mice. Intranasal curcumin (5 mg/kg) has effectively suppressed COX-2 expression which was comparable to dexamethasone (1 mg/kg, i.p.) (Fig. 5), while LOX-5 was significantly increased in Asth and Veh Asth groups but intranasal curcumin treatment did not show any significant effect whereas dexamethasone has effectively reduced LOX-5 level.

Effect of curcumin on MAP kinase activation (phosphorylation) pathway enzymes (ERK, JNK and p38)

Signaling molecules of MAP kinase pathway play crucial role in allergic airway inflammation by activating recruitment of inflammatory cells to the lungs. Therefore, we looked for the activation of three MAP kinases (ERK, JNK and p38) in OVA-induced allergic asthma model. It

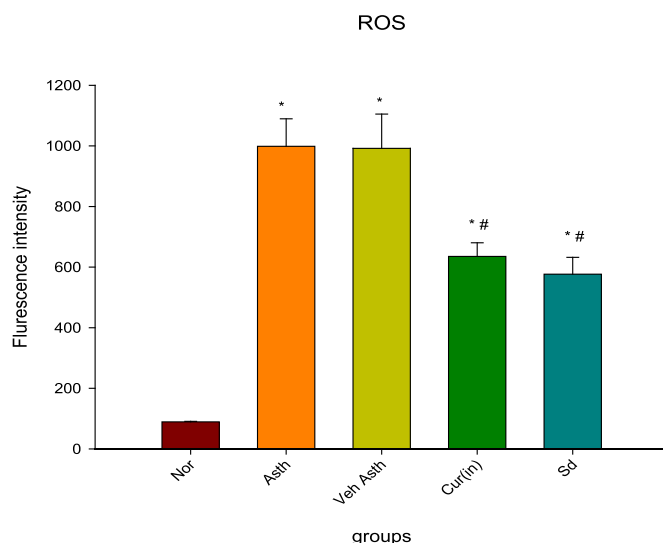


Fig. 4. ROS level in experimental mice: ROS level in the BAL cells was increased in the OVA-exposed mice which was decreased in the intranasal curcumin treatment group. The results are shown in means $\pm$ SEMs (* $p < 0.05$ as compared to Nor, # $p < 0.05$ as compared to Asth and Veh Asth).

was observed that asthmatic mice (Asth and Veh Asth groups) showed enhanced activation and expressions of all the three MAP kinases whereas intranasal curcumin treatment has significantly inhibited activation of p38, ERK and JNK (Fig. 6).

Effect of curcumin on proinflammatory cytokine (TNF- α) level in the BALF

Asthmatic inflammation is well known to be initiated by the secretion of series of pro inflammatory mediators. One of the well known pro inflammatory cytokine is TNF- α . The OVA-exposed mice (Asth and Veh Asth) showed a significant increase in TNF- α level (81% and 86%) in BALF as compared to Nor mice, which has been remarkably suppressed in intranasal curcumin treatment groups (44% and 46%) and was comparable to efficacy of standard drug, dexamethasone (Fig. 8).

Discussion

The present study is experimental evidence demonstrating therapeutic potential of intranasal curcumin in mice model of chronic asthma by regulating MAP Kinases signaling pathway thereby NF- κ B activation and airway inflammation.

TNF- α has been reported to increase the NO production in the airways (Asano et al., 1994). NO strongly promotes the chemotaxis of inflammatory cells in the lungs and favors the development of a Th2 immune response. NO acts on Th1 cells by down regulating IFN- γ production thereby upregulating IL-4 and IL-5 production which in turn increases IgE level and eosinophilic inflammation (Niedbala et al., 2006). As in our previous finding we have seen that intranasal curcumin significantly reduces TNF- α , IgE, IL-4 and IL-5 level and it has also inhibited eosinophil recruitment (Chauhan et al., 2014). Therefore, nitrite level in serum of experimental mice were measured and was found elevated in the asthmatic mice (Asth and Veh Asth groups) which was significantly declined after curcumin treatment (Fig. 3). Our results are consistent with previous reports where curcumin attenuated OVA-induced inflammation in mice by suppressing iNOS expression and NO release by modulating Th1/Th2 balance and inflammation (Moon et al., 2008).

Inflammatory cells such as eosinophils, alveolar macrophages, and neutrophils from asthmatics produce more reactive oxygen species (ROS) as compared to normal subjects (Calhoun et al., 1990). ROS are involved in airway smooth muscle contraction, impairment of β -

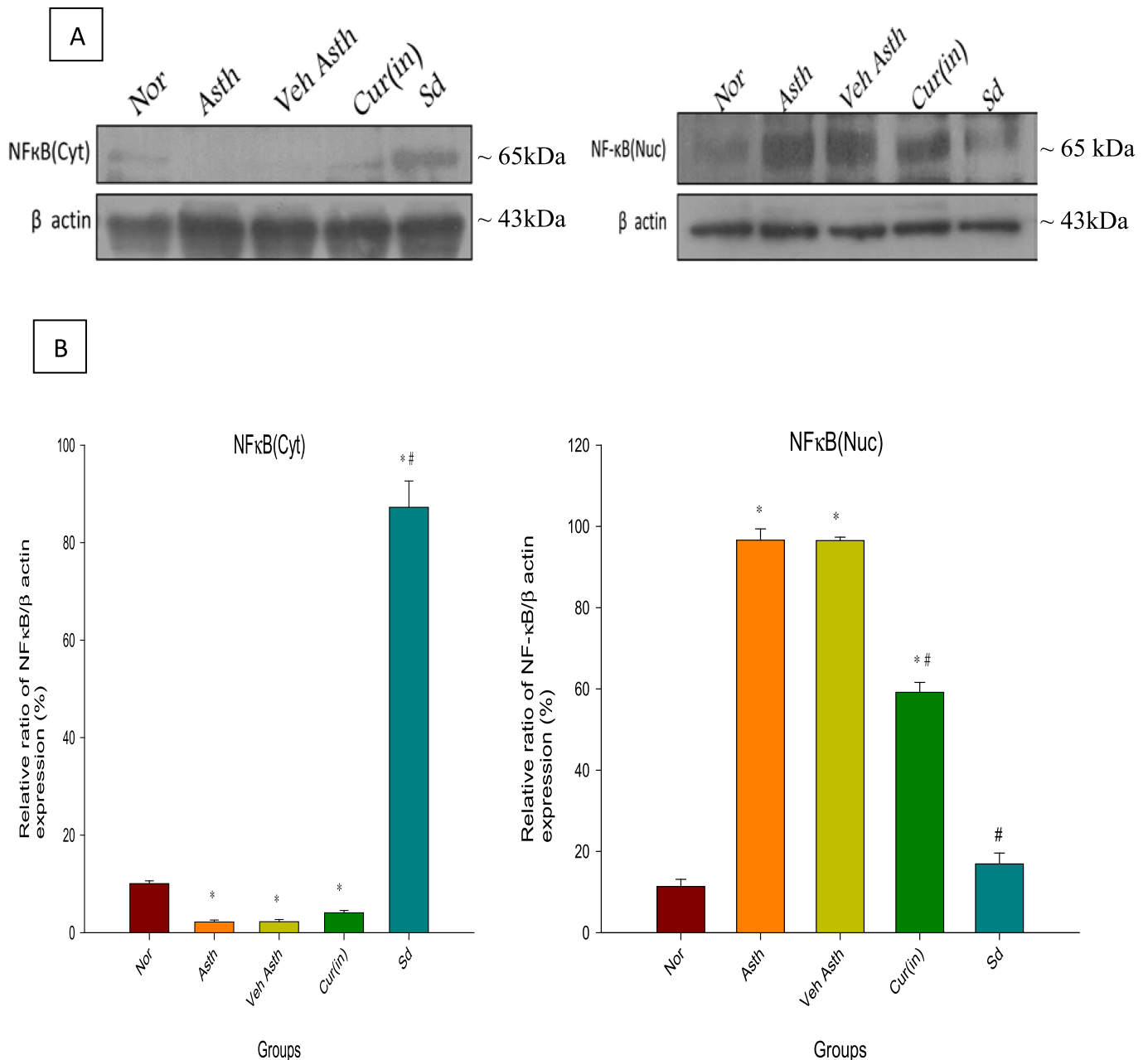


Fig. 5. Effect of intranasal curcumin on the protein expression of NF-κB p65 in lung tissues. Western blot analyses of NF-κB p65 in nuclear (Nuc) and cytosolic (Cyt) protein fraction from lung tissues (A). NF-κB p65 protein levels were quantified and plotted in terms of integrated optical density (B). The relative protein content was calculated as the ratio of the integrated optical density of each protein to that of β actin. The ratio is arbitrarily presented as 100%. The results are shown in means ± SEMs (* $p < 0.05$ as compared to Nor, # $p < 0.05$ as compared to Asth and Veh Asth).

adrenergic receptors, decreased number and function of epithelial cilia, increased mucus production, altered release of inflammatory mediators, influx of inflammatory cells and increased vascular permeability (Henricks and Nijkamp, 2001). The present study demonstrated that ROS production was enhanced in Asth and Veh Asth groups, whereas intranasal curcumin treatment has significantly attenuated ROS generation similar to dexamethasone treated group (Fig. 4).

To study mechanistic pathways involved in analyzing the activity by intranasal curcumin, MAPK pathway leading to NF-κB activation was explored. In response to inflammatory stimuli, intracellular signaling pathways are activated that carry the signals needed to activate the production of inflammatory mediators. Activation of receptors triggers the major intracellular signaling pathway like MAPK pathway leading

to activation of NF-κB. The nuclear factor (NF-κB) plays a critical role in several signal transduction pathways involved in chronic inflammatory diseases such as asthma, arthritis and cancers (Barnes and Karin, 1997). This multiprotein complex (NF-κB) exists in the cytoplasm in an inactive form associated with regulatory proteins called inhibitors of κB (IκB). The expression of several genes such as cyclooxygenase-2 (COX-2), matrix metalloproteinase-9 (MMP-9), inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF), interleukin-8 (IL-8), eotaxin, cell surface adhesion molecules, and anti-apoptotic proteins are regulated by NF-κB (Amit and Ben-Neriah, 2003). Here, we found increased levels of nuclear NF-κB induced by OVA-inhalation which was significantly attenuated in curcumin treatment group (Fig. 5). Significantly higher expression of NF-κB in asthmatic group was observed (Asth and veh

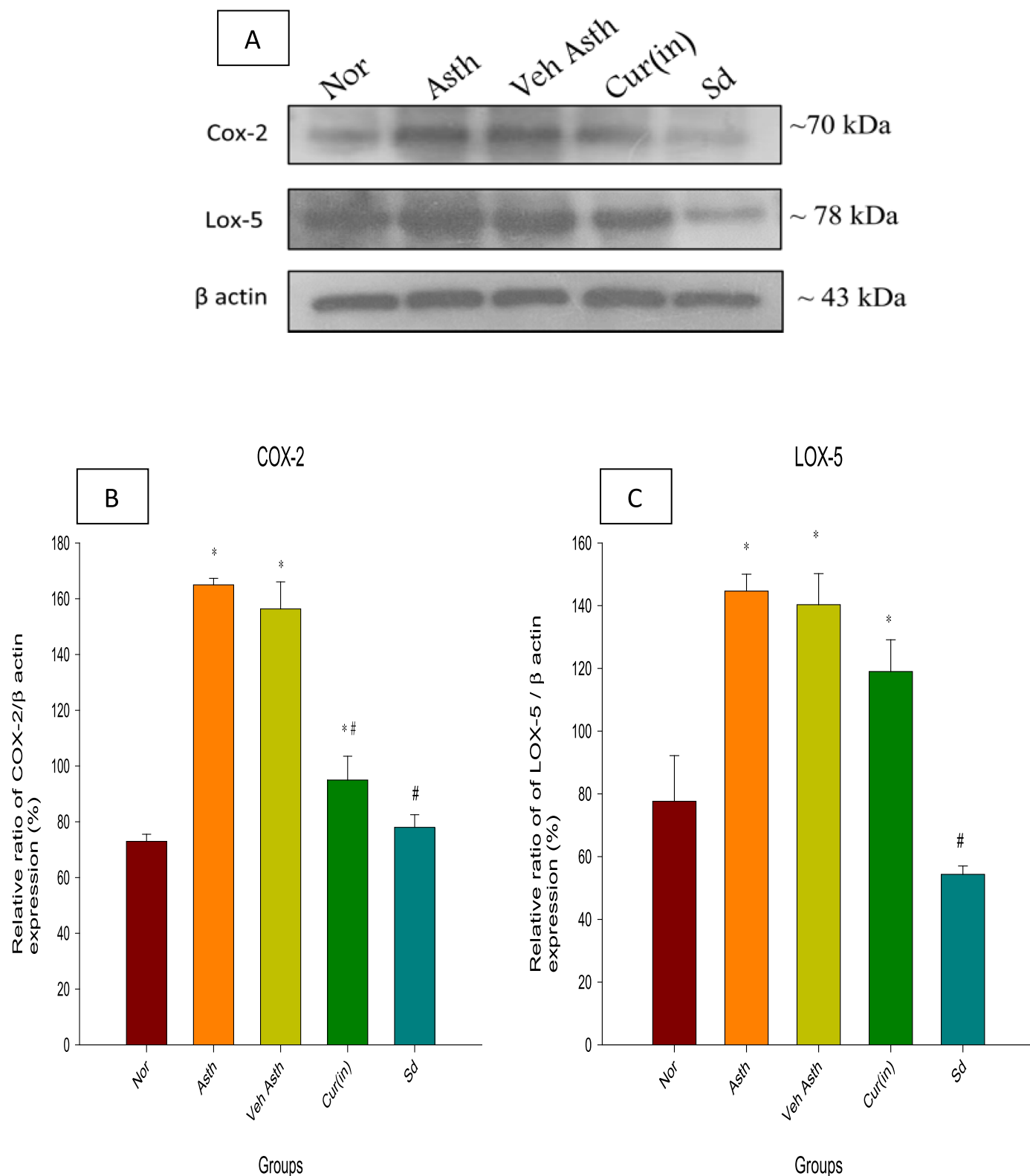


Fig. 6. Effect of intranasal curcumin on the protein expression of COX-2 and LOX-5 in lungs. Western blot analyses of COX-2, LOX-5 and β actin protein extracts from lung tissues (A). Quantification of COX-2 (B) and LOX-5 protein levels (C). The relative protein content was calculated as the ratio of the integrated optical density of each protein to that of β actin. The ratio is arbitrarily presented as 100%. The results are shown in mean $\pm$ SEM (* p < 0.05 as compared to Nor, # p < 0.05 as compared to Asth and Veh Asth).

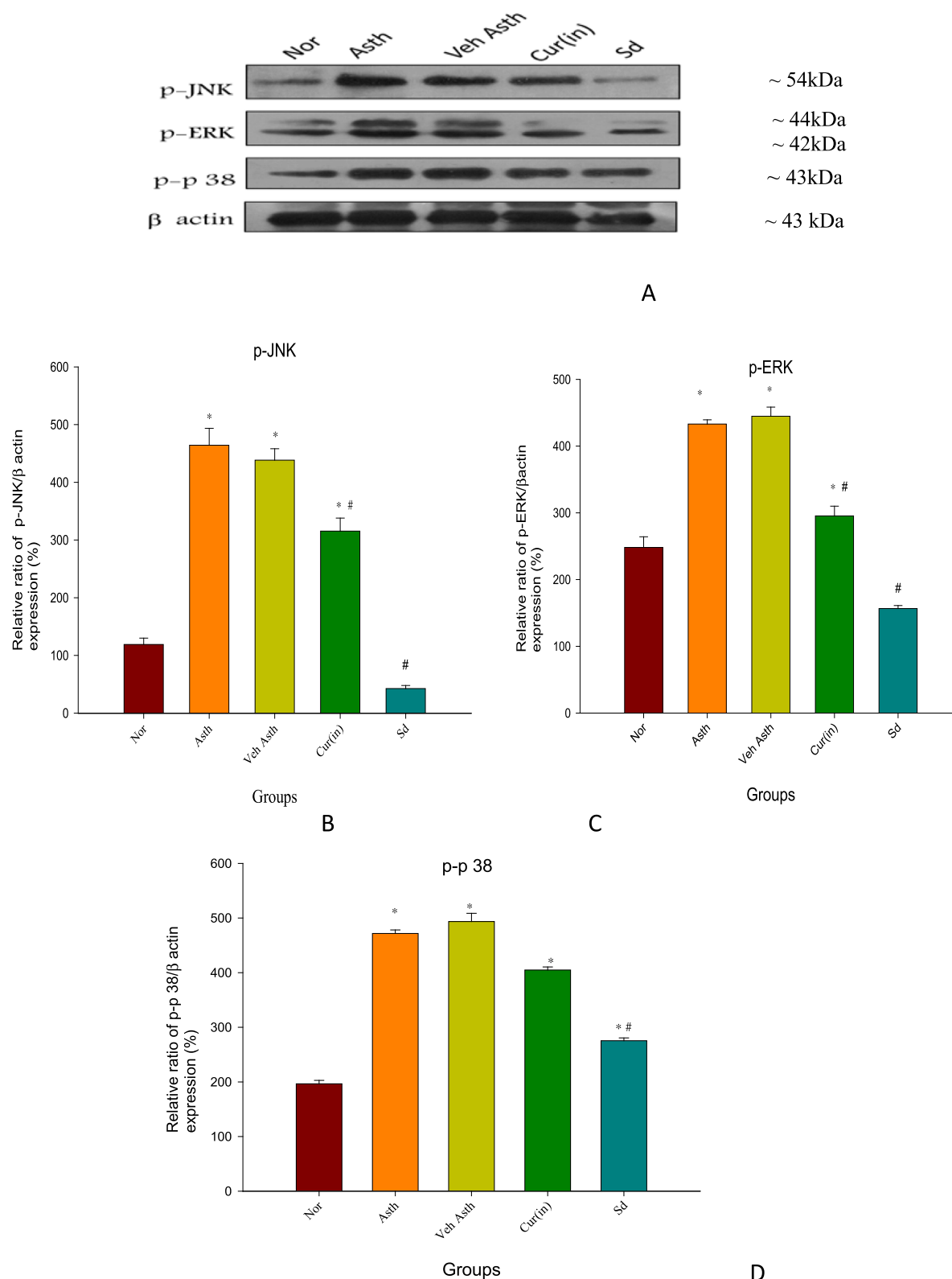


Fig. 7. Effect of intranasal curcumin on the protein expression of p-ERK, p-JNK and p38 MAPK in lung tissues. Western blot analyses of p-ERK, p-JNK, p38 and β actin protein extracts from lung tissues (A). Quantification of p-ERK protein levels (B). Quantification of p-JNK protein levels (C). Quantification of p 38 protein levels (D). The relative protein content was calculated as the ratio of the integrated optical density of each protein to that of β actin. The ratio is arbitrarily presented as 100%. The results are shown in mean $\pm$ SEM (* p < 0.05 as compared to Nor, # p < 0.05 as compared to Asth and Veh Asth).

Asth groups) in nuclear fraction and less in cytosolic fraction (Fig. 5). The results of this study have demonstrated that OVA-induced NF-κB protein expression in nucleus was enhanced and reduced in the cytosol

due to activation of NF-κB and its translocation to the nucleus. Therefore, upregulation of protein expression in nucleus is high and low in cytosol, which may be further associated with the activation of

transcription factors involved in chronic asthma. ROS has also been reported to be directly implicated as second messengers in NF- κ B activation pathway (Schreck et al., 1991). Reduced NF- κ B activity after curcumin treatment was also found which could be correlated to reduced ROS level, affecting its translocation into nucleus. These findings suggest that intranasal curcumin is either inhibiting the activation of NF- κ B by suppressing the ROS activity directly or by blocking its translocation into the nucleus.

Cyclooxygenase and lipoxygenases are two important enzymes that catalyze metabolism of arachidonic acid via prostaglandin and leukotriene synthesis in inflammation. Here, in present study intranasal curcumin significantly has suppressed the OVA-induced COX-2 expression in mice model of chronic asthma (Fig. 6). As OVA-induced nuclear translocation of NF- κ B is inhibited by curcumin treatment and this could be correlated with the inhibition of COX-2 expression. The COX-2 inhibition was consistent with the earlier study where COX-2 activity and decreased PGD2 level was affected by curcumin treatment in the BALF of murine lungs (Subhashini et al., 2016).

Biologically active metabolites of the 5-LOX cascade are hydroxyl eicosanoid tetraenoic acids (HETEs), leukotrienes (LTs), and lipoxins. Leukotrienes are potent mediators of inflammation; these include LTB₄ and the cysteinyl LTs (LTC₄, LTD₄, and LTE₄) (Sala et al., 1998; Radmark, 2000; Penrose et al., 1999). We observed that intranasal curcumin (5 mg/kg) has significantly inhibited COX-2 expression but not LOX-5 (Fig. 6). It can be suggested here that by regulating the NF- κ B signaling pathway, curcumin plays an important role in the regulation of inflammation through the inhibition of the COX-2, NO and ROS.

MAPKs have a critical role in the expression and activation of inflammatory mediators in the airways where both resident and infiltrating cells play a crucial role (Lee and Yang, 2013; Johnson and Lapadat, 2002). In mammals, MAPK signaling modules are divided into at least 3 groups: extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH₂-terminal kinase (JNK). Each MAPK module plays a discrete yet complementary role in attenuating allergic airway inflammation. Recently, the anti-inflammatory effects of MAPK kinase inhibitor have been demonstrated in *in vivo* animal models of asthma. Development of inhibitors targeting MAPK could be an attractive strategy for the treatment of asthma (Duan and Wong, 2006). We have seen here that signaling kinases p38, ERK42/44 and JNK were activated/phosphorylated in OVA-induced chronic asthma (Asth and Veh Asth). Intranasal curcumin treatment has ameliorated activation/phosphorylation of all the three kinases (Fig. 7).

TNF- α is a pro-inflammatory cytokine that has been implicated in many aspects of the airway pathology in asthma. It is pleiotropic cytokine that exerts a variety of effects, such as growth promotion, growth inhibition, angiogenesis, cytotoxicity, inflammation and immunomodulation in inflammatory conditions. Therefore, objective was to evaluate the effect of intranasal curcumin on this pro-inflammatory cytokine. Apart from the Th1 and Th2 cytokines, immunoregulation, intranasal curcumin administration inhibited the level of TNF- α , a potent pro-inflammatory cytokine. Preliminary studies on asthmatic patients have demonstrated an improvement in lung function, AHR, quality of life, and exacerbation rate after treatment with anti-TNF therapy (Matera et al., 2010). In present study, Intranasal curcumin treatment resulted in the significant inhibition in TNF- α level which was enhanced after OVA induction (Fig. 8).

Conclusion

Results indicated that intranasal curcumin attenuates OVA-induced chronic asthma via MAPK and NF- κ B pathways. This may account for the reduction in COX-2, ROS and NO level by inhibition of NF- κ B-mediated transcriptional activation and prevention of p38, JNK, and ERK phosphorylation. By inhibiting COX-2 protein expression intranasal curcumin may be exerting its activity by reducing the production of prostaglandins (PGs) induced inflammatory process. It did

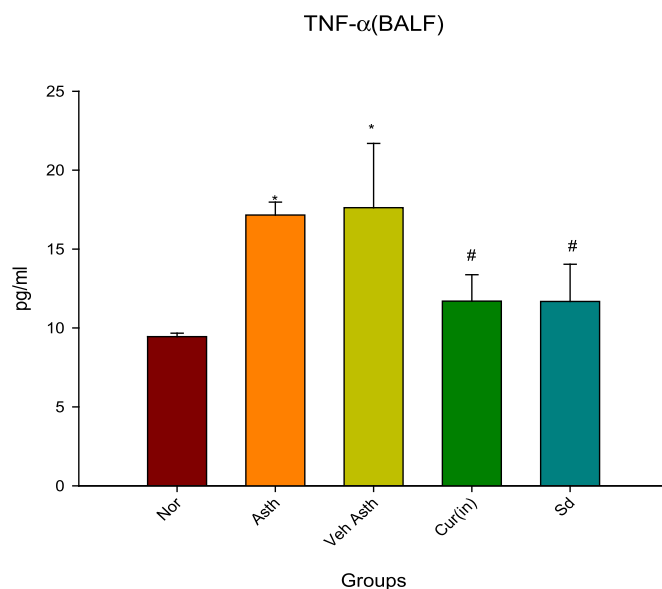


Fig. 8. TNF- α cytokine profile in BALF: TNF- α cytokine levels were determined by ELISA. TNF- α level was increased in the OVA-exposed mice and the increased level was decreased in the intranasal curcumin treatment group.

not show any effect on LOX-5 expression whereas dexamethasone has exerted significant effect to some extent. The specific regulation of LOX-5 and COX-2 by curcumin is not fully established; however, existing evidence indicates that curcumin regulates LOX and COX-2 predominantly at the transcriptional level and, to a certain extent, the posttranslational level. Thus, the curcumin-selective transcriptional regulatory action of COX-2, and dual COX/LOX inhibitory potential of this naturally occurring agent provides distinctive advantages over synthetic COX/LOX inhibitors, such as nonsteroidal anti-inflammatory drugs. NF- κ B and MAPK phosphorylation may be the specific molecular target to study protective effects of intranasal curcumin against OVA-induced chronic asthma.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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References

- Alagusundaram, M., 2016. Nasal drug delivery system-an overview. *Int. J. Res. Pharm.* 1, 454–465.
- Alaimo, A., Gorjod, R.M., Kotler, M.L., 2011. The extrinsic and intrinsic apoptotic pathways are involved in manganese toxicity in rat astrocytoma C6 cells. *Neurochem. Int.* 59, 297–308.
- Amit, S., Ben-Neriah, Y., 2003. NF- κ B activation in cancer: a challenge for ubiquitination- and proteasome-based therapeutic approach. *Semin. Cancer Biol.* 13, 15–28.
- Anand, P., Kunnumakkara, A.B., Newman, R.A., Aggarwal, B., 2007. Bioavailability of curcumin: problems and promises. *Mol. Pharm.* 4, 807–818.
- Asano, K., Chee, C.B., Gaston, B., Lilly, C.M., Gerard, C., Drazen, J.M., Stamler, J.S., 1994. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. *Proc. Natl. Acad. Sci.* 91, 10089–10093.
- Barnes, P.J., Karin, M., 1997. Nuclear factor- κ B: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* 336, 1066–1071.
- Basnet, P., Hussain, H., Tho, I., Skalko-Basnet, N., 2012. Liposomal delivery system enhances anti-inflammatory properties of curcumin. *J. Pharm. Sci.* 101 (2), 598–609.

- Calhoun, W.J., Bush, R.K., Salisbury, S.M., Stevens, C.A., 1990. Enhanced reactive oxygen species metabolism of airspace cells and airway inflammation follow antigen challenge in human asthma. *J. Allergy Clin. Immunol.* 86, 306–313.
- Chauhan, P.S., Dash, D., Singh, R., 2014. Intranasal curcumin attenuates airway remodeling in murine model of chronic asthma. *Int. J. Immunopharmacol.* 21 (1), 63–75.
- Dobelis, I.N., 1986. *Magic and Medicine of Plants* 702 Reader's Digest Association, Pleasantville, NY.
- Duan, W., Wong, W.F., 2006. Targeting mitogen-activated protein kinases for asthma. *Curr. Drug Targets* 7, 691–698.
- Guzel, N.A., Hazar, S., Erbas, D., 2007. Effects of different resistance exercise protocols on nitric oxide, lipid peroxidation and creatine kinase activity in sedentary males. *J. Sports Sci. Med.* 6, 417–422.
- Henricks, P.A., Nijkamp, F.P., 2001. Reactive oxygen species as mediators in asthma. *Pulm. Pharmacol. Ther.* 14, 409–421.
- Johnson, G.L., Lapadat, R., 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298, 1911–1912.
- Kim, D.Y., Ryu, S.Y., Lim, J.E., Lee, Y.S., Ro, J.Y., 2007. Anti-inflammatory mechanism of simvastatin in mouse allergic asthma model. *Eur. J. Pharmacol.* 557, 76–86.
- Lee, I.T., Yang, C.M., 2013. Inflammatory signalings involved in airway and pulmonary diseases. *Med. Inflamm.* 1–12.
- Lin, J.K., Pan, M.H., Lin-Shiau, S.Y., 2000. Recent studies on the biofunctions and biotransformations of curcumin. *Biofactors* 13, 153–158.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Matera, M.G., Calzetta, L., Cazzola, M., 2010. TNF- α inhibitors in asthma and COPD: we must not throw the baby out with the bath water. *Pulm. Pharmacol. Ther.* 23, 121–128.
- Moon, D.O., Kim, M.O., Lee, H.J., Choi, Y.H., Park, Y.M., Heo, M.S., Kim, G.Y., 2008. Curcumin attenuates ovalbumin-induced airway inflammation by regulating nitric oxide. *Biochem. Biophys. Res. Commun.* 375, 275–279.
- Niedbala, W., Cai, B., Liew, F.Y., 2006. Role of nitric oxide in the regulation of T cell functions. *Ann. Rheum. Dis.* 65, iii37–iii40.
- Penrose, J.F., Austen, K.F., Lam, B.K., 1999. Leukotrienes: Biosynthetic Pathways, Release and Receptor-mediated Actions with Relevance to Disease States. *Inflammation Basic Principles and Clinical Correlates*. pp. 361–372.
- Pires, A., Fortuna, A., Alves, G., & Falcao, A., 2009. Intranasal drug delivery: how, why and what for? *J. Pharm. Pharm. Sci.* 2009;12(3):288–311. Review.
- Radmark, O.P., 2000. The molecular biology and regulation of 5-lipoxygenase. *Am. J. Respir. Crit. Care. Med.* 161, S11–S15.
- Sala, A., Zarini, S., Bolla, M., 1998. Leukotrienes: lipid bioeffectors of inflammatory reactions. *Biochemistry* 63, 84–92.
- Schreck, R., Rieber, P., Baeuerle, P.A., 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J.* 10, 2247.
- Subhashini, Chauhan, P.S., Dash, D., Paul, B.N., Singh, R., 2016. Intranasal curcumin ameliorates airway inflammation and obstruction by regulating MAPKinase activation (p38, Erk and JNK) and prostaglandin D2 release in murine model of asthma. *Int. Immunopharmacol.* 31, 200–206.
- Subhashini, Chauhan, P.S., Kumari, S., Kumar, J.P., Chawla, R., Dash, D., Singh, M., Singh, R., 2013. Intranasal curcumin and its evaluation in murine model of asthma. *Int. Immunopharmacol.* 17, 733–743.
- Vallabhapurapu, S., Karin, M., 2009. Regulation and function of NF-kB transcription factors in the immune system. *Annu. Rev. Immunol.* 27, 693–733.
- Wang, Y.J., Pan, M.H., Cheng, A.L., Lin, L.L., Ho, Y.S., Hsieh, C.Y., Lin, J.K., 1997. Stability of curcumin in buffer solutions and characterization of its degradation products. *J. Pharm. Biomed. Anal.* 15, 1867–1876.