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# Ethyl 3′,4′,5′-trimethoxythionocinnamate modulates NF-κB and Nrf2 transcription factors

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ABSTRACT

Recently, we identified a novel cinnamate analog, ethyl 3′,4′,5′-trimethoxythionocinnamate (ETMTC) as a potent inhibitor of cell adhesion molecules (CAMs), such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin. However, its mechanism of action has not been elucidated so far. Since, nuclear factor-kappa B (NF-κB) is the major transcription factor involved in the regulation of ICAM-1, VCAM-1 and E-selectin expression, we determined the status of NF-κB activation in ETMTC treated human endothelial cells. Here, we demonstrate that ETMTC inhibits TNF-α-induced nuclear translocation and activation of NF-κB by inhibiting phosphorylation and degradation of IκBα. The inhibition of IκBα phosphorylation and degradation by ETMTC was found to be due to its ability to inhibit IκB kinase activity. In addition, oxidative stress is known to regulate NF-κB activation through TNF-α signaling cascade, therefore, we examined the effect of ETMTC on TNF-α-induced reactive oxygen species generation. We observed that ETMTC significantly inhibits TNF-α-induced reactive oxygen species generation in endothelial cells. To further elucidate the anti-oxidant potential of ETMTC, we examined its effect on induction of anti-oxidant genes viz. glutamate–cysteine ligase, modifier subunit (GCLM), heme oxygenase-1 (HO1) and NAD (P)H:quinone oxidoreductase 1 (NQO1) in human bronchial epithelial cells. Interestingly, ETMTC significantly induces the anti-oxidant genes viz. GCLM, HO1 and NQO1 by activating nuclear factor-erythroid 2 p45-related factor 2 (Nrf2). Thus, ETMTC could be useful towards developing potent anti-inflammatory molecules.



## 1. Introduction

Inflammation is a hallmark of many diseases like asthma, chronic obstructive pulmonary disease, acute respiratory distress syndrome, rheumatoid arthritis, atherosclerosis and cancer (Baldwin, 1996; Springer, 1994; Suchard et al., 2010). It is mediated by the expression of cell adhesion molecules viz. ICAM-1, VCAM-1 and E-selectin on leukocytes. Pro-inflammatory cytokines like TNF-α, IL-1β or bacterial lipopolysaccharides (LPS) activate redox sensitive transcription factor, NF-κB and induce the surface expression of CAMs (Ciencewicki et al., 2008; Garg and Aggarwal, 2002; Ghosh and Karin, 2002). NF-κB is sequestered in the cytoplasm with its inhibitory molecule (IκB). Rapid phosphorylation and degradation of IκBα allows NF-κB to translocate into the nucleus and regulate transcription of the targeted genes. Emerging evidence suggests that TNF-α signaling causes oxidative stress by producing reactive oxygen species which in turn activates NF-κB (Baeuerle and Baltimore, 1996; Baldwin, 1996, 2001; Rahman and MacNee, 1998). Antioxidants and free radical quenchers have also been shown to block the NF-κB activation (Verhasselt et al., 1999; Wakamatsu et al., 2005; Weber et al., 1995).

Nrf2 is a basic-leucine zipper (b-ZIP) transcription factor present in the cytoplasm; upon its activation in response to inflammatory stimulus, environmental toxicant, oxidative and electrophilic stress, it detaches from its cytosolic inhibitor, Kelch-like ECH-associated protein 1 (Keap1) and translocates to nucleus and induces the expression of several anti-oxidant enzymes. (Nguyen et al., 2009; Nguyen et al., 2003; Nioi et al., 2003; Surh, 2008; Thimmulappa et al., 2002). Emerging literature suggests that there is a crosstalk between NF-κB and Nrf2. Sulforaphane, a well known Nrf2 activator inhibits NF-κB in various cell lines (Heiss and Gerhauser, 2005; Heiss et al., 2001; Surh, 2008; Xu et al., 2005). Similarly, curcumin, a well documented NF-κB inhibitor also activates Nrf2 in several types of cultured cells. It possesses strong anti-inflammatory and antioxidant activities (Balogun et al., 2003; Pugazhenthi et al., 2007).

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Activation of Nrf2 suppressed TNF-α-induced monocyte chemoattractant protein (MCP)-1 and VCAM-1 expression and inhibited TNF-α-induced adhesion of monocytic U937 cell to endothelial cells (Chen et al., 2006). Disruption of Nrf2 enhances upregulation of NF-κB activity, pro-inflammatory cytokines, and ICAM-1 expression and leads to pathogenesis of many inflammatory diseases (Jin et al., 2008; Thimmulappa et al., 2006a). Therefore, pharmacological inhibition of CAMs on endothelial cells is a promising strategy for therapeutic intervention against inflammatory disorders, it could be through inhibition of NF-κB and/or activation of Nrf2 (Sussan et al., 2009; Thimmulappa et al., 2006b; Xu et al., 2005).

Previously, we have reported a novel aromatic ester which exhibited potent ICAM-1 inhibitory activity on endothelial cells (Kumar et al., 2005a). We have also reported that its sulfur analogs (thionocoumarins), when compared to its oxygenated analogs (coumarins), were more potent inhibitors of ICAM-1 expression on endothelial cells (Kumar et al., 2005b). Recently, we designed, synthesized and evaluated the ICAM-1 inhibitory activity of its thio/thiono cinnamate analogs and identified ETMTC as a potent inhibitor of CAMs (Kumar et al., 2011a). In the present study, we demonstrate the molecular mechanism of action of ETMTC.

## 2. Material & methods

### 2.1. Materials

Anti-E-selectin, Anti-ICAM-1, Anti-VCAM-1 antibodies and TNF-α, were purchased from Pharmingen, USA. Anti-mouse-IgG-FITC was purchased from Becton & Dickinson, USA. Anti-p65, Anti- IκBα and Anti-Keap1, Anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology, USA. Anti-DJ-1, Anti-HO1 and Anti-NQO1antibodies were purchased from Abcam USA. M199, L-glutamine, antibiotic and anti-mycotic solution, endothelial cell growth factor, trypsin, Pucks saline, HEPES, o-phenylenediamine dihydrochloride, ficoll-hypaque, cetitrimethyl ammonium bromide, 3-amino-1,2,4 triazole and anti-mouse IgG-HRP were purchased from Sigma Chemical Co., USA. The ICAM-1, VCAM-1, E-selectin & β-actin primer sets were synthesized by Genset Corp., Japan. Fetal calf serum was purchased from Biological Industries, Israel.

### 2.2. Cells and cell culture

Endothelial cells were isolated from human umbilical cord using mild trypsinization (Kumar et al., 2005a). At confluence the cells were subcultured using 0.05% trypsin-0.01 M EDTA solution and cells were used between three and four passages. Human bronchial epithelial (Beas-2B) cells were cultured in DMEM (pH 7.4) supplemented with 10% (v/v) FBS, 100 mg/l gentamicin and genetisin. The concentration of DMSO did not exceed 0.1%. RNA was isolated and gene expression was measured after 16 h.

### 2.3. Time kinetics of ICAM-1 inhibition by ETMTC

To determine the time kinetics of ICAM-1 inhibition by ETMTC, endothelial cells were treated with 70 μM ETMTC for 1–6 h prior to, simultaneously or 1–2 h after induction with TNF-α (10 ng/ml) and were further incubated for 16 h. Cell-ELISA was used for measuring the expression of ICAM-1 on the surface of endothelial cells (Kumar et al., 2007a).

### 2.4. Total RNA isolation and reverse transcription polymerase chain reaction

Endothelial cells ($2 \times 10^6$) were incubated with or without ETMTC (70 μM) for 2 h followed by induction with TNF-α (10 ng/ml) for 4 h. Total RNA was isolated from treated endothelial cells and expression of the transcripts for ICAM-1, VCAM-1 and E-selectin was evaluated by RT-PCR (Kumar et al., 2007b) by using synthesized primers according to the published cDNA sequences to yield products of 555 bp (ICAM-1), 533 bp (VCAM-1), 479 bp (E-selectin) and 661 bp (β-actin). The RT-PCR was performed following the manufacturer's protocol (RT-PCR system, Promega Madison). The PCR products were analyzed in 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Nrf2-regulated gene expression was performed by using random hexamers and MultiScribe reverse transcriptase according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT-PCR analyses of NQO1, HO1, and GCLM were performed by using Assay-on-Demand primers and probe sets from Applied Biosystems. Assays were performed using the ABI 7000 Taqman system (Applied Biosystems, Foster City, CA). β-actin was used for normalization (Kumar et al., 2011b).

### 2.5. Preparation of nuclear extracts

Endothelial cells ($2 \times 10^6$) were incubated with or without ETMTC (70 μM) for 2 h followed by induction with TNF-α (10 ng/ml) for 30 min (Kumar et al., 2007c). The cells were dislodged using a cell scraper, and centrifuged at 300*g* for 10 min. The cell pellet was resuspended in cell lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 1 mM DTT, 0.5% Nonidet P40, 0.1 mM EGTA and 0.1 mM EDTA) and allowed to swell on ice for 5 min. Following centrifugation at 3300*g* for 15 min, the supernatant was collected as cytoplasmic extract and stored at −70 °C. The nuclear pellet was resuspended in nuclear extraction buffer (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF and 1 mM DTT) and incubated for 30 min at 4 °C. The extracted nuclei were pelleted at 25,000 g (15 min at 4 °C) and the supernatant was collected as nuclear extract. The protein concentration in the extracts was determined by bicinchoninic acid (BCA) method.

### 2.6. Preparation of total cell lysate

Endothelial cells or Beas-2B cells ($2 \times 10^6$) were incubated with or without ETMTC (70 μM) for 2 h followed by induction with TNF-α (10 ng/ml) for 5 min (Kumar et al., 2007c). The cells were washed with PBS, dislodged using a cell scraper, and centrifuged at 300*g* for 10 min. The cell pellet was resuspended in RIPA buffer (Tris-HCl 50 mM, NaCl 150 mM, 1 mM PMSF, 1 mM DTT, 1% Nonidet P40, 1 mM EGTA, 1 mM EDTA and cocktail of protein inhibitors) and allowed to swell on ice for 30 min. Following centrifugation at 25,000*g* for 30 min, the supernatant was collected as total cell lysate and stored at −70 °C. The protein concentration in the extracts was determined by BCA protein estimation method (Kumar et al., 2007a).

### 2.7. Immunoblot analysis

All immunoblots were performed using protocols as described previously (Kumar et al., 2007a; Malhotra et al., 2008).

### 2.8. NF-κB activation assay

To determine NF-κB activation, the electrophoretic mobility shift assay (EMSA) was performed as described earlier with some