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Isoliquiritigenin inhibits IκB kinase activity and ROS generation to block TNF-α induced expression of cell adhesion molecules on human endothelial cells

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ABSTRACT

Isoliquiritigenin (ILTG) is a flavonoid with chalcone structure (4,2′,4′-trihydroxychalcone), an active component present in plants like *Glycyrrhiza* and *Dalbergia* which showed various biological activities including anti-inflammatory, anti-carcinogenic and antihistamic. As very little is known in regard to the underlying mechanism involved in explaining the various activities of the compound, we carried out a detailed study on the effect of ILTG on the expression of cell adhesion molecules on human primary endothelial cells. We demonstrate here that ILTG inhibits TNF-α induced adhesion of neutrophils to endothelial monolayer by blocking the expression of ICAM-1, VCAM-1 and E-selectin. Since NF-κB is a major transcription factor involved in the transcriptional regulation of cell adhesion molecules, thus we studied the status of NF-κB activation in ILTG treated endothelial cells. We demonstrate that ILTG inhibits the translocation and activation of nuclear factor-κB (NF-κB) by blocking the phosphorylation and subsequent degradation of IκBα. As oxidative stress is also known to regulate the activation of NF-κB to modulate TNF-α signaling cascade, we tested the effect of ILTG on reactive oxygen species (ROS). We found that it inhibits TNF-α induced ROS production in endothelial cells. These results have important implications for using ILTG or its derivatives towards the development of effective anti-inflammatory molecules.

1. Introduction

The cell adhesion molecules are important for binding of leukocytes to the endothelial cells and in the infiltration of inflammatory cells into tissues [1]. Various inflammatory mediators such as TNF-α, IL-1β and bacterial lipopolysaccharide (LPS), increase the expression of cell adhesion molecules (CAMs) including ICAM-1, VCAM-1 and E-selectin on endothelial cells [2]. Nuclear factor-κB (NF-κB) has been implicated in the transcriptional activation of the genes encoding CAMs [3,4]. 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 target genes. Emerging evidence suggests that TNF-α signaling cascade may cause oxidative stress due to the production of reactive

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Abbreviations: CAMs, cell adhesion molecules; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift assay; HUVECs, human umbilical cord vein endothelial cells; NEMO, NF-κB essential modulator

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oxygen species, which in turn temporally regulate NF-κB activity by IκB kinase and subsequent degradation of IκBα [5–8]. Also a number of antioxidants and free radical quenchers have also been shown to block the NF-κB activation [9]. Thus the centrality of NF-κB as a key mediator involved in regulation of a plethora of inflammatory responses makes the idea of identification of small molecules as inhibitors of NF-κB and of cell adhesion molecules, quite useful therapeutic approach [10–12].

Isoliquiritigenin (ILTG) is a flavonoid with chalcone structure (4,2′,4′-trihydroxychalcone), an active component present in plants like *Glycyrrhiza* and *Dalbergia*. It has been evaluated for its various biological activities including anti-inflammatory, anti-oxidant activity and has been shown to prevent histamine release from rat peritoneal exudate cells induced with antigen–antibody reaction and also with calcium ionophore [13,14]. Its ability to inhibit phosphodiesterase in rat aortic rings helps to relax smooth muscles [15]. In addition, ILTG also suppresses metastasis and proliferation of carcinoma cells and prevents azoxymethane induced colonic abberent crypt focus and tumor formation in mice [16,17] and it also induces apoptosis in gastric cancer cells [18]. Recently, it has been reported that ILTG inhibited the Cox and iNOS activity and exhibit vasorelaxant effect [19,20].

Previously, we have reported that a chalcone derivative, 2′-hydroxychalcone inhibited NF-κB translocation and blocked TNF-α and LPS induced adhesion of neutrophils to human umbilical vein endothelial cells [10]. Tanaka et al. have reported different classes of compounds including ILTG for their ability to inhibit constitutive expression of VCAM-1 on murine endothelial cells and ICAM-1 on mouse myeloid leukemia cells [21]. Further studies on structure activity relationship between substitution pattern of hydroxyl group and VCAM-1 inhibitory activity suggests that chalcone with 2′-hydroxyl group on B-ring and 4′-hydroxyl groups on A-ring are potentially active in decreasing the cell surface level of VCAM-1 expression [21]. Also, Takano–Ishikawa reported that chalcones with hydroxyl residue on 4′ position or 3′,4′-dihydroxy groups on B-ring exhibit significant E-selectin inhibitory activity on HUVECs [22]. These results indicated that chalcones with hydroxyl substitution at different positions have variable effects on the expression of cell adhesion molecules. ILTG has a unique structure having hydroxyl substitutions at 4 position in A-ring and 2′ and 4′ positions in B-ring thus it could possess higher inhibitory activity. However, no detail study has been carried out to evaluate its inhibitory effect on induced expression of CAMs on human primary endothelial cells. In addition, very little is known in regard to the mechanism of action that manifests in the form of the anti-inflammatory activity of ILTG.

Here, we have not only undertaken a detail study on the effect of ILTG on the induced expression of ICAM-1, VCAM-1 and E-selectin on HUVECs, but also tried to unravel the underlying mechanism. We report that ILTG inhibits TNF-α induced adhesion of neutrophils to endothelial monolayer by inhibiting the expression of ICAM-1, VCAM-1 and E-selectin. We also demonstrate that it inhibits CAMs at the level of transcription by blocking the translocation and activation of NF-κB. To elucidate the mechanism further, we have shown that it blocks NF-κB activity by inhibiting IκB kinase activity and TNF-α induced ROS production in endothelial cells.

2. Materials and methods

2.1. Materials

Anti-E-selectin, anti-ICAM-1, anti-VCAM-1 antibodies and TNF-α, were purchased from BD Pharmingen, USA. ILTG, M-199, L-glutamine, antibiotic and antimycotic solution, endothelial cell growth factor, trypsin, puck's 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 and β-actin primer sets were synthesized by Genset Corp., Japan. Fetal calf serum was purchased from Biological Industries, Israel. Anti-mouse-IgG-FITC was purchased from Becton & Dickinson, USA. Anti-p65, anti-IκBα, phospho-specific IκBα, anti-glyceraldehyde-3-phosphate dehydrogenase and anti-β tubulin antibodies were purchased from Santa Cruz Biotechnology, USA.

2.2. Human endothelial cell culture

Endothelial cells were isolated from human umbilical cord using mild trypsinization [23]. The cells were grown in M-199 medium supplemented with 20% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, endothelial cell growth factor (50 μg/ml) and heparin (5 U/ml).

2.3. Cell viability assay

The cytotoxicity of ILTG was analyzed by using trypan blue exclusion test as described [10], and was further confirmed by colorimetric MTT assay as described [12].

2.4. Neutrophil adhesion assay

Neutrophil adhesion assay was performed under static conditions as described previously [10,12]. Neutrophils were isolated from peripheral blood of healthy individuals [10,12]. Endothelial cells were incubated with or without ILTG for 2 h followed by induction with TNF-α (10 ng/ml) for 6 h. Endothelial monolayer was washed and neutrophils (6×10^4/well) were added to the monolayer and were allowed to adhere for 1 h at 37 °C. The non-adherent neutrophils were removed by washing. The neutrophils bound to endothelial cells were assayed by adding a substrate solution consisting of *o*-phenylenediamine dihydrochloride, 0.1% cetitrimethyl ammonium bromide and 3-amino-1,2,4-triazole (1 mM). The absorbance was measured at 490 nm using an automated microplate reader (Spectramax 190, Molecular Devices, USA). The absorbance is directly proportional to number of cells adhere to endothelial monolayer. Thus high absorbance illustrates that high number of neutrophils adhere to endothelial monolayer.

2.5. Cell-ELISA for measurement of ICAM-1, VCAM-1 and E-selectin

Cell-ELISA was used for measuring the expression of ICAM-1, VCAM-1 and E-selectin on surface of endothelial cells [10,12]. Briefly, endothelial cells were incubated with or without ILTG

at desired concentrations for the required period followed by induction with TNF-α for 16 h for ICAM-1 and VCAM-1 expression and for 4 h for E-selectin expression. The cells were fixed, following by overnight incubation at 4 °C with ICAM-1 mAb, VCAM-1 mAb or E-selectin mAb, absorbance was measured at 490 nm using an automated microplate reader (Spectramax 190, Molecular Devices, USA).

2.6. Flow cytometry analysis

The cell surface expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells was further confirmed by flow cytometry [10,12]. Briefly, endothelial cells were incubated with or without ILTG (10.0 μg/ml) followed by treatment with TNF-α. Cells were then incubated with anti-ICAM-1, anti-VCAM-1, anti-E-selectin or control IgG antibody cells were washed and stained with FITC conjugated anti-mouse IgG. The cells were analysed for estimating the expression of cell adhesion molecules using a flow cytometer (FACS Vantage, Becton & Dickinson, USA). For each sample, 20,000 events were acquired. Analysis was carried out by using Cell Quest Software (Becton Dickinson, USA).

2.7. Total RNA isolation and reverse transcription polymerase chain reaction

Endothelial cells (2×10^6) were incubated with or without ILTG (10.0 μg/ml) for 2 h. Following induction with TNF-α for 4 h RNA was isolated from treated endothelial cells according to a modified guanidium thiocyanate procedure [24]. The expression of the transcripts for ICAM-1, VCAM-1 and E-selectin was evaluated by RT-PCR as previously described [18]. Primers were synthesized according to published cDNA sequences. The RT-PCR was performed following the manufacturer's protocol (Access RT-PCR system, Promega, Madison, USA).

2.8. Preparation of nuclear extracts

Endothelial cells (2×10^6) were incubated with or without 10.0 μg/ml ILTG for 2 h followed by induction with TNF-α (10 ng/ml) for 30 min as previously described [10].

2.9. Preparation of total cell lysate

Endothelial cells (2×10^6) were incubated with or without 10.0 μg/ml ILTG for 2 h followed by induction with TNF-α (10 ng/ml) for different time points [25]. The cells were scraped, collected and centrifuged at 300 × *g* for 10 min. The cell pellet was resuspended in RIPA buffer [25], and allowed to swell on ice for 30 min. Following centrifugation at 13,000 rpm for 30 min, the supernatant was collected as total cell lysate and stored at −70 °C. The protein concentration in the extracts was estimated by BCA method.

2.10. Western blot analysis for p65

Western blot analysis for p65 was performed from nuclear and cytoplasmic extracts prepared following ILTG treatment and induction by TNF-α as previously described. [7,10]. To ensure that there was no contamination of nuclear extracts with cytoplasmic proteins, western blot was performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) simultaneously for all samples.

2.11. Western blot analysis for IκBα degradation and phosphorylation

In order to evaluate the effect of ILTG on degradation and phosphorylation of IκBα, western blot analysis was performed with total cell lysates prepared from ILTG treated endothelial cells. Briefly, lysates were fractionated on 12% SDS polyacrylamide gel and the proteins were transferred to Hybond-C membrane (Amersham, UK) and were incubated with polyclonal anti-IκBα (1:100) or phospho-specific IκBα (1:100) for 2 h at 37 °C. The membranes were subsequently incubated with HRP-conjugated antibodies. The blots were exposed to peroxidase substrate. Western blot for β-tubulin was performed to ensure that there was equal loading in all the wells.

2.12. NF-κB activation assay

To determine NF-κB activation, electrophoretic mobility shift assay (EMSA) was performed with some modifications as previously described [26]. Briefly, nuclear extracts were prepared from ILTG treated endothelial cells, 20 μg of nuclear extract was incubated with 40–80 fmoles of ^{32}P-end labelled double-stranded NF-κB oligonucleotide. The DNA-protein complexes were analysed by electrophoresis on a 4% native polyacrylamide gel using Tris-glycine buffer, and visualised by autoradiography. EMSA was also performed for AP-1 and Oct-1 to find out the effect ILTG has on these transcription factors using radiolabelled AP-1 and Oct-1 oligonucleotide.

2.13. NF-κB dependent reporter gene assay

The effect of ILTG on TNF-α induced NF-κB dependent reporter gene transcription was measured as previously described [25]. Briefly, human epithelial A549 cells (2×10^4 cells/well) were plated in 24 well plates. Next day cells were transfected with pNF-κB-d2EGFP (1.0 μg) using Lipofectamine 2000, 22 h after tranfection cells were treated with 10.0 μg/ml ILTG, 2 h after drug treatment, cells were induced with recombinant human TNF-α (10 ng/ml) for inducing the expression of GFP. Green fluorescence was recorded after 6 h by using fluorescence microscope. Images were captured using Nikon camera and analysed for mean fluorescence intensity by using software Image pro plus (Media Cybernetics).

2.14. IκB kinase assay

Immunoprecipitation was performed as previously reported [27], with some modifications. Briefly, confluent cells were incubated with or without ILTG and were stimulated with recombinant human TNF-α (10 ng/ml) for 15 min. The cells were washed with cold PBS and lysed using lysis buffer [27]. The lysate was centrifuged at 13,000 rpm for 45 min, the supernatant was collected as total cell lysate and stored at −70 °C. Endogenous IKK complexes were immunoprecipitated from lysates; 100 μg of cell lysate was incubated with 2 μg anti-IKK-β antibody (H-744, Santa Cruz Biotechnology, Santa Cruz,

CA, USA) in 100 μl lysis buffer supplemented with 250 mM NaCl. After incubation for 4 h on ice, 20 μl protein A beads (50%, v/v) were added, and the mixture was incubated under rotation for an additional 1 h at 4 °C.

Kinase assay was performed as previously described [27]. The reaction mixture consisted of kinase buffer [27], 2 μg GST-IκBα(1–54), 5 μM ATP, and 1 μCi [γ-^{32}P]ATP in a volume of 30 μl. Kinase reactions were performed at 37 °C for 30 min, then the reaction mixtures were subjected to SDS-PAGE and autoradiography. Western blot was performed for β-tubulin to ensure that there was equal loading in all the wells.

2.15. *Measurement of intracellular ROS generation by flow cytometry*

The TNF-α induced intracellular ROS generation in endothelial cells was measured by probe dichlorofluorescein diacetate (DCF-DA) (Molecular Probes, Inc., USA) as previously reported [28].

2.16. *Statistical analysis*

Results are given as mean ± S.D. Independent two-tailed Student's *t*-test was performed. Differences were considered statistically significant for $P < 0.05$. All statistical analysis was performed using software Microcal Origin (ver 3.0; Microcal Software Inc., Northampton, MA) and Cell Quest Software (Becton & Dickinson, USA).

3. Results

3.1. *ILTG is non-toxic to cells*

The cyto-toxicity experiments in this study were performed at 10.0–12.5 μg/ml concentration. Further, we examined the cytotoxic effect of isoliquiritigeinin up to 22.5 μg/ml concentration more than 96% cells were viable at this concentration (data not shown).

3.2. *ILTG inhibits adhesion of neutrophils to endothelial monolayer*

As detected by colorimetric assay, there was low adherence of neutrophils on unstimulated endothelial cells. This adherence was induced more than three-fold by stimulation with TNF-α (Fig. 1). Interestingly, ILTG significantly inhibited the adhesion of neutrophils to endothelium in a concentration dependent manner and a maximum of 90% inhibition was obtained at 12.5 μg/ml concentration.

3.3. *ILTG inhibits the TNF-α induced expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells*

As the expression of cell adhesion molecules on endothelial cells is a prerequisite for adhesion of neutrophils, we investigated the effect of ILTG on TNF-α induced ICAM-1, VCAM-1 and E-selectin expression. Our cell-ELISA results demonstrated that ICAM-1, VCAM-1 and E-selectin were expressed at low levels on unstimulated endothelial cells

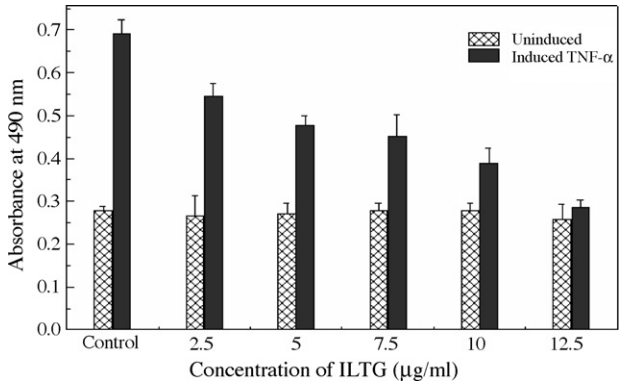

Fig. 1 – ILTG inhibits adhesion of neutrophils to endothelial monolayer. Endothelial cells were incubated with or without indicated concentrations of ILTG for 2 h prior to induction without (hatched bars) or with TNF-α (10 ng/ml) (closed bars) for 6 h. The adhesion of neutrophils on the cells was measured by colorimetric assay as described in Section 2. The data presented are representative of three independent experiments done in triplicate. Values shown are mean ± S.D. of quadruplicate wells.

and there was over four to six-fold increase in their expression upon stimulation with TNF-α (Fig. 2, panel A, i, ii, iii). Pretreatment of endothelial cells with ILTG had no effect on the constitutively expressed levels of ICAM-1, VCAM-1 or E-selectin. The inhibitory activity of ILTG on ICAM-1 expression was first evident at a concentration 0.625 μg/ml with maximal inhibition at a concentration of 10.0 μg/ml (Fig. 2, panel A, i). The inhibition pattern for VCAM-1 was first evident at a concentration 0.625 μg/ml with maximal inhibition at a concentration of 10.0 μg/ml (Fig. 2, panel A, ii). In case of E-selectin, inhibition was first observed at a concentration 2.5 μg/ml with maximal inhibition at a concentration 10.0 μg/ml (Fig. 2, panel A, iii).

The inhibitory activity of ILTG on ICAM-1, VCAM-1 and E-selectin expression was further confirmed by flow cytometry (Fig. 2, panel B, i, ii, iii). The unstimulated cells expressed low levels of ICAM-1 and undetectable levels of VCAM-1 and E-selectin. Upon stimulation with TNF-α, a substantial increase (six- to eight-folds) in the expression of all these three molecules was observed (Fig. 2, panel B, i, ii, iii). Pretreatment of endothelial cells with ILTG (10.0 μg/ml) significantly inhibited the TNF-α induced expression of ICAM-1, VCAM-1 and E-selectin (Fig. 2, panel B, i, ii, iii). Thus, ILTG inhibited the induced expression of cell adhesion molecules as measured using cell-ELISA and confirmed by flow cytometry.

To determine the kinetics of inhibition, endothelial cells were incubated with 10.0 μg/ml of ILTG for 1–4 h prior to, simultaneously or 1–2 h after induction with TNF-α for 16 h. ILTG inhibited ICAM-1 expression when added prior to or simultaneously with TNF-α induction. However, when it was added after TNF-α induction, the inhibition of ICAM-1 expression was not significant (Fig. 3). These results, therefore, indicate that ILTG may be interfering with early signaling events in response to TNF-α.

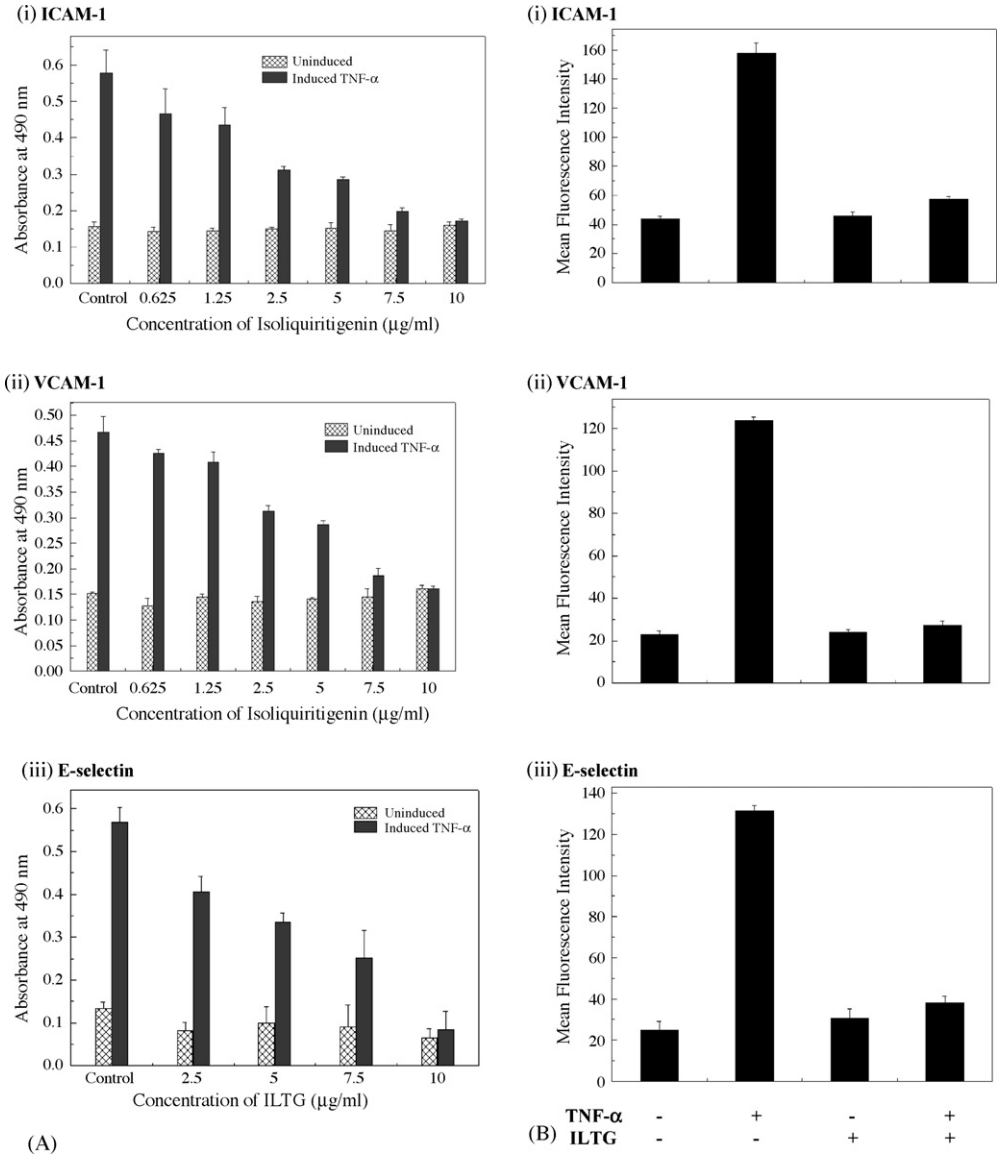

Fig. 2 – Concentration dependent inhibition of TNF-α induced ICAM-1, VCAM-1 and E-selectin expression by ILTG: (A) endothelial cells were incubated with or without indicated concentrations of ILTG for 2 h prior to induction without (hatched bars) or with TNF-α (10 ng/ml) (closed bars) for 16 h for ICAM-1, VCAM-1 and 4 h for E-selectin, and level on the cells was measured by ELISA as described in Section 2. Flow cytometric analysis of inhibition of TNF-α induced ICAM-1, VCAM-1 and E-selectin expression by ILTG: (B) expression of cell adhesion molecules was measured by flow cytometry as described in Section 2. Cell Quest Software was used for statistical analysis ($p < 0.01$). The data presented as mean ± S.D. of three independent experiments after auto-fluorescence was subtracted from treated conditions.

3.4. *ILTG decreases transcript levels of ICAM-1, VCAM-1 and E-selectin*

To understand the mechanisms responsible for inhibition of ICAM-1, VCAM-1 and E-selectin by ILTG, we examined whether ILTG blocks the induction of their transcript levels. As shown in Fig. 4, the unstimulated endothelial cells or cells treated with ILTG alone, there were low levels of ICAM-1 mRNA, and undetectable levels of VCAM-1 and E-selectin mRNA. Stimulation with TNF-α led to a marked increase in ICAM-1, VCAM-1 and E-selectin transcripts (Fig. 4), while pre-treatment with ILTG led to a significant reduction in their induced transcript levels (Fig. 4). Neither TNF-α nor ILTG altered constitutive β-actin mRNA levels (Fig. 4). These results indicate that ILTG inhibits the transcription of ICAM-1, VCAM-1 and E-selectin genes.

3.5. *ILTG inhibits TNF-α induced nuclear translocation of p65*

Previous studies have demonstrated that NF-κB is a key transcription factor for TNF-α induced expression of ICAM-1,

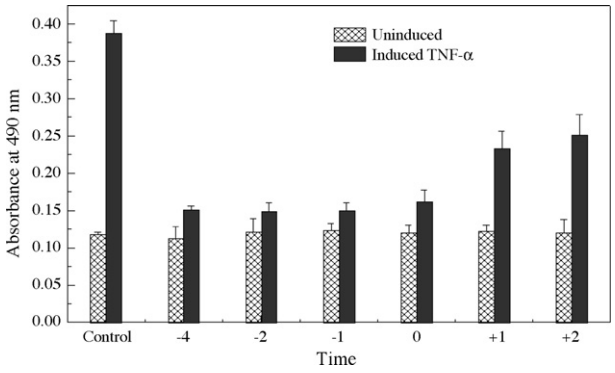

Fig. 3 – Inhibition by ILTG is time dependent: endothelial cells were incubated with or without 10.0 μg/ml ILTG for indicated time periods. This was followed by induction without (hatched bars) or with (closed bars) TNF-α (10 ng/ml) for 16 h. The ICAM-1 level on the cells was measured by cell ELISA as described in Section 2. The data are representative of three independent experiments done in triplicate. Values shown are mean ± S.D.

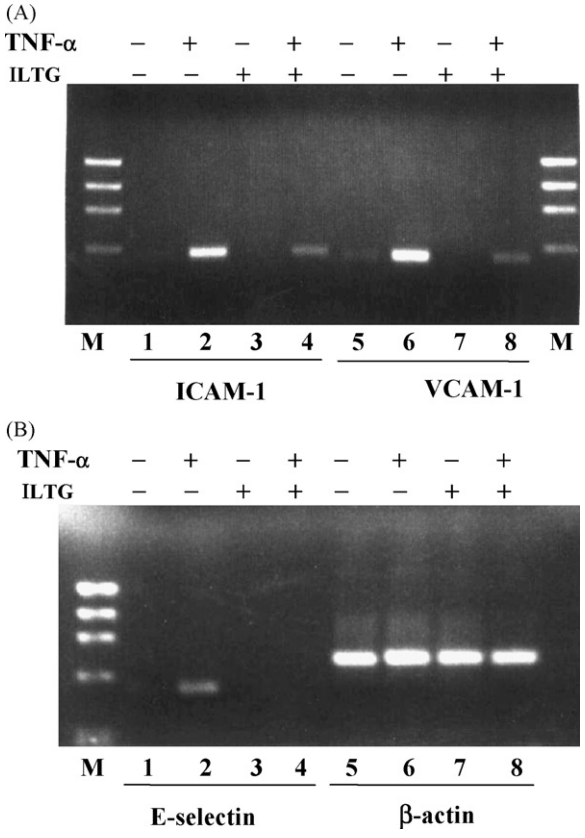

Fig. 4 – Analysis of ICAM-1, VCAM-1 and E-selectin transcript levels in ILTG treated cells: Total RNA from the ILTG treated cells was isolated and analysed by RT-PCR as described in Section 2. The data are representative of three independent experiments. (A) ICAM-1 and VCAM-1; (B) E-selectin and β-actin. Lanes, M: marker φ X 174 Hae III digest; lanes 1 and 5: unstimulated cells; lanes 2 and 6: stimulated with TNF-α; lanes 3 and 7: ILTG; lanes 4 and 8: stimulated with TNF-α after ILTG pre-treatment for 2 h.

VCAM-1 and E-selectin on endothelial cells [4]. The activation of NF-κB requires the translocation of the p65 subunit of NF-κB from the cytoplasm to the nucleus. We, therefore, measured the levels of p65 in the cytoplasm and in the nucleus of ILTG treated cells using western blot. It was observed that there were low levels of p65 in the nucleus of the unstimulated cells or cells treated with ILTG alone (Fig. 5A, lanes 1 and 3) while high levels were observed in the cytoplasm (Fig. 5B, lanes 5 and 8). Upon treatment with TNF-α, the level of p65 in the cytoplasm decreased (Fig. 5B, lane 6) while its level increased in the nucleus (Fig. 5A, lane 2). On the other hand, upon treatment of the cells with ILTG prior to induction with TNF-α, the level of p65 did not decrease in the cytoplasm (Fig. 5B, lane 8) and there was no concomitant increase in the p65 levels in the nucleus (Fig. 5A, lane 4). Equal loading of protein amount was visualized by performing western blot for GAPDH (Fig. 5C and D). Performing this western blot for nuclear protein also ensured that there was no contamination of nuclear extracts with cytoplasmic proteins. These results therefore indicate that ILTG interferes with the translocation of p65 from cytoplasm to the nucleus, and hence may be responsible for preventing the induction of ICAM-1, VCAM-1 and E-selectin.

3.6. *ILTG inhibits TNF-α induced activation of NF-κB but not AP-1 and Oct-1*

To study the effect of ILTG on NF-κB activation, electrophoretic mobility shift assay was performed. As shown in Fig. 5E, there was a low level of NF-κB in unstimulated cells (lane 2). Upon stimulation with TNF-α there was an increased level of NF-κB, thus causing substantial retardation in the mobility of the labelled oligonucleotide (lane 3). The specificity of the NF-κB DNA complex induced by TNF-α was confirmed in control experiments. Incubation with excess unlabelled NF-κB inhibited the formation of the complex, whereas competition with an excess of an irrelevant oligonucleotide, SP-1 did not inhibit the complex (compare lane 7 with lane 8). ILTG alone had no effect on the basal level of NF-κB (lane 4). In contrast, the treatment of cells with ILTG prior to induction with TNF-α caused a substantial decrease in the level of NF-κB binding at a concentration of 10.0 μg/ml (lane 5). Further, we checked the binding of NF-κB to DNA in the presence of ILTG, for this, nuclear extract from TNF-α induced endothelial cells was incubated with radiolabelled NF-κB oligos in the presence of ILTG. We found that ILTG had no effect on NF-κB binding to DNA (Fig. 5E, lane 6). As cell adhesion molecules are regulated by other transcription factors also, like AP-1 and Oct-1 we wanted to see the effect ILTG has on these transcription factors. As seen in Fig. 5F and G, ILTG does not seem to affect induced levels of AP-1 and Oct-1. Thus, the inhibitory activity of ILTG is specific to NF-κB.

To confirm further, we performed reporter gene assay, by transfecting A549, a lung epithelial cell line, with pNF-κB-d2EGFP. It was observed that TNF-α induces GFP expression by three-fold over the control. However, pre-treatment of A549 cells with ILTG prior to TNF-α stimulation significantly inhibited the induced expression of GFP (Fig. 5H). These results therefore, demonstrate that ILTG inhibits the TNF-α induced NF-κB activation.

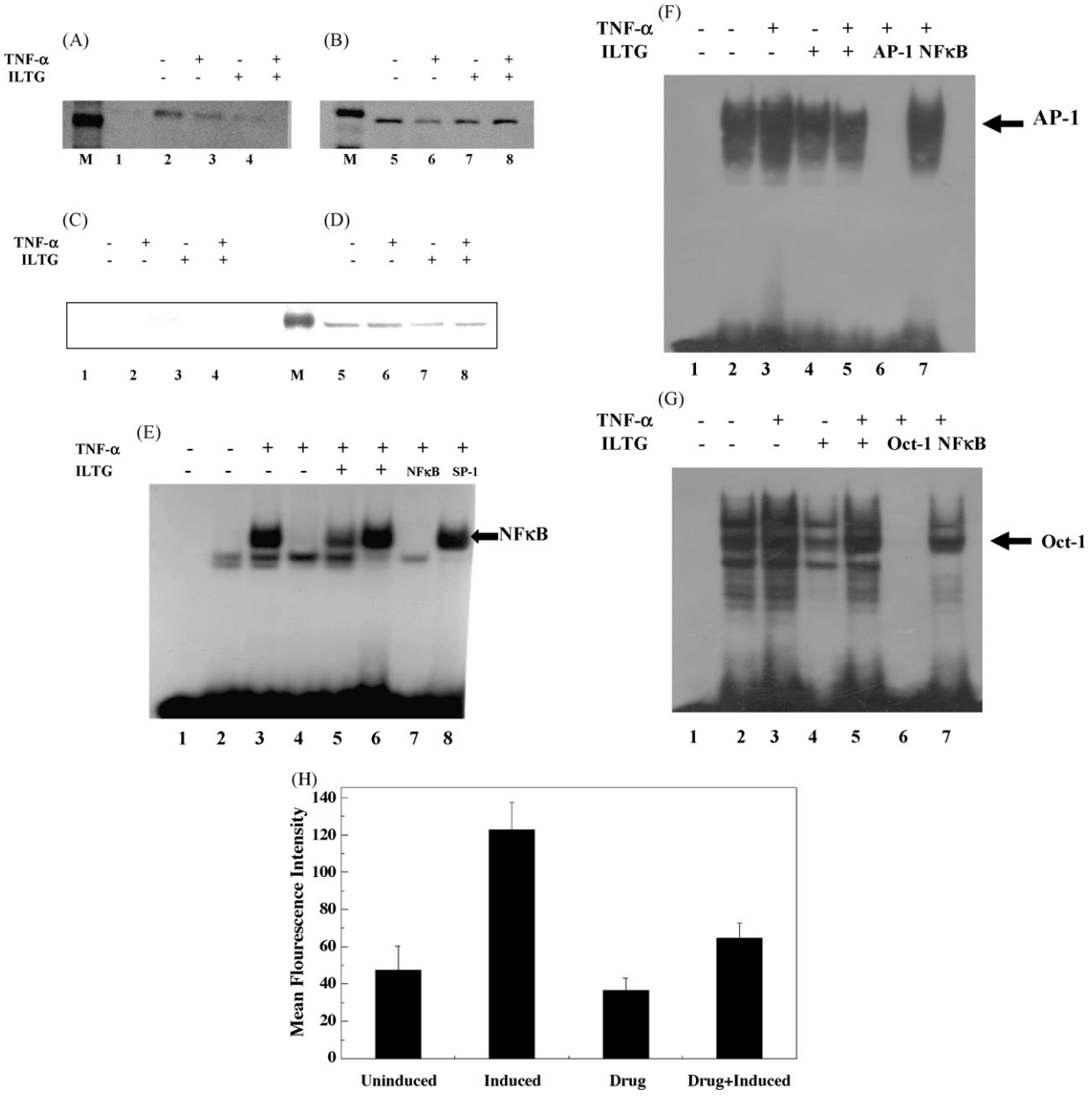

Fig. 5 – Effect of ILTG on p65 translocation: the nuclear and cytoplasmic extracts were prepared and analysed for p65 translocation as described in Section 2. (A) Nuclear extracts and (B) cytoplasmic extracts. Lane M, marker, bio-rad broad range; lanes 1 and 5, unstimulated cells; lanes 2 and 6, stimulated with TNF-α; lanes 3 and 7, ILTG alone; lanes 4 and 8, stimulated with TNF-α after ILTG pre-treatment for 2 h. (C) and (D) are western blot for glyceraldehyde-3-phosphate dehydrogense (GAPDH) for nuclear and cytoplasmic extracts, respectively, to ensure that there is no contamination of cytoplasmic proteins with nuclear proteins (lanes 1–8). (E)–(G) ILTG inhibits NF-κB activation but not AP-1 or Oct-1: EMSA was performed with double stranded NF-κB or AP-1 or Oct-1 as described in Section 2. Lane 1, free probe; lane 2, unstimulated cells; lane 3, stimulated with TNF-α; lane 4, ILTG alone; lane 5, stimulated with TNF-α after ILTG pre-treatment for 2 h; lane 6, nuclear extract from TNF-α stimulated endothelial cells was incubated with ILTG to see its effect on DNA binding; lane 7, cold chase with specific oligos; lane 8, cold chase with non specific oligos. (H) ILTG inhibits NF-κB dependent reporter gene expression. A549 cells were transfected with pNF-κB-d2EGFP as mentioned in Section 2. Bar 1, unstimulated cells; bar 2, stimulated with TNF-α; bar 3, ILTG alone; bar 4, stimulated with TNF-α after ILTG pre-treatment for 2 h. The data presented is one of the three independent experiments.

3.7. *ILTG inhibits TNF-α induced IκBα degradation*

Translocation of NF-κB from cytoplasm to the nucleus is preceded by the phosphorylation and subsequent degradation of IκBα. To determine the effect of ILTG on IκBα degradation, total cell lysate was prepared from the ILTG treated cells. Using western blot analysis, we demonstrated that the degradation of IκBα took place in a time dependent manner with maximum

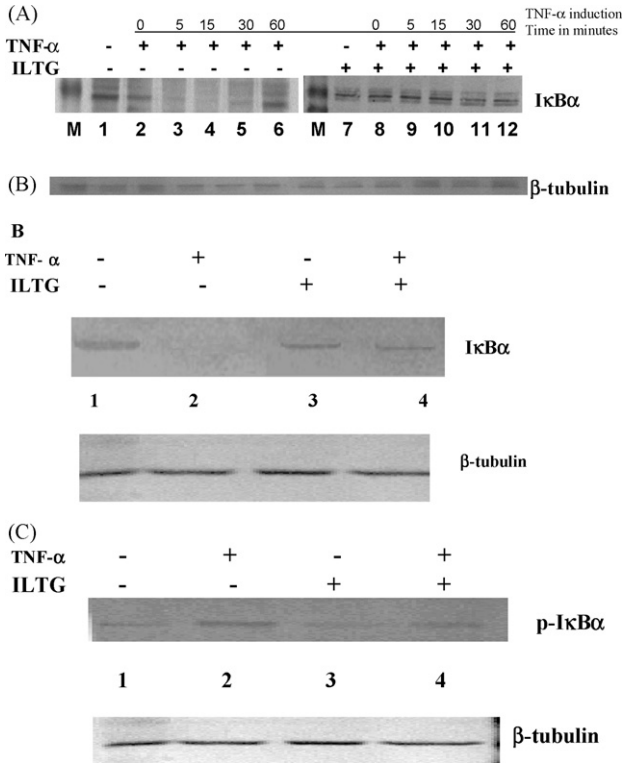

Fig. 6 – Effect of ILTG on TNFα induced IκBα degradation and phosphorylation: total cell lysate was prepared and analysed for IκBα degradation and phosphorylation as described in Section 2. (A) Time kinetics of IκBα degradation, (B) IκBα degradation, (C) IκBα phosphorylation, lane 1, unstimulated cells; lane 2, stimulated with TNF-α; lane 3, ILTG alone; lane 4, stimulated with TNF-α after ILTG pre-treatment for 2 h (B and C). The data presented is one of the three independent experiments. Western blot was done for β-tubulin to ensure that there was equal loading in all the wells.

degradation at 15 min after the TNF-α induction. Interestingly, after 30 min of TNF-α induction the levels of IκBα starts to increase and at 60 min of TNF-α induction to its reached at normal (Fig. 6A, left panel). The pre-treatment of endothelial cells with ILTG inhibited IκBα degradation in a time dependent manner (Fig. 6A, right panel). As shown in Fig. 6B, upon induction with TNF-α for 15 min the intensity of IκBα was significantly reduced (compare lane 1 versus lane 2). In contrast, pre-treatment of cells with ILTG prior to induction with TNF-α significantly inhibited the degradation of IκBα (lane 4). ILTG alone had no effect on the basal level of IκBα (lane 3).

3.8. *ILTG inhibits TNF-α induced IκBα phosphorylation*

As IκBα degradation is dependent on its phosphorylation, we determined the status of its phosphorylation upon pre-treatment with ILTG. As shown in Fig. 6C, the intensity of phosphorylated IκBα significantly increased after induction with TNF-α (compare lane 2 versus lane 1). Interestingly, treatment of endothelial cells with ILTG prior to induction

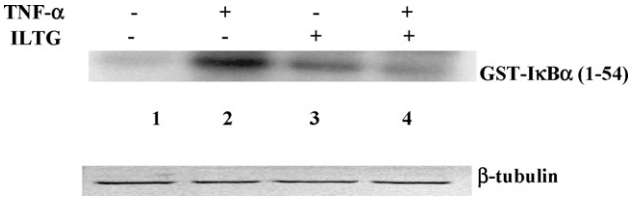

Fig. 7 – ILTG inhibits TNF-α induced kinase activity of IKK: endothelial cells were treated with ILTG (10.0 μg/ml) and stimulated with TNF-α, total cell lysates were prepared. The ability of immunoprecipitates assayed to directly phosphorylate GST-IκBα fusion proteins in presence of [γ^{32}P] ATP *in vitro* kinase assay was performed as mentioned in Section 2. Lane 1, unstimulated cells; lane 2, stimulated with TNF-α; lane 3, ILTG alone; lane 4, stimulated with TNF-α after ILTG pre-treatment for 2 h Western blot was done for β-tubulin to ensure that there was equal loading in all the wells.

with TNF-α significantly inhibited the intensity of phosphorylated IκBα (compare lane 2 versus lane 4). ILTG alone did not effect the basal levels of phosphorylated IκBα (lane 3).

3.9. *ILTG inhibits TNF-α induced kinase activity of IκB kinase (IKK)*

It has been shown that IKK is required not only for TNF-α induced phoshporylation and degradation of IκBα but also for NF-κB activation [25]. Since ILTG inhibits IκBα phosphorylation and NF-κB activation, we examined the effect of ILTG on TNF-α

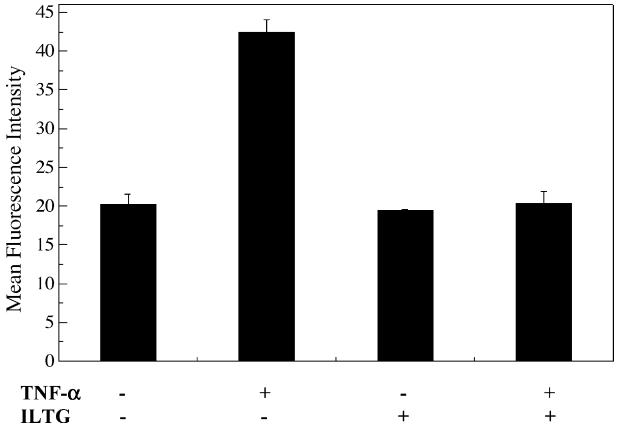

Fig. 8 – ILTG inhibits TNFα induced ROS production: confluent human endothelial cells were treated with ILTG (10.0 μg/ml) for 2 h and then cells were loaded with DCF-DA dye (10 μM) for 30 min and cells were stimulated with TNF-α (10 ng/ml) for 30 min. Cells were washed with ice-cold PBS and collected for acquisition as mentioned in Section 2. The data presented as mean ± S.D. of three independent experiments after auto-fluorescence was subtracted from treated conditions. Bar 1, unstimulated cells; bar 2, stimulated with TNF-α; bar 3, ILTG alone; bar 4, stimulated with TNF-α after ILTG pre-treatment for 2 h. Cell Quest Software was used for statistical analysis ($p < 0.05$).

induced activation of IKK. Stimulation of cells with TNF-α increased the IκB kinase activity (Fig. 7, lane 2). In contrast, pre-treatment of cells with ILTG (10.0 μg/ml) for 2 h before TNF-α stimulation resulted in a decrease in the kinase activity of IKK (Fig. 7, compare lane 2 versus lane 4). There was a slight decrease in the basal level of kinase activity when the cells were treated with ILTG only (compare lane 1 versus lane 3). This shows that ILTG may inhibit kinase activity of IKK.

3.10. *ILTG inhibits intracellular ROS generation*

Emerging evidence suggests that reactive oxygen species (ROS) can contribute to diverse signaling pathways. TNF-α induced free radical generation like H_2O_2 activates inflammatory signaling pathway, including NF-κB in vascular cells [29]. As ILTG inhibits TNF-α induced NF-κB activation, therefore, we examined the effect of ILTG on TNF-α induced ROS generation in endothelial cells. The TNF-α induced intracellular ROS generation in endothelial cells was measured by probe dichlorofluorescein diacetate (DCF-DA). As shown in Fig. 8, there was an approximately two-folds increase in generation of free radicals upon stimulation of cells with TNF-α. In contrast, treatment of endothelial cells with ILTG prior to induction with TNF-α significantly inhibited ROS generation, while ILTG alone had no effect.

4. Discussion

The extracts of *Glycyrrhiza* and *Dalbergia* were previously known to posses various medicinal activities including anti-inflammatory and anti-oxidant activity. Isoliquiritigenin (ILTG), a chalcone is one of the major active components in these extracts. Earlier comparative studies on various molecules with chalcones structure have indicated that ILTG contains a unique structure (4,2′,4′-trihydroxychalcone) and could possess higher anti-inflammatory activity [10,21,22]. In the present study, we have demonstrated that ILTG inhibited the adhesion of neutrophils to endothelial monolayer by blocking TNF-α induced expression of ICAM-1, VCAM-1 and E-selectin on primary endothelial cells. One of the critical aspects of the present study was to compare the effectiveness of ILTG as inhibitor of expression of cell adhesion molecules with our previously reported 2′-hydroxychalcone [10]. We found that ILTG inhibited CAMs at a lower concentration (10.0 μg/ml or 40 μM) in comparison to 2′-hydroxychalcone which required a concentration of 60 μM [10]. Similar to 2′-hydroxychalcone, the effect ILTG has on ICAM-1 was found to be reversible as the treated cells were fully capable of responding to TNF-α induction (data not shown). Thus, ILTG treatment did not cause any permanent change in the endothelial cells. We also found that ILTG was more effective when added prior to or simultaneously with TNF-α (Fig. 3). These results suggested that ILTG may be interfering at early stages of signaling events leading to the expression of CAM upon induction with TNF-α.

To elucidate the mechanism further, we have demonstrated that it effectively inhibited TNF-α induced transcription of ICAM-1, VCAM-1 and E-selectin (Fig. 4), and it was also seen to block the translocation and activation of NF-κB at a 33% lower concentration than that of 2′-hydroxychalcone [10]. Further, when we performed EMSA using labelled AP-1 and Oct-1, it was also seen that ILTG did not affect these transcription factors, thus, inhibitory activity of ILTG seems to be specific to NF-κB. Recently, Hsu et al. [30] have reported that isoliquiritigenin induced apoptosis in human hepatoma cells. Apparently, their findings seem similar to ours in terms of the ability of isoliquirtigenin to inhibit NF-κB dependent gene expression. However, unlike their study, where they have investigated the effect of this compound on the constitutive levels of NF-κB protein expression and its DNA-binding activity in human hepatoma cells, we have shown that ILTG is able to inhibit nuclear translocation and activation of NF-κB upon stimulation with TNF-α by inhibiting IκBα degradation in primary endothelial cells. Hence, our work is quite distinct as we report here the effect of ILTG on NF-κB signaling pathway in primary cells. We have also seen that the NF-κB inhibition by ILTG is not cell specific, as NF-κB inhibition was also observed in A549, a human lung epithelial cell line (data not shown). These results were further confirmed by performing reporter gene assay where the effect of ILTG on the expression GFP was seen in A549 cells transiently transfected with a construct having GFP under NF-κB regulation. We found that ILTG quite effective in blocking NF-κB dependent GFP expression (Fig. 5H). As, NF-κB activation by TNF-α requires phosphorylation of IκBα at 32nd serine residue by IκB kinase complex (IKK) [3–5], we wanted to study the effect ILTG has on the phosphorylation and degradation of IκBα using specific antibody that recognizes the phosphorylation status of serine 32. Indeed we found that the compound inhibited the degradation and phoshorylation of IκBα (Fig. 6B and C). We have also found that ILTG inhibited TNF-α induced IκB kinase activation in endothelial cells (Fig. 7), moreover, when we incubated IKK immunocomplex with ILTG we found that kinase activity of IKK was inhibited in a concentration dependent manner (data not shown). These results though preliminary suggest that ILTG may be directly interacting with IKK. Recently it has been shown that cysteine 179 that lies between the two serine residues in the activation loop of the kinase can be a site for modification by IKK inhibitors such as parthenolide [31]. It would be interesting to investigate whether ILTG works in a similar way. Nonetheless, in order to establish that the inhibitory activity ILTG is due to its ability to physically interact with IKK much more work is required.

It has been shown that apart from direct recruitment of IKK complex to activate NF-κB, TNF-α also activates it by the generation of oxidative stress in inflammatory signaling pathway [29]. Hence we wanted to look at the effect ILTG has on ROS production in primary endothelial cells. Here also we found that this chalcone inhibited TNF-α induced generation of ROS (Fig. 8).

Based on our above findings we are proposing ILTG, a modified chalcone, to be an effective inhibitor of NF-κB signaling and of cell adhesion molecules at a 33% lesser concentration than our previously reported 2′-hydroxychalcone. The concentration at which ILTG works is even lesser when compared to other known NF-κB inhibitors that work at much higher concentrations, ranging from 100 to 1000 μM [32–34]. Also, N-acetylcysteine and pyrrolidone dithiocarbamate are effective in inhibiting the TNF-α induced ROS production

at very high concentrations 30 mM and 100 μM, respectively, in endothelial cells [35].

Recently, Lee et al., reported that cardamomin (2′,4′-dihydroxy-6′methoxychalcone), a natural chalcone analog from *Alpinia conchigera*, blocked NF-κB activation via inhibiting the IκBα degradation and phosphorylation in RAW264.7 cells [36]. The fact that some of their findings are quite similar to ours further supports our claim regarding the mechanism of action of chalcones. ILTG has been successfully tested in animal models for their anti-oxidant, anti-inflammatory, and anti-carcinogenic properties [11,37].

In our attempt to investigate its mechanism of action we have found for the first time that ILTG inhibit NF-κB not only by IKK kinase activity but also by inhibiting ROS generation upon stimulation with TNF-α in primary human endothelial cells. Thus, the possibility that ILTG could be effective in blocking the induction of other protein kinases like protein kinase C and protein tyrosine kinase or a cyclic AMP-independent protein kinase-A can be also explored.

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REFERENCES

[1] Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994;76:301–14.
[2] Mantovani A, Bussolino F, Introna M. Cytokine regulation of endothelial cell function: from molecular level to bedside. Immunol Today 1997;18:231–40.
[3] Baldwin AS. The transcription factor NF-κB and human disease. J Clin Invest 2001;107:3–6.
[4] Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine inducible enhancers. FASEB J 1995;9:899–909.
[5] Ghosh S, Karin M. Missing pieces in the NF-κB puzzle. Cell 2002;109:S81–96.
[6] Beg AA, Finco TS, Nanternet PV, Baldwin Jr AS. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκB-α: a mechanism for NF-κB activation. Mol Cell Biol 1993;13:3301–10.
[7] Los M, Schenk H, Hexel K, Baeuerle PA, Droge W, Schulze-Osthoff K. IL-2 gene expression and NF-kappa B activation through CD28 requires reactive oxygen production by 5-lipoxygenase. EMBO J 1995;14:3731–40.
[8] Bonizzi G, Piette J, Merville MP, Bours V. Cell type-specific role for reactive oxygen species in nuclear factor-kappaB activation by interleukin-1. Biochem Pharmacol 2000;59:7–11.
[9] Schreck R, Meier B, Mannel DN, Droge W, Baeuerle PA. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. J Exp Med 1992;175:1181–94.
[10] Madan B, Batra S, Ghosh B. 2′-Hydroxychalcone inhibits NF-κB and blocks TNF-α and LPS induced adhesion of neutrophils to human umbilical vein endothelial cells. Mol Pharmacol 2000;58:526–34.
[11] Kumar S, Singh BK, Kalra N, Kumar V, Kumar A, Raj HG, et al. Novel thiocoumarins as inhibitors of TNF-alpha induced ICAM-1 expression on human umbilical vein endothelial cells (HUVECs) and microsomal lipid peroxidation. Bioorg Med Chem 2005;13:1605–13.
[12] Kumar S, Arya P, Mukherjee C, Singh BK, Singh N, Parmar VS, et al. Novel aromatic ester from *Piper longum* and its analogs inhibit expression of cell adhesion molecules on endothelial cells. Biochemistry 2005;44:15944–52.
[13] Kakegawa H, Matsumoto H, Satoh T. Inhibitory effects of some natural products on the activation of hyaluronidase and their anti-allergic actions. Chem Pharm Bull 1992;40:1439–42.
[14] Vaya J, Belinky PA, Aviram M. Antioxidant constituents from licorice roots: isolation, structure elucidation and antioxidative capacity toward LDL oxidation. Free Radic Biol Med 1997;23:302–13.
[15] Wegener JW, Nawrath H. Differential effects of ILTG and YC-1 in rat aortic smooth muscle. Eur J Pharmacol 1997;323:89–91.
[16] Yamazaki S, Morita T, Endo H, Hamamoto T, Baba M, Joichi Y, et al. ILTG suppresses pulmonary metastasis of mouse renal cell carcinoma. Cancer Lett 2002;183:23–30.
[17] Baba M, Asano R, Takigami I, Takahashi T, Ohmura M, Okada Y, et al. Studies on cancer chemoprevention by traditional folk medicines. XXV. Inhibitory effect of ILTG on azoxymethane-induced murine colon aberrant crypt focus formation and carcinogenesis. Biol Pharm Bull 2002;25:247–50.
[18] Ma J, Fu NY, Pang DB, Wu WY, Xu AL. Apoptosis induced by ILTG in human gastric cancer MGC-803 cells. Planta Med 2001;67:754–7.
[19] Takahashi T, Takasuka N, Iigo M, Baba M, Nishino H, Hiroyuki Tsuda H, et al. ILTG, a flavonoid from licorice, reduces prostaglandin E2 and nitric oxide, causes apoptosis, and suppresses aberrant crypt foci development. Cancer Sci 2004;95:448–53.
[20] Yu SM, Kuo SC. Vasorelaxant effect of ILTG, a novel soluble guanylate cyclase activator, in rat aorta. Br J Pharmacol 1995;114:1587–94.
[21] Tanaka S, Sakata Y, Morimoto K, Tambe Y, Watanable Y, Honda G, et al. Influence of natural and synthetic compounds on cell surface expression of cell adhesion molecules, ICAM-1 and VCAM-1. Planta Med 2001;67:108–13.
[22] Takano-Ishikawa Y, Goto M, Yamaki K. Inhibitory effect of various flavonoids on E-selectin expression on human umbilical vein endothelial cells stimulated by tumor necrosis factor-α. Phytotherapy Res 2003;17:1224–7.
[23] Gupta B, Ghosh B. *Curcuma longa* inhibits TNF-α induced expression of adhesion molecules on human umbilical vein endothelial cells. Int J Immunopharmacol 1999;21:745–57.
[24] Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal Biochem 1987;162:156–9.
[25] Chaturvedi MM, Kumar A, Darnay BG, Chainy GBN, Agarwal S, Agarwal BB. Sanguinarine (pseudochelerythrine) is a potent inhibitor of NF-κB activation, IκB-α phosphorylation, and degradation. J Biol Chem 1997;272:30129–34.
[26] Marrugo J, Marsh DG, Ghosh B. The conserved lymphokine element-O in the IL5 promoter binds to a high mobility group protein. Mol Immunol 1996;33:1119–25.

[27] Mercurio FH, Zhu BW, Murray A, Shevchenko BL, Bennett J, Li DB, et al. IKK-1 and IKK-2: cytokine-activated IκB kinase essential for NFκB activation. Science 1997;278: 860–6.
[28] Li WG, Gavrila D, Liu X, Wang L, Gunnlaugsson S, Stoll LL, et al. Ghrelin inhibits proinflammatory responses and nuclear factor κB activation in human endothelial cells. Circulation 2004;109:2221–6.
[29] Garg AK, Agrawal BB. Reactive oxygen intermediates in TNF-α signaling. Mol Immunol 2002;39:509–17.
[30] Hsu L, Kuo PL, Lin LT, Lin CC. Isoliquiritigenin inhibits cell proliferation and induces apoptosis in human hepatoma cells. Planta Med 2005;71:130–4.
[31] Liang M, Bardhan S, Emily A, Rosman D, Beutler J, Porco J, et al. Inhibition of transcription factor NF-κB signaling proteins IKKβ and p65 through specific cystine residues by epoxyquonone a monomer: correlation with its anticancer cell growth activity. Biochem Pharmcol 2006;71: 634–45.
[32] Sakai A. Diclophenac inhibits endothelial cell adhesion molecule expression induced with lipopolysaccharide. Life Sci 1996;58:2377–87.
[33] Weber C, Erl W, Pietsch A, Strobel M, Ziegler-Heitbrock HWL, Weber PC. Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-kB mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. Arterioscler Thromb Vasc Biol 1994;14:1665–73.
[34] Pierce JW, Read MA, Ding H, Luscinskas FW, Collins T. Salicylates inhibit I kappa B-alpha phosphorylation, endothelial-leukocyte adhesion molecule expression, and neutrophils transmigration. J Immunol 1996;156:3961–9.
[35] Rahman A, Kefer J, Bando M, Niles WD, Malik AB. E-selectin expression in human endothelial cells by TNFα-induced oxidant generation and NF-κB activation. Am J Physiol 1998;275:L533–44.
[36] Lee JH, Jung HS, Giang PM, Jin X, Lee S, Son PT, et al. Blockade of nuclear factor-κB signaling pathway and anti-inflammatory activity of cardamomin, a chalcone analog from *Alpinia conchigera*. J Pharmacol Exp Ther 2006;316: 271–8.
[37] Fujioka T, Murakami K, Kubota T, Kodama R, Honda S, Nasu M. Sofalcone for treatment of *Helicobacter pylori* infection. J Gastroenterol 1996;31:56–8.