Am J Cardiovasc Dis 2011;1(1):16-30
www.AJCD.us /ISSN: 2160-200X/AJCD1103002

Original Article

Control of eotaxin-1 expression and release by resveratrol and its metabolites in culture human pulmonary artery endothelial cells

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Received March 23, 2011; April 19, 2011; Epub April 26, 2011; published June 1, 2011

Abstract: Population studies suggest that moderate red wine intake correlates with reduced risk of cardiovascular disease (CVD); cardioprotection may attribute to consumption of red wine polyphenol resveratrol. Since inflammation plays a key role in CVD, we investigated modulation of inflammation by resveratrol and its metabolites by determining the expression and release of chemokine, eotaxin-1, in cultured human pulmonary artery endothelial cells (HPAEC) treated with proinflammatory cytokines IL-13 and TNF-α. Up-regulation of eotaxin-1 gene expression by IL-13 or TNF-α was confirmed by RT-PCR, by reporter assays using eotaxin-1 gene promoter constructs, and by the changes in transcriptional factors STAT6 and NF-κB. Exposure to resveratrol suppressed IL-13 and TNF-α induced eotaxin-1 gene expression as well as attenuated the eotaxin-1 promoter activity, in coordination with inhibition of expression of JAK-1, reduction in phosphorylated-STAT6 and decreased p65 subunit of NF-κB. In addition, quantitative determination of eotaxin-1 release using enzyme-linked immunosorbent assay (ELISA) showed increased eotaxin-1 release in response to treatment by IL-13 and TNF-α, which was effectively inhibited by resveratrol. Whether resveratrol metabolites affected eotaxin-1 was also tested; piceatannol showed potency similar to resveratrol. We propose that control of eotaxin-1 expression and release by proinflammatory cytokines in HPAEC may be considered as an *in vitro* model for screening and discovering polyphenols with anti-inflammatory activities and cardioprotective potentials.

Keywords: Resveratrol, piceatannol, eotaxin-1, STAT6, NF-κB

Introduction

Atherosclerosis (AS) is a major risk factor for CVD, inflammation plays a prominent role in AS [1-3]. According to the "response-to-injury" hypothesis first introduced by Ross and coworkers in the mid 1970s, AS is a progressive disease initiated by coalescence of inflammation and a dysfunctional endothelium [4-7]. Immune cells migrate to the endothelium at the injured, inflamed site, in response to effectors elaborated by the immune cells *per se* or through their dynamic, reciprocal interplay with endothelial cells [8-10]. Therefore, in recent years considerable efforts have been directed towards the discovery of agents, particularly ones derived from dietary sources, with anti-inflammatory potentials as an adjunctive approach to prevent damaging effects of CVD [11-14].

The chemokines are low molecular weight chemotactic cytokines grouped according to the spacing of the first two cysteine residues into C, CC, CXC and CXXXC subfamilies [15, 16]. Eotaxin, a CC chemokine discovered in the ovalbumin-sensitized guinea pig inflammation model [17], exists in humans as eotaxin-1/CCL11, eotaxin-2/CCL24 and eotaxin-3/CCL26 [18-20]. The synthesis and secretion of eotaxin by dermal fibroblast and bronchial epithelial cells [21-23], play a significant role in inflammation ascribed to these cell types [24-27]. For example, eotaxin is robustly expressed in the epithelium of asthmatic mice, and acts to recruit eosinophils to the site of inflammation by interacting with its cognate receptor CCR3 [28, 29]. Studies on mechanism of expression of eotaxin have identified participation by STAT6 and NF-κB [30, 31]; binding sites for these transcription factors

have been located in the eotaxin gene promoter [30]. Exposure to cytokines IL-4 or IL-13 induces the phosphorylation and nuclear translocation of STAT6, in coordination with up-regulation of eotaxin expression [31-33]. TNF-α treatment results in increased phosphorylation and degradation of IκBα, accompanied by nuclear translocation of NF-κB, and concomitant with increased eotaxin expression [27, 34, 35].

Resveratrol, a polyphenol found abundantly in grapes, red wine, and various food items [36], exhibits chemopreventive and chemotherapeutic activities [36-38], and also confers protection against oxidative stress, CVD [39-41], and inflammation [42-45]. Anti-inflammatory and anti-carcinogenic effects of resveratrol may attribute to suppression of transcription factors, e.g., NF-κB [46, 47], AP-1 [46], and STAT3 [47, 48]. Thus, resveratrol reportedly inhibit TNF-α induced phosphorylation of the NF-κB-p65 subunit, and inhibits activation of IκB kinase (IKK) accompanied by attenuated translocation of NF-κB to the nucleus [46].

Few studies have investigated the control of eotaxin-1 expression and release in culture HPAEC [25]. Because endothelial cells and inflammation play critical role in the pathogenesis of AS [7, 49], we tested whether resveratrol may modulate inflammation in CVD by studying their effects on eotaxin-1 expression in HPAEC treated with proinflammatory cytokines IL-13 and TNF-a as mediated by transcription factors, STAT6 and NF-κB. Since use of resveratrol for chronic disease prevention and treatment has been marred by issues of limited bioavailability and biotransformation to other metabolites with ill-defined biological properties [50-52], the effects of resveratrol metabolites on eotaxin expression and secretion were also included in our analysis.

Materials and methods

Reagents

Resveratrol (*trans*-3, 5, 4'-trihydroxystilbene) was obtained from LKT Laboratories (St Paul, MN, USA) and piceatannol was obtained from A.G. Scientific, Inc. (San Diego, CA, USA). Piceid, and 3-O- and 4'-O-glucuronide derivatives of resveratrol were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Primary and secondary antibodies were obtained from various commercial vendors. Fetal bovine serum, RPMI-1640, streptomycin and penicillin were obtained from Cellgro, Inc (Herndon, VA, USA). All other chemicals and solvents used were of analytical grade. Plasmids containing eotaxin-1 promoters, pEotx 1363 and pEotx 300 were generously provided by Dr. Robert Schleimer, Professor and Chief of Allergy-Immunology at Northerwestern Feinberg School of Medicine.

Cell culture

HPAEC cells (isolated from normal human pulmonary arteries and cryopreserved at passage 2) were obtained from Cell Applications, Inc. (San Diego, CA, USA) and maintained using endothelial cell media supplemented with subculture Reagent kit. Cells were passaged using instructions provided by the manufacturer. Only passage 5 cells were used for experiments.

Treatment of HPAEC by cytokines, resveratrol, and its metabolites

The cells were seeded in 6- or 24-well plates at a density of 1×10^5cells/ml and incubated for 12-14 h in serum-containing cultured media. The cells were then switched to serum-free media and maintained overnight in a CO_2 incubator. Next, the cells were treated with recombinant human IL-13 or TNF-α (PeproTech Inc., Rocky Hill, NJ, USA), alone or in combination, for an additional 4 h. To test the effects of resveratrol or its metabolites, cells were pretreated with resveratrol or metabolites at the dose indicated, for 1 h prior to the addition of cytokines. Control and treated cells were harvested and changes in specific gene expression were evaluated by enzyme-linked immunosorbent assay (ELISA), RT-PCR and Western blot analysis.

RT-PCR analysis and determination of gene-specific mRNA expression

Total RNA was extracted from HPAEC using the TriZol reagent (Invitrogen, Carlsbad, USA). Isolated RNA (0.5μg) was reverse transcribed (RT) with one-step RT-polymerase chain reaction (PCR) kit (Promega Corp., Madison, WI, USA). The PCR primer sequences used were: eotaxin-1, forward 5'-CTC CAA CAT GAA GGT CTC C-3', reverse 5'-CAT GCC CTT TGG ACT GA-3'; GAPDH, forward 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3', reverse 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'. The expression of GAPDH was used as a control for normalizing mRNA expression results. PCR condition for eotaxin-1 mRNA expres-

sion: denaturation, 95°C, 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. Correspondingly, PCR condition for GAPDH: denaturation at 95°C, 5 min, followed by 28 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

Transient transfection of HPAEC with eotaxin-1 promoter linked to luciferase reporter

Cells were grown to 50-70% confluence in 24-well plates. A transfection solution was prepared by adding 0.5 µg pGL-3 controls or eotaxin-1 promoter plasmid DNA dissolved in 50µl serum-free medium, to lipofectamine-2000 solution prepared by dissolving 2 µl lipofectamine-2000 reagents (Invitrogen, Carlsbad, CA, USA) in 50 µl serum-free medium, and incubating the transfection solution for 20 min at room temperature. Transient transfection experiments were initiated by removing media from HPAEC-containing wells, followed by addition of a mixture containing 400 µl serum-free medium and 100 µl transfection solution, and incubation at 37°C for 5 h in a CO_2 humidified incubator. The transfection mixture was removed from individual wells, replaced with 1 ml serum-containing culture media, and cells were incubated for an additional 48 h before treating for 1 h with resveratrol or piceatannol, and addition of IL-13 and TNF-α, single or combined, and incubation for another 4 h before harvest. The control and cytokine/polyphenol-treated HPAEC was next processed for determination of luciferase using the dual-luciferase reporter assay kit (Promega Corp., Madison, WI, USA). Briefly, the cells were lysed in 50 µl passive lysis buffer provided by the manufacturer, incubated at room temperature for 15 min, and aliquots (20 µl lysate) were added to luminometer tubes, mixed with 50 µl of Luciferase Assay Reagent and readings recorded. Next, 50 µl of Stop & Glo® Reagent was added and a second reading was taken. The luciferase results were normalized using the pRL-CMV (Promega Corp., Madison, WI, USA) activity.

Preparation of cell lysates and Western blot analysis

For immunoblotting experiments, cells were harvested by centrifugation and lysed in ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton® X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol and 10 µl/ml protease inhibitor cocktail). The lysates were centrifuged and the clear supernatants were aliquoted and stored at –70°C, for determination of protein content using Coomassie Protein Assay kit (Pierce, Rockford, IL, USA) and BSA as standard. Control and treated cell extracts containing 10 µg total proteins were electrophoresed on 10% SDS–polyacrylamide gel, separated proteins were transferred to nitrocellulose membranes and membranes were blocked for 1 h with 3% non-fat powder milk dissolved in TBST buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 0.05% Tween-20). The blots were incubated with primary antibodies for 12-15 h, 4˚C and with horseradish peroxidase-conjugated secondary antibodies, diluted in TBST. Various immunoreactive bands were identified by enhanced chemiluminescence (ECL), and the intensity of signals was quantified by densitometry and expressed as a ratio to the expression of actin as loading control.

Determination of secreted eotaxin-1 in culture media

Eotaxin-1 in cell culture media was assayed using ELISA (R&D Systems, Inc., Minneapolis, MN, USA). Briefly, 96-well plates (Maxisorb, Nunc) were coated with capture antibody (100 ml/well) overnight at room temperature, washed with phosphate buffered saline supplement with Tween-20 (PBST), blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature and repeated washing. Aliquots (300 µl) of culture media from control and treated HPAEC were added to the antibody-coated plates, incubated first at room temperature for 2 h, and then with added detection antibody and an additional 2 h at room temperature. Individual wells were next incubated with streptavidin-conjugated horseradish peroxidase and substrate solution, for 30 min at room temperature, in the dark. The reaction was terminated and absorbance at 450 nm was measured using Tecan Sunrise plate reader (Phoenix Research Products, Hayward, CA, USA). Eotaxin-1 levels were determined using a standard curve generated with the reference eotaxin-1 supplied by the manufacturer.

Statistical analysis

Results were presented as means ± SEM and analyzed using the nonparametric t-test. A two-

tailed *p value* < 0.05 was considered statistically significant.

Results

Eotaxin-1 mRNA levels in HPAEC are increased by exposure to IL-13 and TNF-a; the induction is partially inhibited by addition of resveratrol

Eotaxin is initially thought to play a specific role in functioning of eosinophils, however, recent studies show that eotaxin is also secreted by HPAEC and is localized in AS lesions [53-55]. Eotaxin expression reportedly is induced by cytokines in multiple cell types including fibroblasts and airway epithelial cells; by contrast, little information is currently available on regulation of eotaxin in HPAEC, notably in response to proinflammatory cytokines. To investigate the control of eotaxin by cytokines IL-13 and TNF-a, HPAEC cells at 50-60% confluence were serum-starved overnight and treated with increasing dose of IL-13 and TNF-α, alone or in combination for 3 h. Changes in the level of eotaxin-1 mRNA were assayed by RT-PCR and quantified by densitometry. Minimally detectable eotaxin-1 mRNA was evident in untreated HPAEC, while a copious increase in eotaxin-1 mRNA occurred in cells treated with 50-100 ng/ml IL-13 (3- to 11-fold increase), or 10 ng/ml TNF-α (12- to 14-fold increase); maximum induction was observed in cells exposed to 50 ng/ml IL-13 combined with 10 ng/ml TNF-α (~22-fold increase) (**Figure 1A**). Time course analysis of change in eotaxin-1 mRNA showed ample increase at 3 h in IL-13- or TNF-α-exposed HPAEC, followed by a precipitous decline at 6 h of treatment (**Figure 1B**). In cells treated with both cytokines, the eotaxin-1 mRNA increase at 3 h was additive, while the decrease at 6 h observed in single cytokine-treated cells (from 12.3-fold at 3 h to 3.8-fold at 6 h for IL-13, and 15-fold at 3 h to 8.8-fold at 6 h for TNF-a) was largely abolished (elevated ~26-fold at both 3 and 6 h). These results suggest that IL-13 and TNF-a exert complex effects on eotaxin-1 mRNA expression in HPAEC.

Resveratrol reportedly display potent anti-inflammatory activities [56, 57], and accordingly might modulate the proinflammatory cytokine-induction of eotaxin-1 mRNA in HPAEC. To test this hypothesis, HPAEC was pretreated with resveratrol and then stimulated with IL-13 (50 ng/ml) or TNF-α (10 ng/ml), as a single agent or combined. Changes in eotaxin-1 mRNA were determined by RT-PCR. The increase in eotaxin-1 mRNA expression in combined cytokine-exposed cells was inhibited by 18% with pre-treatment by 25 μM resveratrol (**Figure 1C**), suggesting that the red wine polyphenol partially attenuated the induction of eotaxin-1 mRNA expression by the combination of IL-13 and TNF-α.

Eotaxin-1 gene promoter activity is induced by IL-13/TNF-α and modulated by resveratrol

Studies have shown that STAT6 contributes to IL-13-mediated induction of eotaxin-1 expression by binding to its gene-specific DNA responsive elements [30, 31]. Similarly, exposure to TNF-α effects nuclear translocation of NF-κB and its subsequent interaction with NF-κB-responsive genes including the eotaxin-1, at its promoter sequences overlapping with those targeted by the STAT6 [34]. To investigate transcriptional control of eotaxin by IL-13 and TNF-α in HPAEC, transfection experiments were performed using the eotaxin promoter construct (pEotx) containing binding site for STAT6, NF-kB and AP-1 as illustrated in Figure 2A. Moreover, to define region of the promoter responsible for transcriptional activation by IL-13 and TNF-α., HPAEC was transfected with control plasmid pGL3 or reporter plasmids containing eotaxin-1 gene promoter spanning its upstream 1363 nucleotides (denoted pEotx-1363 containing AP-1 plus STAT6/NF-kB binding sites) or alternatively upstream 300 nucleotides (denoted pEotx-300 containing STAT6/NF-kB binding sites), followed by assay of luciferase activity. Results in Figure 2B showed that treatment by the combination of IL-13 (50 ng/ml) and TNF-α (10 ng/ml) greatly increased luciferase activity compared to control, being maximally observed using pEotx-300 and to a lesser degree also with pEotx-1363 (**Figure 2B**). The addition of 25 μM resveratrol significantly inhibited the 1363-bp eotaxin-1 gene promoter activity (by >60%) and also reduced the 300-bp eotaxin-1 gene promoter-driven effects (by ~35%) (**Figure 2B**). The promoter analysis results suggest that the increase in eotaxin-1 mRNA levels by the combined chemokines was in part mediated by transcriptional control targeting the eotaxin-1 gene promoter, an effect that was substantially modulated by resveratrol.

Role of transcription factors on eotaxin-1 mRNA expression induced by IL-13 and TNF-α and

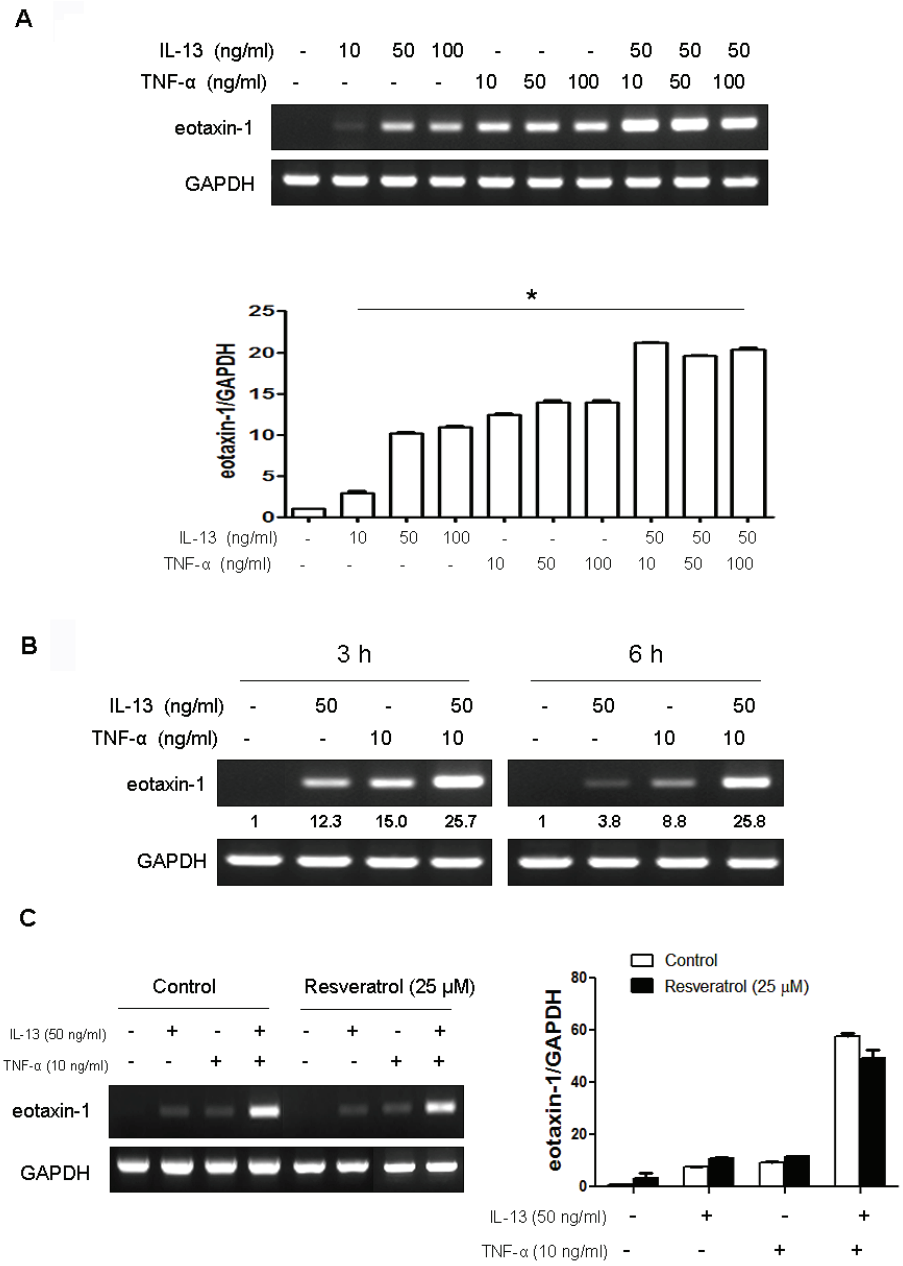

Figure 1 Control of eotaxin-1 expression in HPAEC. (A) Dose-dependent effect of IL-13 and TNF-α on the induction of eotaxin-1 expression. Cultured HPAEC were treated with different concentrations of IL-13 and TNF-α, individually or combined. Total RNA was isolated from control and treated HPAEC at 3 h following stimulation and eotaxin-1 mRNA was assayed by RT-PCR. The PCR products were separated on agarose gels according to size and visualized by ethidium bromide staining (top panel). Eotaxin-1 mRNA levels following treatments were quantified and presented as fold differences against control shown in the bottom panel. Asterisks (*) indicate statistically significant difference between cytokine-treated groups compared with vehicle controls. (B) Time-dependent effect of IL-13 and TNF-α on the expression of eotaxin-1 mRNA. Following treatment, control and treated HPAEC cells were harvest at 3 or 6 h, the changes on eotaxin-1 expression was assayed by RT-PCR. Densitometric data presented under the respective eotaxin -1 PCR signals were normalized against the value of GAPDH for each treatment condition; a value of 1.0 was set as the baseline for normalized eotaxin-1 level in control cells. (C) Modulation of IL-13 and TNF-α induced eotaxin-1 expression by resveratrol. HPAEC was pretreated by 25 μM resveratrol for 1 h and stimulated with IL-13 (50 ng/ml), TNF-α (10 ng/ml), alone or in combination, for 3 h. Eotaxin-1 mRNA levels were assayed by RT-PCR, quantified by densitometry, and expressed as fold differences by normalization against GAPDH.

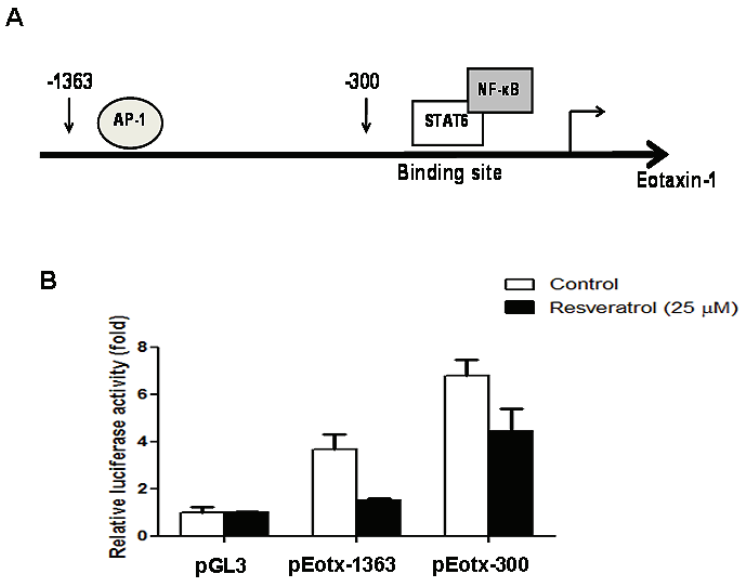

Figure 2 Control of eotaxin promoter. (A) Eotaxin-1 promoter construct and binding site for putative response elements in this promoter. (B) Effect of resveratrol on the induction of eotaxin-1 gene promoter activity in response to treatment by IL-13/TNF-α. HPAEC cells were transfected with control plasmid (pGL3) or plasmids harboring the eotaxin-1 promoter-luciferase reporter (pEotx-1363, 300). After transient transfection as described in Materials and Methods followed by an additional 48 h culture, the cells were pretreated with resveratrol for 1 h and stimulated with IL-13 (50 ng/ml) and TNF-α (10 ng/ml) for 4 h. The luciferase activity was normalized to pRL-CMV activity and calculated as a fold against control. Values are expressed as mean±SEM for three experiments.

control by resveratol

Previous studies showed that STAT6 and NF-κB participated in transcription control of eotaxin-1 in cultured human fibroblasts and epithelial cells by proinflammatory cytokines [30, 34]. To determine whether these transcription factors also played a role in IL-13 and TNF-α-induced eotaxin-1 expression in HPAEC, time-dependent changes on STAT6 and NF-κB protein expression were assayed by immunoblot analysis. Combined cytokines caused a 4- to 6-fold increase in Y641-phosphorylated STAT6 at 10 min which remained variably elevated throughout the 60 min experiment; by contrast, total STAT6 and NF-κB subunits were largely unchanged while JAK-1 acting upstream of STAT6 phosphorylation actually steadily declined (**Figure 3A**). Since the eotaxin-1 gene promoter spanning the upstream 1363 nucleotides also contained sequences targeted by AP-1, under the control of ERK and JNK, immunoblot analysis was also performed on changes in phosphorylated ERK and JNK, which showed marked increases as early as 10 min, were sustained at 20 min and subsequently declined in combined cytokine-treated HPAEC (**Figure 3A**).

Since the eotaxin-1 gene promoter activity was effectively attenuated by resveratrol (**Figure 2**), we tested whether resveratrol also affected protein expression of STAT6 and NF-κB subunits. Figure 3B showed that increase in phosphorylated STAT6/STAT6 ratio elicited by 50 ng/ml IL-13 (arbitrary value of 19.95±0.27, compared to 1.0 in untreated HPAEC) or combined IL-13 and 10 ng/ml TNF-α (32.90±1.07) was ~50% inhibited by 50 μM resveratrol. JAK-1, an upstream activator of STAT6 showing no change in HPAEC treated with IL-13 or TNF-α, alone or combined, was reduced by 40-70% in response to resveratrol in untreated and cytokine-stimulated cells. Moreover, pretreatment by resveratrol lowered NF-κB p65 expression to barely detectable levels, in contrast to the essentially unchanging level of NF-κB p50, in all conditions tested (**Figure 3B**). Addition of 50 μM resveratrol significantly inhibited JNK phosphorylation induced in 10 ng/ml TNF-α or combined cytokine exposed cells, and also blocked ERK phosphorylation in HPAEC exposed to 50 ng/ml IL-13. Interestingly, cells treated with combined IL-13 and TNF-α showed reduced phosphorylation of ERK (**Figure 3B**).

IL-13 and TNF-a induces copious increase in eotaxin-1 secretion, which is effectively inhibited by resveratrol

Time course study was performed on eotaxin-1 release in HPAEC incubated with 50 ng/ml of IL-13, 10 ng/ml of TNF-α, or their combination. Secreted eotaxin-1, barely detectable in the media of unstimulated cells at both 24 and 48 h, was markedly increased in cells stimulated with either cytokine (IL-13: 12.68±0.23 and

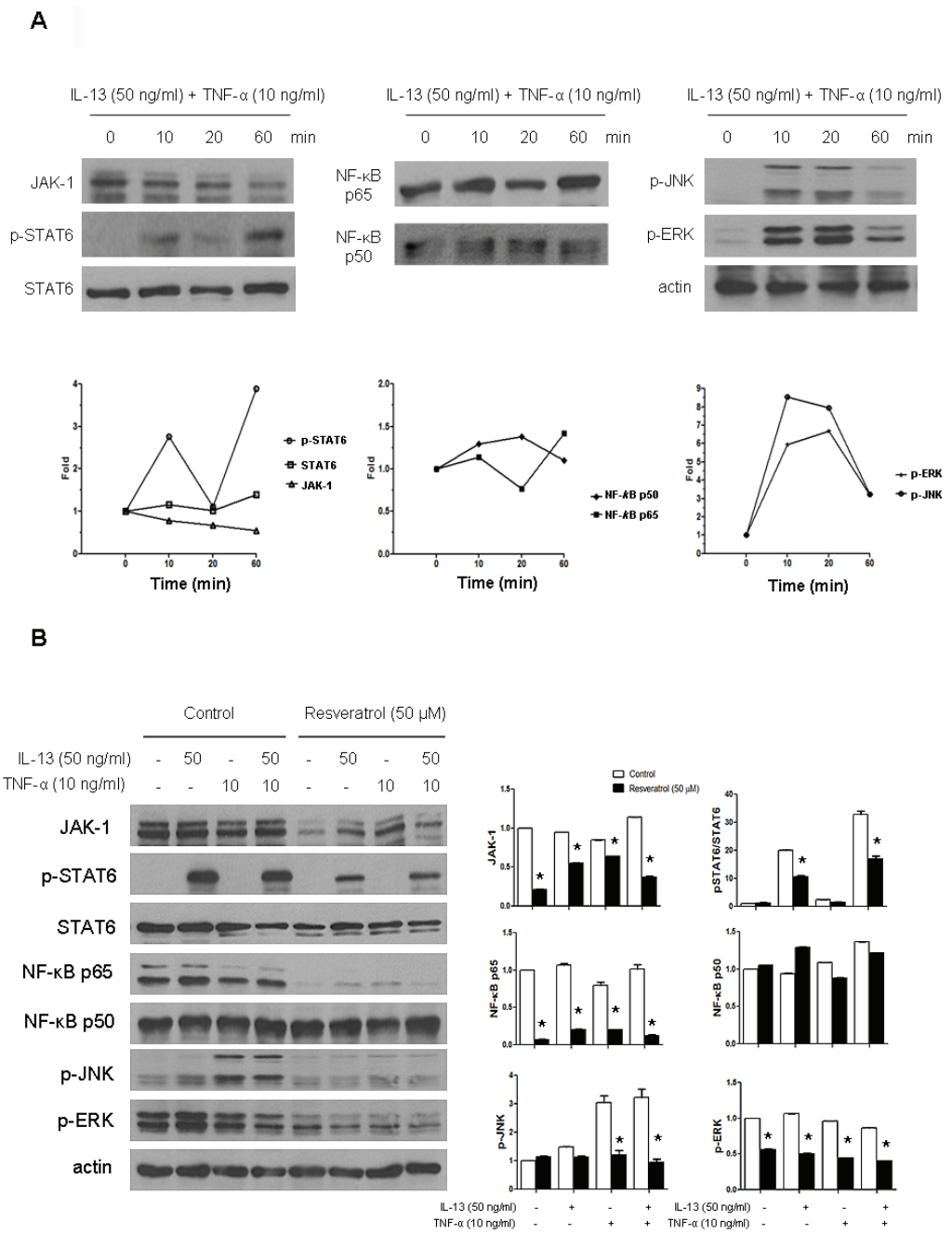

Figure 3 Effects of IL-13 and TNF-α on eotaxin-1 transcriptional control via changes on JAK-1/STAT6, NF-κB and p-JNK/p-ERK expression. (A) Western blot analysis was performed to measure the changes in protein expression of JAK -1, p-STAT6/STAT6 and two subunit of NF-κB as well as p-JNK/p-ERK on cells treated with combined 50 ng/ml IL-13 and 10 ng/ml TNF-α for 0, 10, 20, and 60 min. Intensity of the protein bands from control and treated cells was normalized to actin and the difference in protein expression at each time points were expressed as a fold difference against time 0 (lower panel). (B) Effects of resveratrol on IL-13 and TNF-α induced protein changes were investigated. HPAEC cells were pretreated by resveratrol for 1 h and stimulated with IL-13 (50 ng/ml), TNF-α (10 ng/ml), alone or combined, for 3 h. Changes in protein levels of JAK-1, p-STAT6, STAT6, NF-κB p65, NF-κB p50, p-JNK and p-ERK were determined by western blots. The intensity of the signals corresponding to various proteins analyzed was quantified and expressed as fold differences against actin. Values are expressed as mean±SEM for three experiments. Asterisks (*) indicate statistically significant difference between cells pre-treated with resverartrol followed by exposure to cytokines, alone or in combination compared with cells treated with single or combined cytokines.

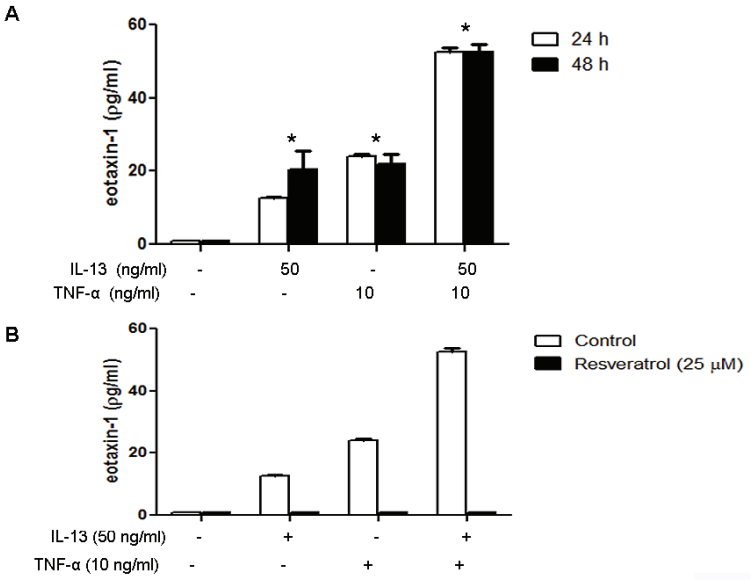

Figure 4 Control of eotaxin-1 secretion in HPAEC. (A) Effect of IL-13 and TNF-α on eotaxin-1 secretion. Cells were treated with IL-13 (50 ng/ml) and TNF-α (10 ng/ml), alone or in combination for 24 and 48 h, and cultured medium were harvested and secreted eotaxin-1 levels from control and treated HPAEC culture media were determined by ELISA. Values are expressed as mean±SEM for three experiments. Asterisks (*) indicate statistically significant difference between 24 h or 48 h cytokine-treated groups compared with vehicle controls at the same time points. (B) Modulation of IL-13 and TNF-α induced eotaxin-1 secretion by resveratrol was investigated. Secreted eotaxin-1 levels were assayed with ELISA. Values are expressed as mean±SEM for three experiments.

20.41±5.23 pg/ml; TNF-α: 24.05±0.68 and 22.00±2.73 pg/ml, at 24 and 48 h, respectively), and synergistically elevated in cells incubated with both cytokines (52.46±1.36 and 52.68±2.05 pg/ml, at 24 and 48 h) (**Figure 4A**). Secreted eotaxin-1, in response to 24 h treatment by 50 ng/ml IL-13 or 10 ng/ml TNF-α, alone or combined, was almost completely abolished by 25 μM resveratrol (**Figure 4B**).

Effects of resveratrol metabolites on IL-13 and TNF-a induced eotaxin-1 mRNA

Little work has been performed on bioefficacy of resveratrol derivatives and/or metabolites, prompting a study of effects of resveratrol metabolites on IL-13 and TNF-a induced eotaxin-1 mRNA in HPAEC. Four resveratrol metabolites: piceid (glycosylated resveratrol), 3-O- and 4'-O-glucuronidated resveratrol (glucuronidated resveratrol) and piceatannol (hydroxylated resveratrol) shown in Figure 5A were tested. Cells were pretreated with resveratrol metabolites followed by stimulation with combined IL-13 (50 ng/ml) and TNF-α (10 ng/ml) and changes in eotaxin-1 mRNA expression were assayed by RT-PCR. The marked increase in eotaxin-1 mRNA level in combined cytokine-stimulated cells was inhibited by 25 μM piceatannol (>20%); less reduction (~10%) resulted from exposure to piceid or 3-O- and 4'-O-glucuronidated resveratrol (**Figure 5B**).

Resveratrol metabolite piceatannol attenuates IL-13/TNF-α induced eotaxin-1 gene promoter activity and eotaxin-1 protein secretion in HPAEC

Since piceatannol inhibited IL-13/TNF-α induced eotaxin-1 mRNA expression, we examined its effects on transcriptional control of eotaxin by IL-13 and TNF-α in HPAEC using reporter assays. Cells were transfected cells with plasmids containing a pEotx-1363, pEotx-300 or vector pGL3. Treatment by combined IL-13 (50 ng/ml) and TNF-α (10 ng/ml) greatly increased luciferase activity; addition of 25 μM piceatannol inhibited the pEotx-1363 and pEotx-300 eotaxin-1 gene promoter activity by ~75% and ~15% (**Figure 6A**), and almost completely inhibited secreted eotaxin-1 into culture media, in response to 50 ng/ml IL-13 or 10 ng/ml TNF-α, alone or combined (**Figure 6B**).

Discussion

In this study, we have examined eotaxin-1 gene expression in cultured HPAEC in response to stimulation by proinflammatory cytokines TNF-α and IL-13. Using RT-PCR analysis we show that the eotaxin-1 mRNA is rapidly and dynamically induced upon stimulation by TNF-α and IL-13 and that this increase is associated with time-dependent and sustained secretion of eotaxin-1 into the culture media. We also show that the

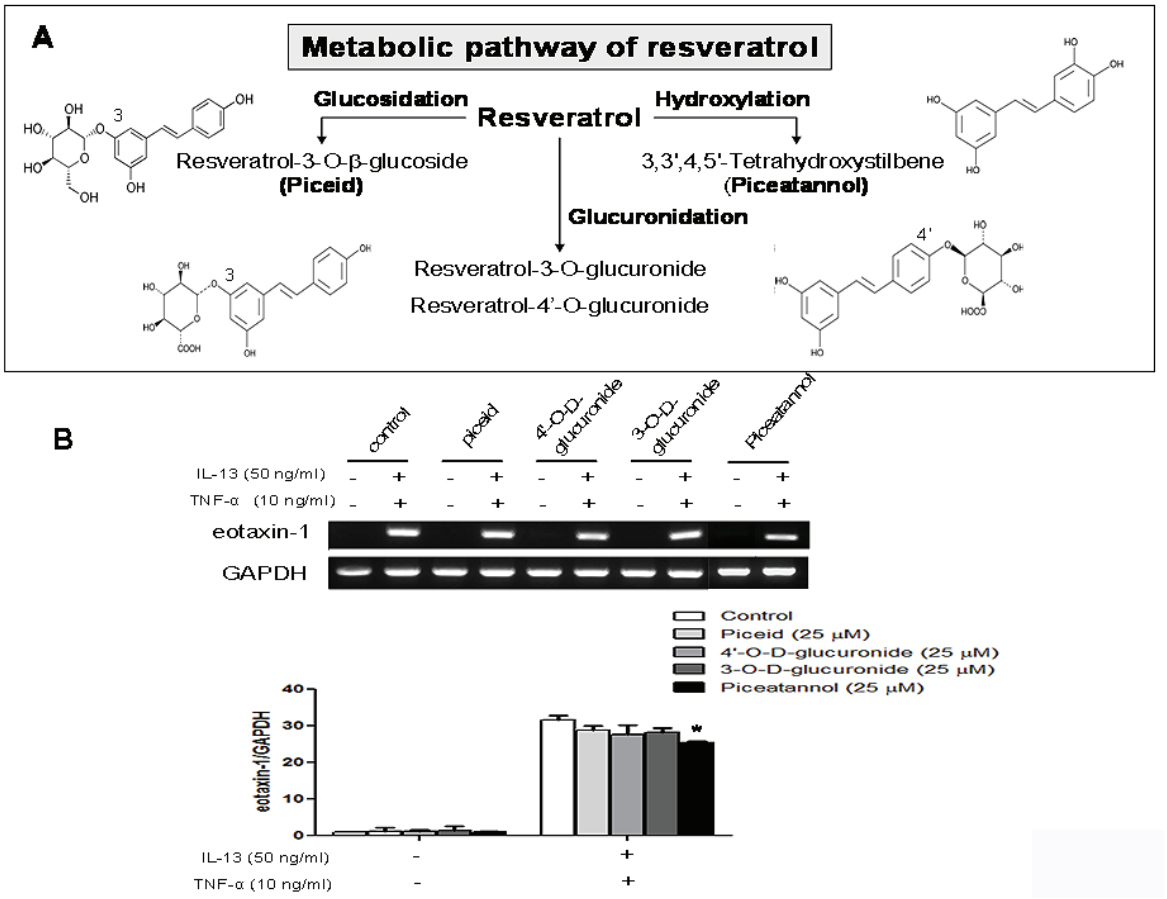

Figure 5 Effect of the metabolites of resveratrol on IL-13 and TNF-α induced eotaxin-1 expression. (A) List of resveratrol metabolites used in this study including piceid, 4'-O-D-glucuronide, 3-O-D-glucuronide and piceatannol. (B) HPAEC cells were pretreated with 25 μM piceid, 4'-O-D-glucuronide, 3-O-D-glucuronide or piceatannol for 1 h and stimulated with IL-13 (50 ng/ml) and TNF-α (10 ng/ml) for 3 h. Changes in mRNA of eotaxin-1 were assayed by RT-PCR and quantified by densitometry and expressed as a fold difference against GAPDH (lower panel). Values are expressed as mean±SEM for three experiments. Asterisks (*) indicate statistically significant difference between cells pre-treated with piceatannol followed by exposure to both IL-13 and TNF-a compared with cells treated with both cytokines.

cytokine-mediated up-regulation of eotaxin-1 gene expression is effectively modulated by red wine polyphenol, resveratrol and its metabolite piceatannol. These findings are significant since evidence points to eotaxin-1, in addition to its established role as an eosinophil-specific chemo-attractant, also plays a role in the cardiovascular system and AS. Notably, increase in eotaxin levels correlates with human AS plaques and endothelial inflammation [54, 58]; eotaxin levels in circulation are elevated in patients with CHD [25, 59, 60]; eotaxin gene polymorphism shows an association with an increased risk for myocardial infarction [61] and with vascular smooth muscle cell proliferation and migration [62]; vascular smooth muscle cells in human atheroma exhibit copious expression of eotaxin suggesting involvement in AS progression [54]. However, molecular mechanisms underlying eotaxin gene expression by endothelial cells, in response to proinflammatory cytokines remain incompletely understood.

As has been previously reported that cytokines, such as TNF-α and IL-4 increase eotaxin expression in vascular endothelial cells [63], fibroblasts [64, 65] and bronchial epithelial cells [63, 66], our studies show that TNF-α and IL-13 induce the eotaxin-1 mRNA expression in HPAEC accompanied by increased secretion of

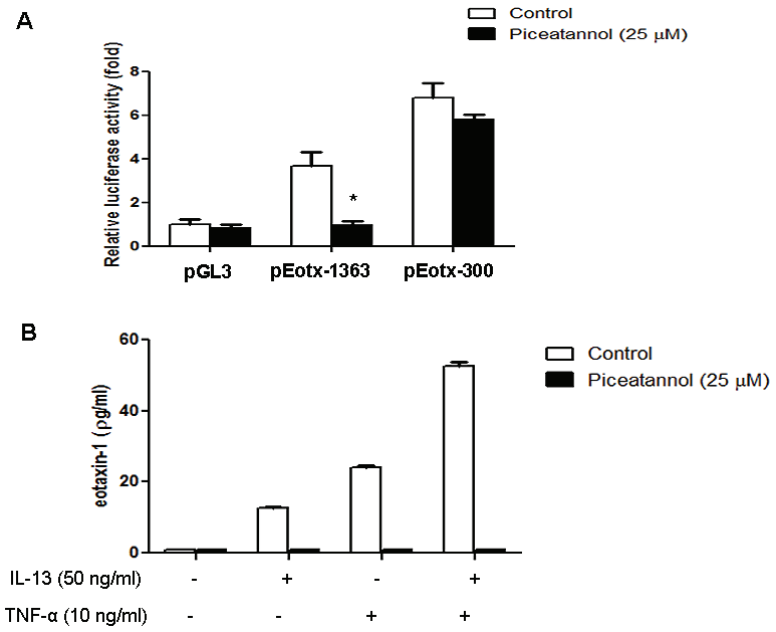

Figure 6 Effect of piceatannol on IL-13 and TNF-α induced eotaxin promoter activity and eotaxin-1 secretion. (A) Effect of piceatannol on the induction of eotaxin-1 gene promoter activity in response to treatment by IL-13/TNF-α. HPAEC cells were transfected with control plasmid (pGL3) or plasmids harboring the eotaxin-1 promoter-luciferase reporter (pEotx-1363, 300). After transient transfection as described in Materials and Methods followed by an additional 48 h culture, the cells were pretreated with piceatannol for 1 h and stimulated with IL-13 (50 ng/ml) and TNF-α (10 ng/ml) for 4 h. The luciferase activity was normalized to pRL-CMV activity and calculated as a fold against control. Values are expressed as mean±SEM for three experiments. Asterisks (*) indicate statistically significant difference between cells pre-treated with piceatannol followed by exposure to both IL-13 and TNF-a compared with cells treated with both cytokines. (B) Modulation of IL-13 and TNF-α induced eotaxin-1 secretion by piceatannol. Cells were treated with IL-13 (50 ng/ml) and TNF-α (10 ng/ml), alone or in combination for 24 h, and cultured medium were harvested and secreted eotaxin-1 levels from control and treated HPAEC culture media were determined by ELISA. Values are expressed as mean±SEM for three experiments.

eotaxin-1 protein into the culture media. TNF-α or IL-13 alone induced maximum increase in eotaxin-1 mRNA at 3 h, followed by a noticeable decline at 4-6 h; in contrast, additive increase in eotaxin-1 mRNA occurred in HPAEC exposed to both TNF-α and IL-13, and moreover, the added combined cytokines also substantially attenuated the eotaxin-1 mRNA decline in HPAEC challenged with either cytokine alone (**Figure 1**). These results suggest that TNF-α or IL-13 regulates eotaxin-1 mRNA transcriptionally while both cytokines control eotaxin-1 gene expression by transcription and post-transcription mechanisms. Consistent with this possibility, eotaxin-1 gene promoter analysis shows a 6-7-fold increase in luciferase reporter activity (**Figure 2B**), which is substantially less than the 26-fold increase in eotaxin-1 mRNA, in HPAEC exposed to both TNF-α and IL-13 (**Figure 1**). It is also noteworthy that previous studies have shown that half-life of eotaxin-1 mRNA was significantly prolonged with IL-4 or TNF-α treatment [67]. Thus, it may be surmised that a similar mechanism operative in HPAEC sustained synthesis and release of eotaxin-1 protein to the culture at 24-48 h (**Figure 4A**) despite a decrease in its mRNA levels at 6 h compared to 3 h (**Figure 1B**).

The molecular mechanisms on the induction of eotaxin-1 expression by IL-13 and TNF-α were further elucidated by focusing on transcription factors STAT6 and NF-κB. In IL-4 or IL-13 stimulated airway epithelial cells [30], STAT6 phosphorylation was found to be an obligatory step preceding its homodimerization, nuclear translocation and binding to STAT6-mediated promoter elements for transcription of cytokine-responsive genes including eotaxin [30, 31, 68, 69]. Our studies showed that in HPAEC challenged with both cytokines, a time-dependent increase in Y641-STAT6 phosphorylation, elevation in phosphorylated STAT6/STAT6 ratio, and upstream JAK-1 expression was observed (**Figure 3**).

Our studies also showed that proinflammatory cytokine mediated induction of eotaxin-1 mRNA and protein secretion was partially and almost completely inhibited by the red wine polyphenol, resveratrol. Hitherto, little information is available on the effect of red wine polyphenols on the regulation of STAT6 expression. We also found that IL-13 and TNF-α-induced STAT6 acti-

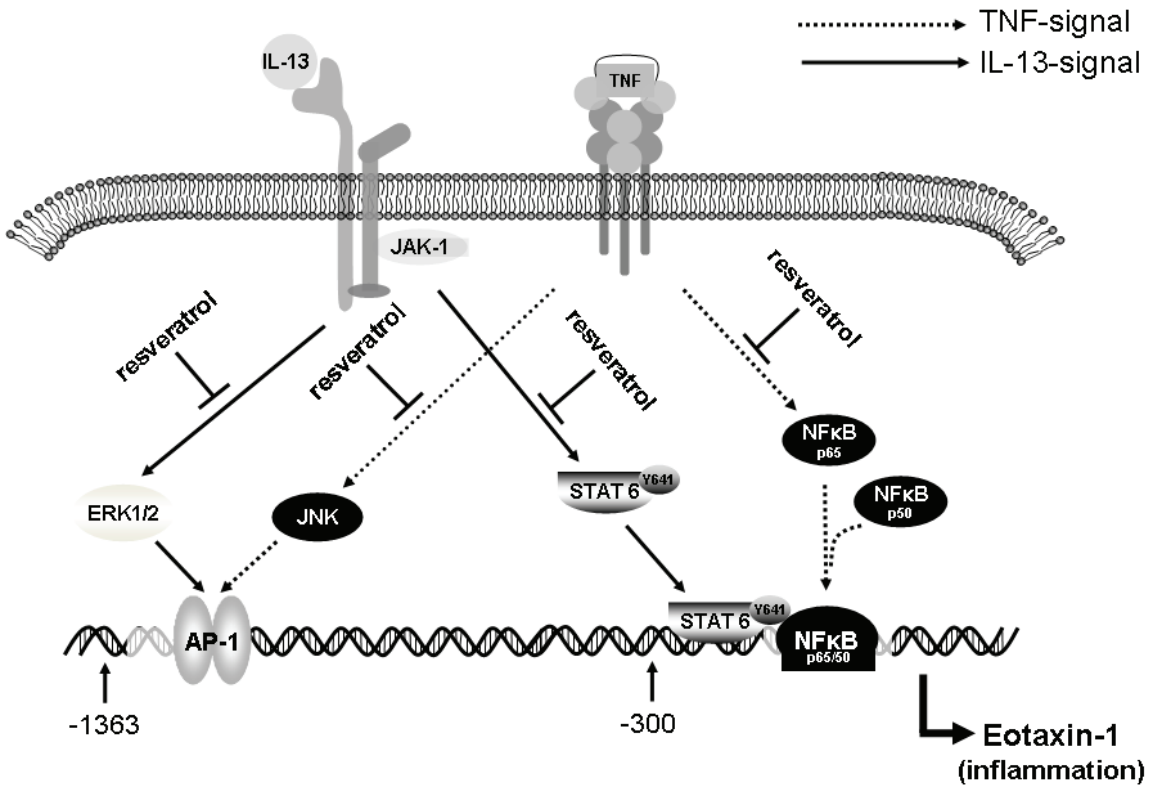

Figure 7 A proposed mechanistic scheme illustrating induction of eotaxin-1 gene transcription by IL-13 and TNF-α and modulation by resveratrol. The proposed mechanism of resveratrol reduced eotaxin-1 promoter activity and expression. The expression of eotaxin-1 induced by IL-13 and TNF-α was suppressed by resveratrol which partially mediated through the inhibition of JAK-1/STAT6 and NF-κB p65 expression and the involvement of AP-1 on the inhibition of directly inhibited eotaxin-1 expression by incompletely defined mechanisms is also considered.

vation presumably via upstream JAK-1 expression resulting in Y641-phosphorylation were significantly decreased by resveratrol (**Figure 3A, B**). Since *in vitro* reporter construct assays have suggested that eotaxin-1 gene promoter contains overlapping elements targeted for binding by NF-κB and STAT6 [34], and because NF-κB also regulates eotaxin-1 expression [65], we analyzed changes in the eotaxin-1 promoter activity and p50 and p65 subunits of NF-κB and showed that the eotaxin-1 promoter activity inducible by IL-13 and TNF-α was reduced by resveratrol (**Figure 2B**) as was induction of NF-κB by IL-13 and TNF-α (**Figure 3B**). Notably, the p65 subunit of NF-κB decreased to below detection levels (**Figure 3B**). These results could explain, at least in part, why the protein expression of eotaxin-1 was almost completely suppressed by treatment with resveratrol, and suggest that both STAT6 and NF-κB targeting the eotaxin-1 gene promoter and driving its transcription were suppressed by resveratrol.

Our studies also attempted to address issues of bioefficacy and biotargeting of resveratrol, in the context of its limited bioavailability and enzymatic conversion to metabolites with largely unknown activities. Since resveratrol has been shown to convert to its glucuronidated and sulfated metabolites in isolated rat intestine studies sections or in human feeding experiments [50, 70-74], and because resveratrol is found in red wine or grape juice mostly as piceid (resveratrol glycosides) [75], we tested effects of piceid, 3-O- and 4'-O-glucuronidated resveratrol and piceatannol on the induction of HPAEC eotaxin-1 by combined IL-13/TNF-α. Only a small reduction in eotaxin-1 mRNA levels by the resveratrol metabolites was observed (**Figure 5**), a notable exception was found in piceatannol

which suppressed the expression and release of eotaxin-1 in HPAEC, induced by IL-13 and TNF-α, as effectively as resveratrol.

In summary, the proinflammatory cytokines TNF -α and IL-13 induce eotaxin-1 mRNA expression and secretion in HPAEC, in coordination with transcriptional control mediated by JAK-1/ STAT6 and NF-κB expression targeting promoter sequences of the eotaxin-1 gene, all of which are effectively suppressed by resveratrol or piceatannol. The mechanistic scheme presented in **Figure 7** provide a framework linking endothelial damage and inflammation with synthesis and release of cytokines and chemokines relevant to AS and CVD and also cardioprotection by red wine polyphenols, resveratrol, piceatannol and resveratrol metabolites.

Acknowledgements

We thank Dr. Robert Schleimer, Professor and Chief of Allergy-Immunology at Northerwestern Feinberg School of Medicine and his colleagues Drs. James and Norton and Satoshi Matsukura for providing us with the plasmids containing eotaxin-1 promoters, respectively, pEotx 1363 and pEotx 300.

Results reported in this communication and studies on cardioprotection by resveratrol in our laboratories over the years were supported in part by: the Intramural Sponsored Research Program of New York Medical College, California Grape Trade Commission, Phillip Morris USA Inc. and Phillip Morris International, to JMW.

The authors have declared no conflict of interest.

Abbreviations: AS, atherosclerosis; CVD, cardiovascular disease; ELISA, enzyme-linked immunosorbent assay; CCL11, CC chemokine ligand 11; STAT6, signal transducer and activators of transcription 6; NF-κB, nuclear factor-kappa B; TNF-α, tumor necrosis factor-alpha; JAK-1, Janus kinase 1; CCR3, chemokine receptor 3; HPAEC, human pulmonary artery endothelial cells; AP-1, transcription factor activator protein 1; STAT3, signal transducer and activators of transcription 3; PBST, phosphate buffered saline supplement with Tween-20; BSA, bovine serum albumin

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