

Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms

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Donnelly, Louise E., Robert Newton, Gina E. Kennedy, Peter S. Fenwick, Rachel H. F. Leung, Kazuhiro Ito, Richard E. K. Russell, and Peter J. Barnes. Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. *Am J Physiol Lung Cell Mol Physiol* 287: L774–L783, 2004. First published June 4, 2004; 10.1152/ajplung.00110.2004.—Resveratrol (3,4',5-trihydroxystilbene) is a polyphenolic stilbene found in the skins of red fruits, including grapes, that may be responsible for some of the health benefits ascribed to consumption of red wine. Resveratrol has been shown to have antioxidant properties and can act as an estrogen agonist. This study examined the anti-inflammatory effects of resveratrol on human airway epithelial cells. Resveratrol and the related molecule quercetin, but not deoxyrhapontin, inhibited IL-8 and granulocyte-macrophage colony-stimulating factor release from A549 cells. Neither the estrogen receptor antagonist tamoxifen nor the glucocorticoid antagonist mifepristone altered the inhibitory effect of resveratrol. The mechanism of resveratrol action was investigated further using luciferase reporter genes stably transfected into A549 cells. Resveratrol and quercetin inhibited NF- κ B-, activator protein-1-, and cAMP response element binding protein-dependent transcription to a greater extent than the glucocorticosteroid dexamethasone. These compounds also had no significant effect on acetylation or deacetylation of core histones. Resveratrol, but not estradiol or *N*-acetyl cysteine, inhibited cytokine-stimulated inducible nitric oxide synthase expression and nitrite production ($IC_{50} = 3.6 \pm 2.9 \mu\text{M}$) in human primary airway epithelial cells. Resveratrol also inhibited granulocyte-macrophage colony-stimulating factor release ($IC_{50} = 0.44 \pm 0.17 \mu\text{M}$), IL-8 release ($IC_{50} = 4.7 \pm 3.3 \mu\text{M}$), and cyclooxygenase-2 expression in these cells. This study demonstrates that resveratrol and quercetin have novel nonsteroidal anti-inflammatory activity that may have applications for the treatment of inflammatory diseases.

inducible nitric oxide synthase; quercetin; red wine

RESVERATROL (3,4',5-trihydroxystilbene) is a polyphenolic compound found in the skins of red fruits (22) (Fig. 1). It is thought that the presence of resveratrol in red wine may, in part, explain the "French paradox" and be responsible for many of the health benefits ascribed to the consumption of red wine (21, 43). Resveratrol has been proposed to exhibit antioxidative (30), antiproliferative (17), and anti-inflammatory properties (47). The anti-inflammatory properties of this molecule have been attributed to the inhibition of cyclooxygenase (COX)-2 transcription (35, 42) and inhibition of COX-1 activity (28) via a peroxidase-mediated mechanism (44). Resveratrol is a molecule with multiple effects on gene transcription, although it is

not an inhibitor of inducible gene transcription per se. Exposure of human umbilical vein endothelial cells to resveratrol or the related molecule quercetin leads to an increase in fibrinolytic protein expression, namely, tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (1). Resveratrol has some structural similarity with estradiol (Fig. 1) and has a weak affinity for the estrogen receptor (20, 33). Resveratrol also has been shown to exhibit antagonism for the aryl hydrocarbon nuclear receptor (6, 8); however, such agonist-antagonist interactions are unlikely to account for all the therapeutic actions of this molecule. More recently, resveratrol has been shown to bind to the peroxisome proliferator-activated receptor (PPAR)- α in murine brain (26), suggesting that resveratrol may be exerting some of its anti-inflammatory capacity via this receptor. Other structurally related plant phenolic compounds, such as quercetin and deoxyrhapontin, may also exhibit some of the properties of resveratrol (Fig. 1). Indeed, the related stilbene quercetin exhibits antioxidant and antiproliferative capacities similar to those of resveratrol (17, 30, 47).

More recently, the anti-inflammatory effects of resveratrol have been associated with inhibition of the transcription factor NF- κ B (34), possibly mediated via the inhibition of I κ B kinase (24). NF- κ B activation is required for the expression of many inflammatory proteins, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8, COX-2, and inducible nitric oxide synthase (iNOS) (4, 39). Therefore, inhibition of NF- κ B could reduce the expression of inflammatory genes and is one mechanism by which the anti-inflammatory agents glucocorticosteroids might elicit their anti-inflammatory effects (38). A second transcription factor, activator protein-1 (AP-1), may also be inhibited by resveratrol (34). NF- κ B and AP-1 may be important in the regulation of many genes that are induced via oxidative stress (29) and might in part explain some of the antioxidative properties of resveratrol. This suggests that an understanding of the mechanism(s) of action of these compounds may lead to novel anti-inflammatory therapies.

The mainstays of anti-inflammatory therapy in inflammatory airway diseases, including asthma, are the glucocorticosteroids. However, there is a small but significant group of asthmatic patients for whom glucocorticosteroids are not effective: either high doses are needed that have systemic side effects, or very rarely patients are completely resistant (31). In contrast, glucocorticosteroids are ineffective at suppressing the

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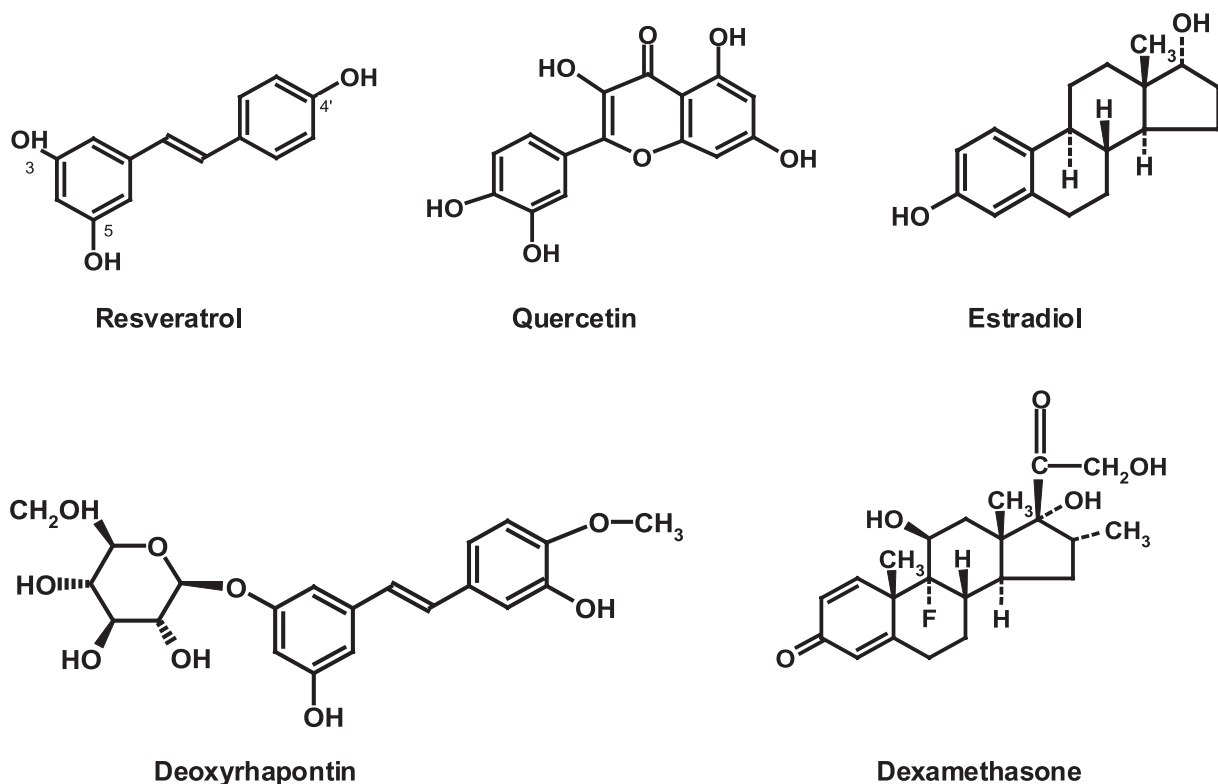


Fig. 1. Structure of resveratrol and related compounds.

airway inflammation of chronic obstructive pulmonary disease (COPD) (11). Recently, we demonstrated that resveratrol inhibited IL-8 and GM-CSF release by alveolar macrophages from patients with COPD (12) under conditions where steroids are largely ineffective (13). Therefore, this study aimed to investigate the mechanism of action of plant-derived polyphenolic compounds, such as resveratrol and quercetin, by examining inflammatory mediator release by human lung epithelial cells.

MATERIALS AND METHODS

Materials

A549 cell lines were obtained from the American Type Culture Collection. DMEM, Ham's F-12 nutrient medium, HBSS, and G-418 were purchased from GIBCO (Paisley, Scotland); 2,3-diaminonaphthalene from Alexis (Nottingham, UK); IL-1 β , TNF- α , IFN- γ , and IL-8 ELISA kits from R & D Systems Europe (Abingdon, Oxon, UK); anti-GM-CSF antibodies from PharMingen (San Diego, CA); avian myeloblastosis virus reverse transcriptase, RNAsin, random primers, Tfx50 reagent, restriction enzymes, and pGL.neo.TATA from Promega (Southampton, UK); pMC1neoPolyA from Stratagene (La Jolla, CA); Taq polymerase, dNTPs, and KCl buffer from Bioline (London, UK); RNA extraction kit from Qiagen (Crawley, UK); and WY-14643 from Tocris Avonmouth (Avon, UK). Resveratrol was a kind gift from Pharmascience (Montreal, PQ, Canada). GW-466471 was a kind gift from GlaxoSmithKline (Stevenage, Herts, UK). Sigma Chemical (Poole, UK) or BDH (Poole, UK) supplied all other reagents.

Methods

Isolation and culture of human primary epithelial cells. Human primary epithelial cells were obtained from explant cultures of tissue obtained from normal human lung transplant donors as described

previously (15). Cells were grown to 90% confluency and cultured in serum-free medium for 24 h before initiation of experiments. All experiments were performed under serum-free conditions. Ethical permission for this study was obtained from the Ethics Committee of the Royal Brompton Hospital.

Culture of A549 cell lines. A549 cells were cultured in DMEM containing 10% (vol/vol) FCS and 2 mM glutamine. Cells were maintained in a humidified incubator at 37°C and 95% (vol/vol) air-5% (vol/vol) CO₂. The cells were provided with fresh medium every 2–3 days. The cells were grown to 90% confluency and cultured in serum-free medium for 24 h before initiation of experiments. All experiments were performed under serum-free conditions.

Measurement of cell viability. Cells were treated with 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in HBSS for 30 min at 37°C. The MTT solution was removed from the surface of the cells, and DMSO was added. The absorbance of the resultant solution was measured at 550 nm, and treated cells were compared with control cells.

Measurement of nitrite and nitrate in cell culture medium. The amount of nitrite and nitrate in cell culture medium was measured using a modification of the method of Misko et al. (37) as described previously (15).

Measurement of GM-CSF and IL-8 in cell culture medium. GM-CSF was measured by sandwich ELISA, as described previously, with a detection limit of 32 pg/ml (15). IL-8 was measured using a commercially available ELISA kit (R & D Systems), according to the manufacturer's instructions, with a detection limit of 32 pg/ml.

Measurement of PGE₂ release from A549 cells. PGE₂ release was measured using RIA as described previously (7).

Measurement of histone deacetylation and histone acetylation activity. Histone deacetylation activity and histone acetylation activity were measured in A549 cells as described previously by Ito et al. (27).

Generation of stable A549 reporter plasmids. A549 cells harboring stable transfectants of the NF- κ B-dependent reporter 6 κ Btkluc.neo

have been described previously (3). An identical protocol was used to generate stable transfectants containing six cAMP regulatory elements (CRE) or six AP-1 sites upstream of a minimal rabbit β -globin promoter driving a luciferase gene (23). The glucocorticoid regulatory element (GRE) reporter was constructed from pGL3basic.neo. This vector was generated by the insertion of a ~1.1-kb *XhoI/SalI* fragment containing the neomycin gene and polyadenylation signals from pMC1neoPolyA into the *SalI* site downstream of and in the same orientation as the luciferase gene in pGL3basic. A modified minimal rabbit β -globin promoter (-42/+3) flanked by *HindIII* sites and containing a C \rightarrow T mutation at position -31 to generate a consensus TATA box was inserted into the *HindIII* site of pGL3.neo to generate pGL3.neo.TATA. This vector was then opened at the *SmaI* site, and a double-stranded oligonucleotide, 5'-GCT GTA CAG GAT GTT CTA GGC TGT ACA GGA TGT TCT AG-3', containing two consensus GRE sites (underlined) was inserted to create pGL3.neo.TATA.2GRE. Stable recombinants in A549 cells were generated as described previously (7). In all cases, before treatment, cells were incubated overnight in serum-free medium in the absence of G-418 selection.

Measurement of luciferase activity. Luciferase activity was measured using the luciferase assay system (Promega). The cell lysates were resuspended in 200 μ l of cell lysis buffer, and 40 μ l of the resulting lysate were used to measure luciferase activity in the presence of 40 μ l of luciferase assay reagent. Emitted light was measured using a luminometer (Turner Designs model TD-20/20, Steptech Instruments, Stevenage, UK).

RNA isolation. RNA was isolated from primary epithelial cells using the Qiagen RNeasy mini kit according to the manufacturer's instructions.

RT-PCR. Reverse transcription was performed on 0.5 μ g of RNA. RNA was heated to 70°C for 5 min and then mixed with 0.01 μ g/ μ l random primers, 1.0 mM dNTP, 1 U/ μ l RNasin, and 0.25 U/ μ l avian myeloblastosis virus reverse transcriptase in 1 \times reverse transcriptase buffer and incubated at 42°C for 1 h and then denatured at 90°C for 4 min. The resultant cDNA was diluted by the addition of 80 μ l of water.

For PCR, 5 μ l of cDNA were incubated in a final volume of 25 μ l containing 1 \times KCl buffer, 2 mM dNTP, 5 ng/ μ l specific primers, and 0.02 u/ μ l *Taq* polymerase. PCR for iNOS, COX-2, and GAPDH was performed as reported previously (16, 40).

Specific primers for tPA gave a specific product of 531 bp: 5'-CCCTGGCAGGCTGCCATCTTT-3' (forward primer) and 5'-GTGTCTCCAGCACACAGCATG-3' (reverse primer). The cycles were as follows: 94°C for 45 s, 56°C for 45 s, and 72°C for 60 s for 32 cycles followed by 72°C for 10 min.

PCR products were identified on 2% (wt/vol) agarose gels. Samples that did not contain reverse transcriptase were used as negative controls.

RESULTS

Effect of Resveratrol and Related Compounds on Inflammatory Mediator Release From A549 Cells

To investigate the mechanism of resveratrol inhibition of inflammatory mediator release, the effect of this drug and the related compounds quercetin and deoxyrhapontin was examined using A549 cells. IL-1 β (1 ng/ml) stimulated these cells to release GM-CSF: 35.7 \pm 1.47 and 741 \pm 270.5 pg/ml in basal and stimulated conditions, respectively (n = 9). Resveratrol inhibited the release of GM-CSF from IL-1 β -stimulated A549 cells by ~75%, with an IC₅₀ of 24.3 \pm 5.5 μ M (n = 9; Fig. 2A). Quercetin also inhibited GM-CSF release from these cells, with less efficacy than resveratrol but with similar potency (~50%), with an IC₅₀ of 21.6 \pm 12.3 μ M (n = 8; Fig. 2B). However, deoxyrhapontin failed to inhibit GM-CSF release from A549 cells (Fig. 2C). Resveratrol at 100 μ M could inhibit IL-8 release from A549 cells that had been stimulated with 1 ng/ml IL-1 β (undetectable and 15.4 \pm 3.8 ng/ml in basal and stimulated conditions, respectively, n = 6) by only ~40% (IC₅₀ = 72 \pm 11 μ M, n = 6; Fig. 3A). Quercetin inhibited IL-8 release from A549 cells to a similar level, with an EC₅₀ of 48 \pm 24 μ M (n = 6; Fig. 3B), whereas deoxyrhapontin had no inhibitory effect (Fig. 3C). Cell viability as determined by MTT assays was unaffected by any of these treatments.

Effect of Estrogens

To investigate whether resveratrol-mediated inhibition of inflammatory mediator release from human airway epithelial cells was via the estrogen receptor, the effects of the estrogen antagonist tamoxifen on resveratrol-mediated inhibition were examined. Tamoxifen at 1–20 μ M has been demonstrated by other workers (9) to affect arachidonic acid release from A549 cells. Tamoxifen (10 μ M) failed to antagonize the resveratrol-mediated inhibition of GM-CSF or IL-8 release from IL-1 β -stimulated A549 cells (Fig. 4, A and B). IL-1 β (1 ng/ml) stimulated these cells to release PGE₂ (0.13 \pm 0.05 and 4.07 \pm

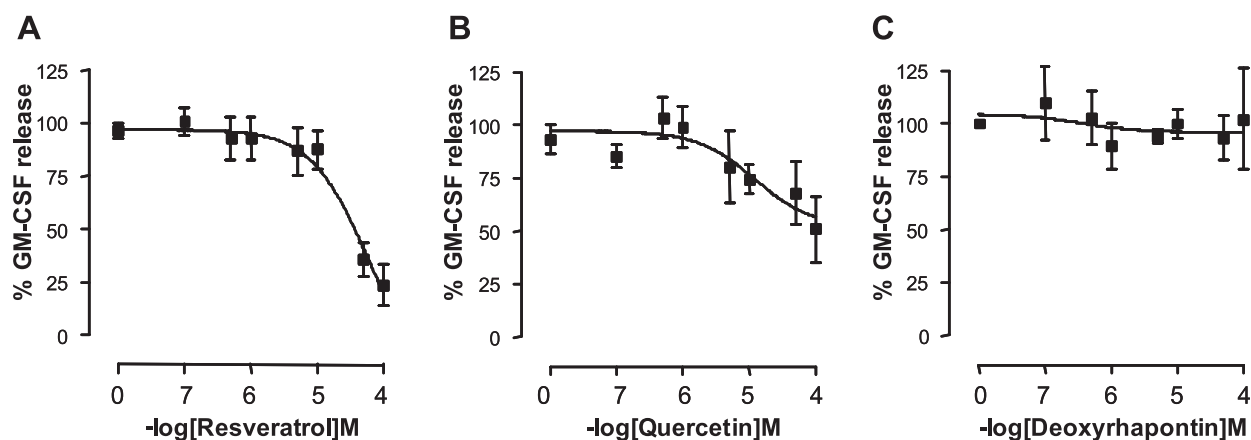


Fig. 2. Effect of resveratrol, quercetin, and deoxyrhapontin on release of granulocyte-macrophage colony-stimulating factor (GM-CSF) from A549 cells. A549 cells were cultured for 24 h in the presence of 1 ng/ml IL-1 β and resveratrol (A), quercetin (B), or deoxyrhapontin (C). Medium was harvested, and GM-CSF was measured. Values are means \pm SE; n = 8–9.

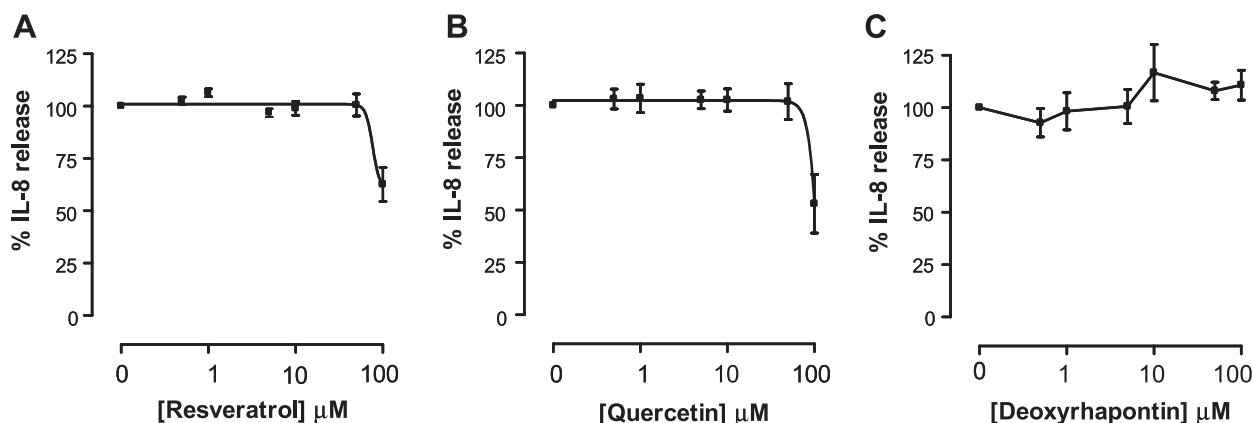


Fig. 3. Effect of resveratrol, quercetin, and deoxyrhapontin on release of IL-8 from A549 cells. A549 cells were cultured for 24 h in the presence of 1 ng/ml IL-1 β and resveratrol (A), quercetin (B), or deoxyrhapontin (C). Medium was harvested, and IL-8 was measured. Values are means \pm SE; $n = 4-8$.

1.26 pg/ml in basal and stimulated conditions, respectively, $n = 4$). Similarly, resveratrol inhibition of IL-1 β -stimulated PGE₂ release (IC₅₀ = 16.1 \pm 3.0 μ M) could not be antagonized by tamoxifen (Fig. 4C). Indeed, tamoxifen further suppressed the release of PGE₂ from these cells. In addition, estradiol was unable to inhibit GM-CSF, IL-8, or PGE₂ release from these cells (Fig. 4), suggesting that the observed inhibitory effects of resveratrol are not mediated via the estrogen receptor.

Effect of Glucocorticoids

Structural similarities between resveratrol and glucocorticosteroids suggested that resveratrol could be acting as a glucocorticosteroid. Mifepristone is a glucocorticosteroid receptor antagonist that exhibits little ability to transactivate glucocorticosteroid receptor-dependent transcription (46) and has a K_i for dexamethasone binding in human airway epithelial cells of ~ 60 nM (32). To investigate the possibility that resveratrol is acting via the glucocorticosteroid receptor, the effect of the glucocorticosteroid antagonist mifepristone (RU-486) was examined. Mifepristone at 10⁻⁸–10⁻⁶ M has been shown to antagonize dexamethasone-mediated repression of COX-2 expression in A549 cells (41). Mifepristone was unable to antag-

onize resveratrol-mediated inhibition of IL-1 β -stimulated IL-8 release from A549 cells (Fig. 5A). This suggested that resveratrol was not exerting its effects via the glucocorticoid receptor. To confirm this observation, A549 cells were stably transfected with a luciferase reporter gene containing 2XGRE sites in front of a luciferase reporter gene. This reporter was activated by dexamethasone (Fig. 5B). By contrast, increasing concentrations of resveratrol failed to activate the luciferase reporter (Fig. 5B). This would suggest that resveratrol is not acting as an agonist for the glucocorticoid receptor.

Effect on Histone Acetylation

To further characterize the mechanism of resveratrol action, its effect on the regulation of gene expression was investigated. One such possible mechanism is the unwinding of DNA from around histone proteins, thereby allowing the binding of transcription factors. This is regulated via acetylation of histones by histone acetylase transferases (HAT). Acetylation of histones alters their conformation, such that DNA unwinds, allowing transcription factors to bind DNA, enabling gene transcription to occur. This regulation can be silenced by the removal of acetyl moieties from histones by histone deacetylases (HDAC). Hence, gene transcription may be regulated through the acti-

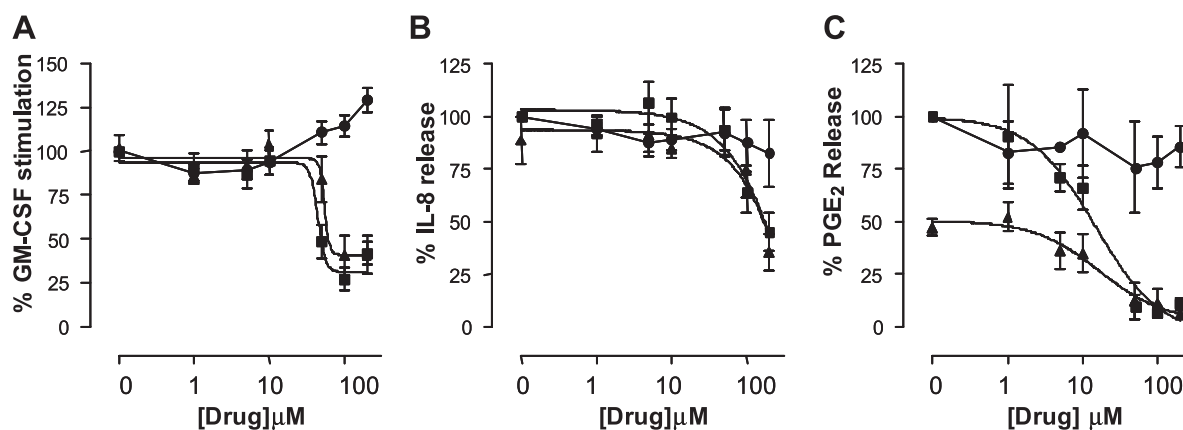
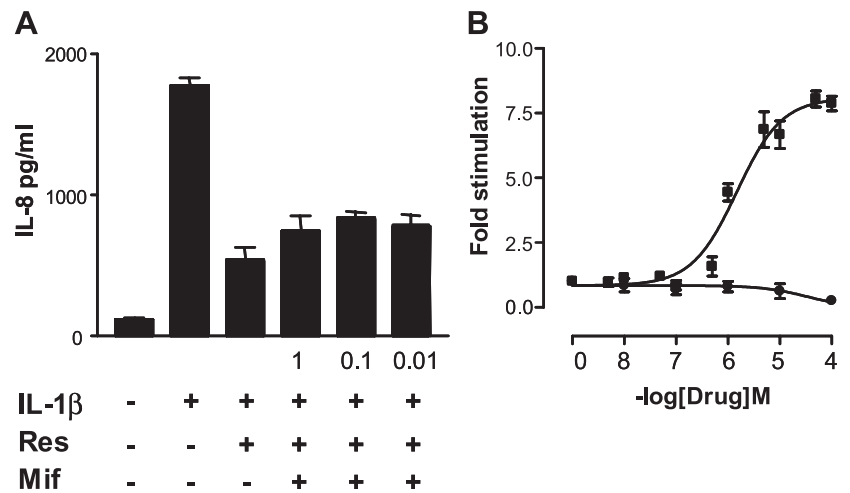


Fig. 4. Effect of estrogens on resveratrol-mediated inhibition of GM-CSF, IL-8, and PGE₂ release from A549 cells. A549 cells were cultured for 24 h in the absence or presence of 1 ng/ml IL-1 β with or without resveratrol (■), estradiol (●), or resveratrol in the presence 10 μ M tamoxifen (▲). Medium was harvested, and GM-CSF and IL-8 release were measured by ELISA and PGE₂ release was measured by RIA. Values are means \pm SE; $n = 4-5$.

Fig. 5. Effect of resveratrol on glucocorticoid activity. A: A549 cells were treated for 24 h in the absence or presence of 1 ng/ml IL-1 β in the absence or presence of 50 μ M resveratrol (Res) with or without 0.01–1 μ M mifepristone (Mif). Medium was harvested, and IL-8 levels were measured by ELISA. B: A549 cells that had been stably transfected with 2XGRE luciferase reporter gene were treated for 6 h with increasing concentrations of dexamethasone (■) or resveratrol (●), cells were harvested, and luciferase activity was measured.



vation and deactivation of these enzymes. Therefore, the effect of resveratrol on the activation of HAT/HDAC in A549 cells was examined. Resveratrol (100 μ M) or quercetin had no effect on HDAC activity (Table 1). Furthermore, there was no effect of resveratrol on HAT activity in these cells (Table 1). These data would suggest that modulation of HDAC/HAT activity was unlikely to be the mechanism by which resveratrol or quercetin was exerting anti-inflammatory effects in these cells.

Effect on Transcription

A luciferase reporter gene containing six κ B sites was stably transfected into A549 cells, and the effect of resveratrol and quercetin on transcription factor activation was investigated. Resveratrol inhibited IL-1 β -induced expression of luciferase in a dose-dependent manner (Fig. 6A), with an IC₅₀ of 41 \pm 10 μ M (n = 7). Moreover, complete inhibition of this reporter gene activity occurred in the presence of 100 μ M resveratrol (Table 2). Quercetin also inhibited IL-1 β -induced expression of luciferase (IC₅₀ = 21 \pm 13 μ M, n = 5; data not shown). Although dexamethasone inhibited expression of the NF- κ B reporter, the maximum inhibition observed was only 41% (IC₅₀ = 10 \pm 6 nM, n = 5; Fig. 6A, Table 2). The effect of these compounds was also examined using an AP-1 luciferase reporter. Cells were stimulated with 10⁻⁷ M PMA in the presence of the test compounds. Again, resveratrol and quercetin inhibited AP-1-dependent luciferase expression [IC₅₀ = 28 \pm 13 μ M (Fig. 6B) and 20 \pm 12 μ M, n = 4–6 (data not shown)]. Dexamethasone inhibited AP-1-dependent luciferase

expression, but again maximum inhibition was less than that seen with resveratrol or quercetin (Fig. 6B, Table 2). Another transcription site implicated in the regulation of inflammatory genes is CRE. Again, a luciferase reporter was generated in A549 cells, and activity was stimulated with IL-1 β . Resveratrol and quercetin inhibited CRE-dependent expression, with IC₅₀ values of 46 \pm 23 μ M (Fig. 6C) and 49 \pm 28 μ M (n = 6; data not shown), respectively. Again dexamethasone inhibited CRE-dependent transcription, but with a maximum inhibition of ~50% in the presence of 100 μ M dexamethasone (Fig. 6C, Table 2). These results would suggest that the plant-derived compounds resveratrol and quercetin inhibit inflammatory gene transcription and, although not as potent as dexamethasone, inhibit transcription factor activity to a greater extent than the steroid used in these studies.

To ensure that the inhibitory effect of resveratrol was not merely occurring through a general inhibition of gene transcription, the effect of resveratrol was examined on a luciferase reporter gene containing a TATA transcription site. Resveratrol failed to inhibit constitutive luciferase expression in this system (data not shown), suggesting that the inhibitory effect of resveratrol on inflammatory gene transcription is not merely a reflection of inhibition of gene transcription per se.

Effect of PPAR- α

The possibility that the anti-inflammatory action of resveratrol could be via activation of the PPAR- α receptor was examined using the PPAR- α agonist WY-14643 and the specific PPAR- α antagonist GW-466471 (48). The PPAR- α agonist WY-14643 was less effective than resveratrol at inhibiting IL-1 β -dependent NF- κ B transcription (EC₅₀ = 57.4 \pm 21.5 μ M, n = 3; Fig. 7A). Moreover, the specific PPAR- α antagonist GW-466471 was unable to reverse the resveratrol-mediated inhibition of IL-1 β -dependent NF- κ B transcription (Fig. 7B). Taken together, these data suggest that resveratrol is not mediating its anti-inflammatory effects in A549 cells via PPAR- α .

Effect of Resveratrol on Inflammatory Mediator Release From Primary Human Airway Epithelial Cells

Human primary epithelial cells were cultured in the presence of 50 ng/ml of IL-1 β , TNF- α , and IFN- γ ("cytomix") in the

Table 1. Effect of resveratrol and quercetin on HDAC and HAT activity in A549 cells

	HDAC Activity, dpm/ μ g protein	HAT Activity, dpm/10 μ g protein
No stimulus	206 \pm 28	18,413 \pm 1,822
IL-1 β (1 ng/ml)	180 \pm 27	27,043 \pm 4,276
IL-1 β + 1 μ M Res	185 \pm 27	33,134 \pm 2,183
IL-1 β + 10 μ M Res	203 \pm 28	26,199 \pm 4,351
IL-1 β + 100 μ M Res	240 \pm 27	24,884 \pm 3,570
IL-1 β + 100 μ M Q	207 \pm 27	26,249 \pm 4,727

Values are means \pm SE; n = 6. HDAC, histone deacetylase transferase; HAT, histone acetyltransferase; Res, resveratrol; Q, quercetin

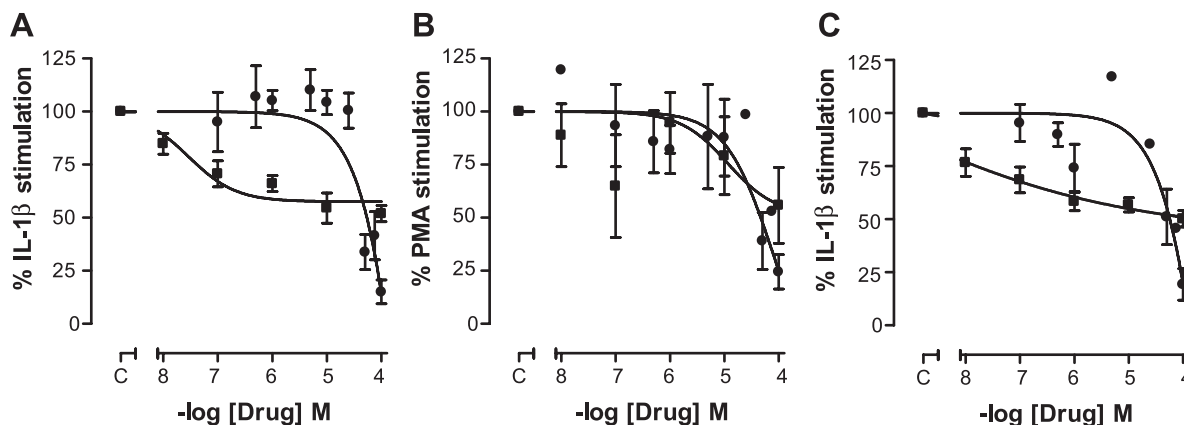


Fig. 6. Effect of resveratrol and dexamethasone on NF- κ B-, AP-1-, and cAMP response element-dependent transcription. A549 cells stably transfected with NF- κ B (A), AP-1 (B), or cAMP response element (C) reporter genes were stimulated with 1 ng/ml IL-1 β or 10⁻⁷ M PMA for 6 h in the presence of increasing concentrations of resveratrol (●) or dexamethasone (■). Cells were harvested, and luciferase activity was measured. Values are means \pm SE; $n = 4-6$.

absence or presence of resveratrol. Cytomix stimulation of these cells at this concentration induces iNOS expression, which can be measured by examining the levels of the NO metabolite nitrite in cell culture medium (16). Resveratrol dose dependently inhibited cytomix-stimulated release of nitrite (Fig. 8A) and nitrate (NO_x; Fig. 8B), with IC₅₀ values of 3.6 \pm 2.9 μ M ($n = 6$) and 0.9 \pm 0.5 μ M ($n = 6$), respectively. Cytomix stimulated release of GM-CSF (30.9 \pm 16.7 and 156.6 \pm 39.8 pg/ml in basal and stimulated conditions, respectively) and release of IL-8 (0.66 \pm 0.17 and 2.01 \pm 0.23 ng/ml in basal and stimulated conditions, respectively) from these cells. Resveratrol also dose dependently inhibited cytomix-stimulated release of GM-CSF and IL-8 from human primary epithelial cells, with IC₅₀ values of 0.44 \pm 0.17 and 4.7 \pm 3.3 μ M, respectively ($n = 5$; Fig. 8, C and D). Cell viability was unchanged in the presence of increasing concentrations of resveratrol (up to 100 μ M) as measured by MTT.

Cytomix exposure of human primary epithelial cells for 4 h led to an induction in iNOS mRNA, and this induction was inhibited by 50 μ M resveratrol (Fig. 9, A and B). In contrast, iNOS mRNA was not altered in the presence of the antioxidant *N*-acetyl cysteine (0.5 mM) or estradiol (50 μ M; Fig. 9, A and B). These concentrations were selected because *N*-acetyl cysteine has been shown to exhibit antioxidant properties on other cell types (14, 30), and if resveratrol was acting as an agonist at the estrogen receptor, the equivalent concentration of estradiol should elicit the same effect. Similarly, the induction of COX-2 mRNA was also reduced by resveratrol, but not by *N*-acetyl cysteine or estradiol (Fig. 9, A and C). This

suggests that the effect of resveratrol cannot be accounted for by its antioxidative or estrogen-like properties. Moreover, resveratrol is not acting as an inhibitor of transcription, because it did not inhibit the basal expression or the twofold induction of tPA with cytomix (Fig. 9, A and D).

DISCUSSION

Resveratrol has been ascribed numerous properties that could be beneficial in the treatment of several chronic inflammatory diseases (19). Many studies have been performed using a variety of cell types from different species to investigate the putative mechanisms of action of this molecule; however, no definitive mechanism to explain how resveratrol can exhibit such diverse activities has been identified. Our study is novel, in that it examines the anti-inflammatory mechanism(s) of resveratrol in cells relevant to human disease and explores all the proposed mechanisms in a single study.

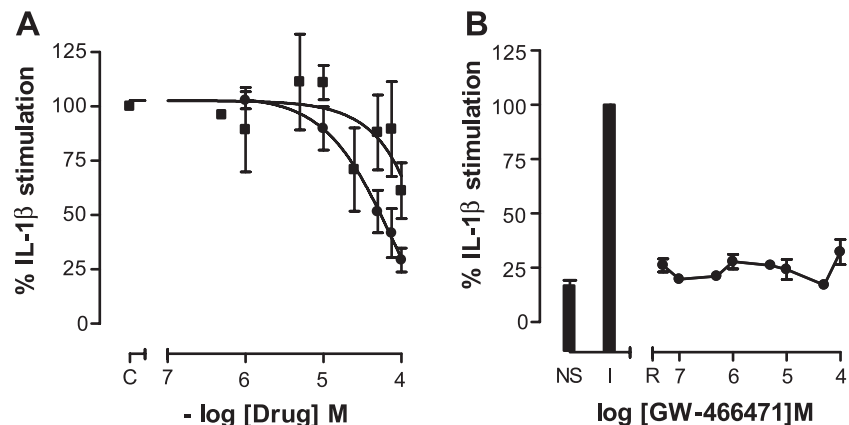
Resveratrol exhibited anti-inflammatory activity in all the systems we examined. Furthermore, it appeared to be more effective, although less potent, than glucocorticoids. Resveratrol inhibited inflammatory mediator release from human airway epithelial cells. Release of inflammatory mediators into the cell culture medium was measured as a surrogate for the production of inflammatory mediators into the lung environment, as observed in inflammatory lung diseases including COPD and asthma. Moreover, resveratrol did not act as an estrogen or glucocorticosteroid. In this study, resveratrol inhibited iNOS and COX-2 gene transcription, together with IL-8 and GM-CSF expression in human primary airway epithelial cells. This study confirms the effects of resveratrol on iNOS expression reported in murine macrophage systems (36, 45). However, this contrasts with the effect reported in rat Kupffer cells, where iNOS transcription was unaffected by up to 100 μ M resveratrol but translation and, hence, activity were reduced (30). In this study, the expression of iNOS protein was not examined directly; however, iNOS activity was also inhibited in human primary airway epithelial cells. There was also no shift in the balance of nitrate and nitrite formation after inhibition via resveratrol. The inhibition of iNOS expression and activity in primary human airway epithelial cells is significant, because steroids are ineffective in this system (16).

Table 2. Effect of resveratrol, quercetin, and dexamethasone on NF- κ B-, TRE-, and CRE-dependent transcription

Reporter	Resveratrol		Quercetin		Dexamethasone	
	EC ₅₀ , μ M	% Inhibition (100 μ M)	EC ₅₀ , μ M	% Inhibition (100 μ M)	EC ₅₀ , nM	% Inhibition (100 μ M)
NF- κ B	41 \pm 10	95 \pm 2	21 \pm 13	97 \pm 0.3	10 \pm 6	41 \pm 3
CRE	46 \pm 23	91 \pm 3	49 \pm 28	69 \pm 22	15 \pm 15	50 \pm 4
TRE	30 \pm 13	89 \pm 6	20 \pm 12	79 \pm 4	7 \pm 6	62 \pm 3

Values are means \pm SE. CRE, CAMP response element; TRE, TPA response element.

Fig. 7. Effect of peroxisome proliferator-activated receptor- α antagonist on resveratrol-mediated inhibition of NF- κ B-dependent transcription. A549 cells stably transfected with NF- κ B reporter genes were stimulated with 1 ng/ml IL-1 β for 6 h in the presence of resveratrol (●) or WY-14643 (■; A) or in the absence (NS) or presence of 1 ng/ml IL- β (I) and 50 μ M resveratrol (R) in the presence of GW-466471 (●; B). Values are means \pm SE; $n = 3$.

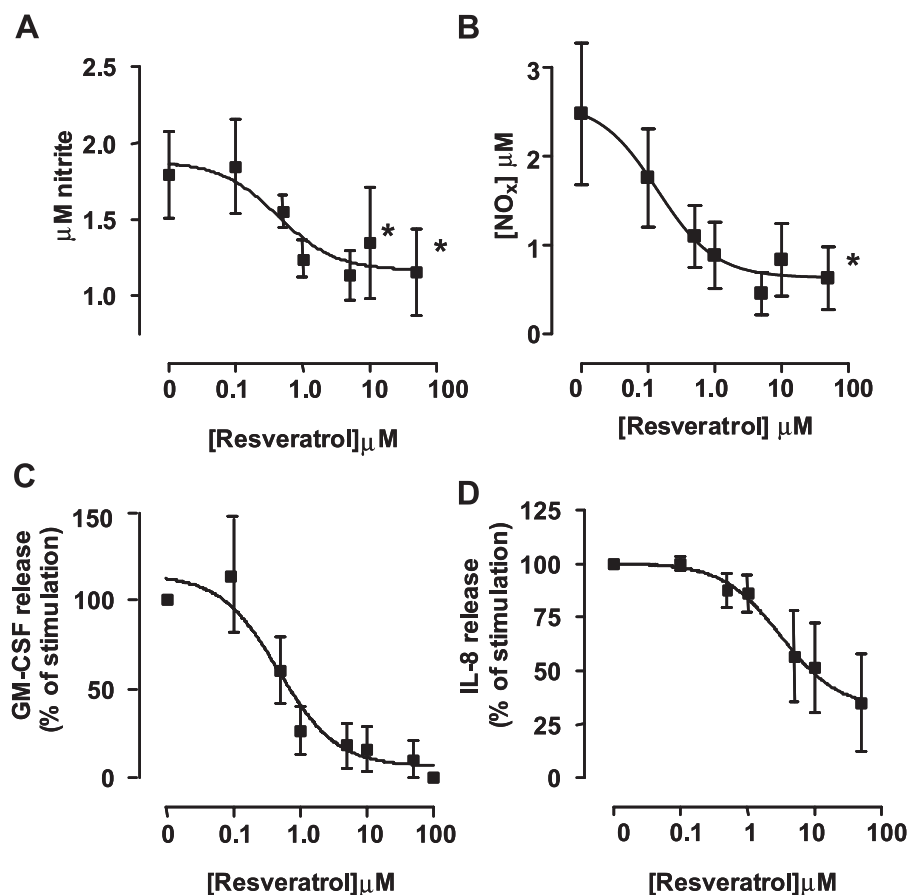


Resveratrol has been shown to inhibit phorbol ester-induced COX-2 transcription in human mammary epithelial cells (42), and this, together with inhibition of NF- κ B activation in the monocytic cell line U937 (34), has been purported to be the mechanism for the anti-inflammatory effects of this compound. This study confirms that COX-2 expression and NF- κ B activation can also be inhibited in human airway epithelial cells by administration of resveratrol; however, this effect of resveratrol is not specific for NF- κ B, inasmuch as resveratrol also inhibited AP-1- and CREB-dependent transcription. Moreover, this is not a general inhibition of inducible gene transcription, because TATA-dependent luciferase activity and expression of tPA in primary cells were unaffected by administration of resveratrol.

There are differences in the efficacy of resveratrol on IL-8 and GM-CSF release from A549 and primary epithelial cells, with inhibition of IL-8 release being less sensitive. There is also a difference in sensitivity between primary epithelial cells and A549 cells, with the cell line being less sensitive to inhibition by resveratrol, despite the use of high concentrations of cytomix required to stimulate the primary cells (16). We previously reported the potency of resveratrol on primary human alveolar macrophages to be less than that observed with A549 cells (12) and nearer to that of primary human airway epithelial cells.

IL-8 and GM-CSF are important in the development of inflammation, because IL-8 is important in the recruitment of

Fig. 8. Effect of resveratrol on inflammatory mediator release from human primary epithelial cells. Human primary epithelial cells were cultured in the absence or presence of cytomix (50 ng/ml) with or without resveratrol for 24 h. Medium was harvested and assayed for nitrite (A), nitrate + nitrite (NO $_x$; B), GM-CSF (C), and IL-8 (D). Values are means \pm SE; $n = 5$ –6 individual subjects. * $P < 0.05$.



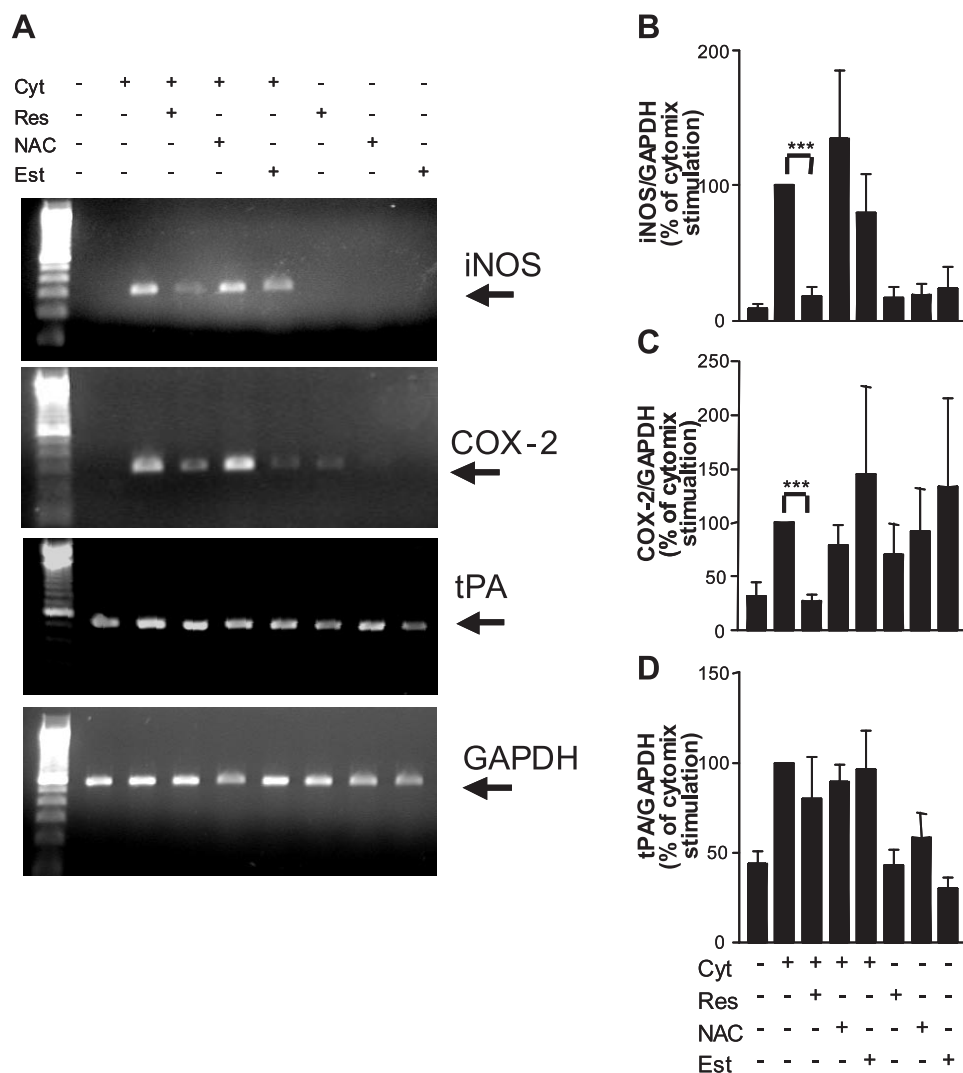


Fig. 9. Effect of resveratrol on inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and tissue plasminogen activator (tPA) transcription in human primary epithelial cells. Cells were cultured for 4 h in the absence (NS) or presence of cytomix (Cyt; 50 ng/ml) with or without 50 μ M resveratrol (Res), 0.5 mM *N*-acetyl cysteine (NAC), or 50 μ M estradiol (Est). RNA was extracted, and RT-PCR was performed for iNOS, COX-2, tPA, and GAPDH. Data are presented as a representative gel (A), iNOS-to-GAPDH ratio (B), COX-2-to-GAPDH ratio (C), and tPA-to-GAPDH ratio (D). Values are means \pm SE ($n = 5$) for data normalized to cytomix-stimulated cells. *** $P < 0.001$.

inflammatory leukocytes, particularly neutrophils, and GM-CSF is a cell survival factor, thus prolonging the resident time of inflammatory cells. The differential inhibitory effects of resveratrol on IL-8 and GM-CSF release further suggest that resveratrol is not simply acting as a general inhibitor of inflammatory mediator release but exhibits some selectivity. The effect of resveratrol and quercetin to inhibit GM-CSF release from A549 cells correlates with their relative efficacies to inhibit NF- κ B reporter activity. However, resveratrol and quercetin were more effective at inhibiting AP-1-dependent transcription. The lack of effect of deoxyrhapontin, which is closely related structurally, to inhibit IL-1 β -stimulated GM-CSF release from A549 cells indicates that there is a strict, structural requirement for this inhibitory activity. Several studies have demonstrated that the hydroxyl moieties at positions 3 and 5 (Fig. 1) are critical for activity of the resveratrol molecule, and this activity is reduced by substitution with a glucosyl group (2, 36).

It would appear that, despite its structure and reported ability to act as a weak estrogen agonist (20), resveratrol is not acting as an estrogen in this system. In the present study, tamoxifen, an estrogen antagonist, did not antagonize the inhibitory effect of resveratrol in A549 cells. Furthermore, estradiol did not

elicit any inhibitory responses in A549 or primary airway epithelial cells. This may relate to the estrogen-independent anti-inflammatory effect of resveratrol reported in MCF-7 breast cancer cells (18). Alternatively, the lack of effect of tamoxifen, an antagonist at estrogen receptor- α and - β , and estradiol in these cells may be due to the reported lack of expression of the estrogen receptor in A549 cells (9, 10); however, type II estrogen binding sites, together with estrogen receptor binding, have been reported in these cells (5). Nevertheless, because estradiol at equivalent concentrations to resveratrol did not attenuate any response in this study, it remains unlikely that the anti-inflammatory effect of resveratrol can be attributed to activation of the estrogen receptor. Similarly, resveratrol is not acting as a glucocorticosteroid, because mifepristone was unable to antagonize resveratrol inhibition of IL-8 release from A549 cells and resveratrol failed to stimulate GRE-dependent luciferase activity. Further evidence suggesting that resveratrol and glucocorticosteroids exert their effects by different mechanisms was demonstrated by the failure of dexamethasone to inhibit the reporter gene assays $>50\%$, whereas resveratrol and quercetin inhibited the responses completely. Resveratrol has also been reported to be more effective than dexamethasone on the adhesion of the monocytic cell line

THP-1 to human umbilical vein endothelial cells via reduced expression of the adhesion molecules ICAM-1 and VCAM-1 on THP-1 cells (2). Recently, resveratrol has been reported to act as an antagonist for the aryl hydrocarbon receptor (6); therefore, the possibility remains that resveratrol could be acting as an agonist/antagonist for other nuclear receptors, the function of which is unknown. More recently, resveratrol has been shown to activate PPAR- α (26); however, the lack of effect of the PPAR- α antagonist to reverse resveratrol-mediated inhibition suggests that it is unlikely that resveratrol is acting via PPAR- α .

A possible mechanism whereby resveratrol and quercetin might be acting as anti-inflammatory agents would be alteration of the HAT-HDAC balance within cells to inhibit inflammatory gene transcription in a manner analogous to the reported effect for glucocorticoids (27). Histone hyperacetylation in Caco-2 human intestinal epithelial cells leads to inhibition of IL-8 release (25). However, resveratrol and quercetin have no effect on the HAT-HDAC balance in A549 cells; therefore, this is an unlikely mechanism for the inhibitory action of their anti-inflammatory effects.

This study demonstrates that plant-derived polyphenolic compounds can act as novel anti-inflammatory agents. Their mechanism of action is not via the estrogen or the glucocorticoid receptor; thus these agents might be beneficial in inflammatory diseases where glucocorticosteroids have proved to be ineffective, such as COPD, steroid-resistant asthma, and arthritis. These compounds may provide candidate molecules for the development of novel anti-inflammatory therapies.

GRANTS

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