Journal of
Cardiology and Vascular Medicine

Research Open Access

Resveratrol or Its Metabolites Modulate TNF-α And IL-13 Mediated Changes In IL-8 and Acidic Mammalian Chitinase In Cultured Human Pulmonary Artery Endothelial Cells

Ching-Jen Yang, Tze-chen Hsieh, Susan C. Olson and Joseph M. Wu*

Department of Biochemistry & Molecular Biology, New York Medical College, Valhalla, New York, U.S.A

***Corresponding author:** Joseph M. Wu, Department of Biochemistry & Molecular Biology, New York Medical College, Valhalla, New York 10595, U.S.A.; Tel: 914-594-4891; Fax: 914-594-4058; Email: Joseph_Wu@nymc.edu

Received Date: July 29, 2013, **Accepted Date:** September 13, 2013, **Published Date:** September 13, 2013

Citation: Joseph M. Wu, et al. (2013) Resveratrol or Its Metabolites Modulate TNF-α And IL-13 Mediated Changes In IL-8 and Acidic Mammalian Chitinase In Cultured Human Pulmonary Artery Endothelial Cells. J Cardio Vasc Med 1: 1-8

Abstract

Chronic inflammation is implicated in the etiology of Cardiovascular Disease (CVD), the leading cause of death among men and women. Accordingly, the discovery of inflammation biomarkers, identification of agents with anti-inflammation properties, and studies of their anti-CVD and cardioprotective mechanisms are of significant interest. Previously, we reported that in cultured Human Pulmonary Artery Endothelial Cells (HPAEC), treatment by grape phytochemicals resveratrol and piceatannol significantly inhibited the induction of expression and release of eotaxin-1 by proinflammatory cytokines IL-13 and TNF-α. In this paper we further tested the effects of resveratrol and its metabolites in controlling inflammatory chemokines IL-8 and AMCase, and on the level and subcellular distribution of transcription factors, NF-κB, STAT3 and STAT6, which are possibly involved in control of IL-8 and AMCase expression. In HPAEC cultures exposed to TNF-α and IL-13, IL-8 mRNA level was dose-dependently suppressed by IL-13 but was copiously up-regulated by TNF-α. AMCase mRNA was decreased in HPAEC treated with high (100 ng/ml) concentration of TNF-α or by the combination of IL-13 and TNF-α. The IL-8 and AMCase changes were correlated with an increase in expression of STAT3 and p50 subunit of NF-κB. Pre-treatment by 25-50 μM resveratrol or its metabolite piceatannol, significantly reduced the induction of IL-8 elicited by combined treatment of IL-13/TNF-α and also suppressed expression and nuclear localization of pSTAT6 and p50/p65 subunits of NF-κB. To investigate the antioxidant properties of resveratrol and its metabolites, changes in phase II detoxification enzyme quinone reductase 1 (NQO1) was determined. Resveratrol-treated HPAEC showed an increase in protein expression of NQO1 with no corresponding change in its mRNA levels; in the mean time, treatment by piceid, or 3-O- and 4'-O-glucuronidated resveratrol inhibited NQO1 mRNA level with no concomitant alteration in its protein expression. On the basis of these results we propose that profile of the expression of IL-8, AMCase, and NQO1 in HPAEC may be used as cell-based screening markers for identifying cardioprotective potential of phytochemicals or their metabolites, with or without challenge by proinflammatory cytokines.

Keywords: IL-8; AMCase; STAT3; STAT6; NF-κB; Resveratrol; Piceatannol; Resveratrol metabolites

Abbreviations: AS: Atherosclerosis; AMCase: Acidic Mammalian Chitinase; BALF: Bronchoalveolar Lavage Fluid; BSA: Bovine Serum Albumin; CVD: Cardiovascular Disease; HPAEC: Human Pulmonary Artery Endothelial Cells; NF-κB, Nuclear Factor-kappa B; NQO1: Quinone Oxidoreductase 1, a phase II detoxification enzyme; OVA: Ovalalbumin; PBST: Phosphate Buffered Saline Supplement with Tween-20; SDS: Sodium Dodecyl Sulphate; STAT3/6: Signal Transducer and Activators of Transcription 3 or 6; TNF-α: Tumor Necrosis Factor-alpha.

Introduction

Atherosclerosis (AS) is a major risk factor for CVD, the leading cause of death among men and women [1]. The endothelium, in addition to serving as the barrier separating circulating blood from peripheral tissues, also plays a critical role in the maintenance of normal vascular functions, and in the development of AS. Accumulating evidence, from both *in vitro* and *in vivo* studies, indicate that sustained inflammation markedly impairs the integrity and function of endothelium [2-5], culminating in a dysfunctional endothelium that facilitates the initiation and progression of AS, for instance, by reducing Nitric Oxide (NO) production. Moreover, impairment of endothelial structure and function also increases the expression of vascular adhesion molecules, which attract and activate platelets and white blood cells, and permit lipid diffusion and entry into the sub-endothelial space where retained lipids are oxidized. This set of events, summarily featured by inflammation-induced blood cell adhesion to endothelial cells followed by transmigration into the subendothelial space, eventually leads to fatty streak, atheroma formation and overt AS [6,7]. Additionally, Reactive Oxygen Species (ROS) plays a critical role in AS via its ability to oxidize LDL-cholesterol and promote inflammation. ROS also oxidizes NO to peroxynitrite, a stronger ROS that enhances the AS process. These observations suggest that agents capable of suppressing inflammation, oxidative stress and LDL oxidation, such as bioactive components found in grapes, are cardioprotective candidates with potential benefits in preventing vascular complications of AS. An added consideration for cardioprotective agents is their effects on biomarkers identified as predictive of inflammation and oxidative response, and relevant to improvement of overall cardiovascular health for individuals in the population.

IL-8 is a member of CXC chemokine family that displays neutrophil chemotactic activity [8]; as part of inflammatory response, IL-8 induces chemotaxis in target cells causing them to migrate toward the site of infection [9]. Exposure to proinflammatory cytokines, e.g., TNF-α induces the IL-8 expression [8,10] while oxidative stress regulates the secretion of IL-8 [8,11]; accordingly, IL-8 could serve as biomarker for monitoring both inflammatory and oxidative response in AS.

AMCase is a 50-kDa prototypic chitinase, an enzyme that contains a 39-kDa chitin-hydrolyzing catalytic domain at the N-terminus, a flexible hinge region, and a chitin-binding domain at the C-terminus [12,13]. AMCase is elevated in Th2-mediated inflammation and this occurs by an IL-13/STAT6–dependent mechanism [14,15]. AMCase is also hyper-expressed in the lungs of asthmatic patients, in animal models of asthma [13, 14, 16, 17], and in the alveolar macrophages derived from OVA-stimulated mice [18]. AMCase has been shown to control the expression of monocyte chemoattractant protein-1 (MCP-1) and eotaxin-1, which are chemokines required for the recruitment of T cells, eosinophils and macrophages in the lung and which contribute to the development of airway hyper respon¬siveness and pathogenesis of asthma [14,19]. Inhibition of AMCase with specific antibodies reduces the inflammatory responses in BALF and lung tissues [14]. Data to date suggest that a major biological function of AMCase is the control of asthma and that changes in AMCase can be used to monitor Th-2/IL-13-mediated immune response and inflammation.

Cardiac NQO1 has been shown to confer protection against menadione toxicity in guinea pig atria and also against oxidative and electrophile injury resulting from exposure to xanthine oxidase/xanthine, 4-hydroxy-2-nonenal or doxorubicin [20]. NQO1 has also been proposed to play a role in safeguarding the genetic integrity of endothelial cells and thus may be used to assess different aspects of the oxidative stress response in CVD [21]. Based on the foregoing findings, we postulate that changes in IL-8, AMCase and NQO1 can be collectively used to investigate the mechanism by which resveratrol or its metabolites confer protection against inflammation and oxidative stress in cardiovascular cells and systems.

Endothelial cells exposed to proinflammatory cytokines elaborate and secrete chemokines as antecedents to the establishment of a proanthrogenic state. Previously, we have used HPAEC cells as a model to examine the expression of chemokine eotaxin-1 in response to treatment by cytokines TNF-α and/or IL-13 and also, the modulation of eotaxin-1 gene control in cytokine-exposed cells pre-treated with grape-derived phytochemical resveratrol and its metabolite piceatannol [22]. In this study, we utilized the same cell system to evaluate the efficacy of resveratrol in modulating changes in IL-8 and AMCase in IL-13/TNF-α-challenged HPAEC in coordination with alterations in transcription factors, STAT3/6 and NF-κB. Due to the rapid conversion of resveratrol to its glucuronidated metabolites and because resveratrol is abundantly present in grape skin, red wine, and other grape-derived products as the glycosylated derivative piceid, we also included 3-O- and 4'-O-glucuronidated resveratrol and piceid in our analysis. In addition, the effects of resveratrol and its metabolites in affecting anti-oxidant enzyme NQO1 mRNA and protein levels were also determined.

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, streptomycin and penicillin were obtained from Cellgro, Inc (Herndon, VA, USA). All other chemicals and solvents used were of analytical grade.

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). Cells were maintained using endothelial cell media supplemented with subculture Reagent kit following instructions provided by the manufacturer [22]. Only passage 5 cells were used for experiments.

Treatment of HPAEC by cytokines, resveratrol, or its metabolites

The cells were seeded in 6- or 24-well plates at a density of 1×10^5cells/ml in serum-containing cultured media. When cells reached 50-60% confluence they were switched to serum-free media and maintained overnight. The cells were treated with indicated dose of recombinant human IL-13 or TNF-α (PeproTech Inc., Rocky Hill, NJ, USA), alone or combined for an additional 6 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 expression of specific genes were evaluated by 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, CA, 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 were as follows: IL-8, forward 5'-TCT GCA GCT CTG TGT GAA GG-3', reverse 5'-TGA ATT CTC AGC CCT CTT CAA-3'; AMCase, forward 5'-CGG GAT CCA TGA CAA AGC TTA TTC TCC TC-3', reverse 5'-GGA GAT GCC AGC AGC TAC TGC AGC-3'; NQO1 forward 5'-GCT AAC GTT GCT CCC TTG AG -3', reverse 5'-AAA GGT CAC ATG GAC GGA AG-3'; STAT3, forward 5'-GCT GCA ACT CCT CCA GTT TC-3', reverse 5'-AGT ATA GCC GCT TCC TGC AA-3'; STAT6, forward 5'-CTG CCA AAG ACC TGT CCA TT-3', reverse 5'-GGT AGG CAT CTG GAG CTC TG-3'; p65 NF-κB, forward 5'-AAT GGC TAC ACA GGA CCA GG-3', reverse 5'-ATC TTG AGC TCG GCA GTG TT-3'; p50 NF-κB, forward 5'-CAC CTA GCT GCC AAA GAA GG-3', reverse 5'-TCA GCC AGC TGT TTC ATG TC-3'; GAPDH, forward 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3', reverse 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'. PCR reaction conditions for all genes (except AMCase) were as follows: denaturation at 95°C, 5 min, followed by 29 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The reaction conditions for AMCase gene reaction were: 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. The expression of GAPDH was used as an internal control for normalizing mRNA expression results.

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% 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. Protein content of cell lysates was determined by Coomassie Protein Assay kit (Pierce, Rockford, IL, USA) using BSA as standard. Proteins (10 μg) in extracts prepared from control and treated cells were separated by electrophoresis on 10% SDS–polyacrylamide gel, followed by transfer to a nitrocellulose membrane and blocked with 3% nonfat dried milk in TBST buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 0.05% Tween-20) for 1 h. The blots were incubated with various primary antibodies at 4°C for 12-15 h and then with appropriate secondary antibodies conjugated to horseradish peroxidase dissolved in TBST. Expression of actin was used as loading control. The specific immunoreactive bands were identified by Enhanced Chemi Luminescence (ECL), and the intensity of signals was quantified by densitometry (using imageJ) and expressed as a ratio relative to expression of actin.

Preparation of cytosolic and nuclear fractions from control and treated HPAEC

Subcellular fractionation was performed using the nuclear extraction kit obtained from Active Motif, Inc. (Carlsbad, CA, USA), as described [23]. In brief, the cells were lysed in hypotonic buffer and lysates were centrifuged at 14,000 x g for 30 seconds to obtain the supernatant (cytosolic fraction) and nuclear pellet. The nuclear pellets were re-suspended in complete lysis buffer, rocked at 150 rpm for 30 min, and centrifuged at 14,000 x g for 10 min. The supernatant was collected and designated as the nuclear fraction.

Statistical analyses

Results were analyzed by nonparametric t-test. Difference in statistical significance was set at $P < 0.05$.

Results

IL-13 and TNF-α exert different effects on mRNA expression of IL-8, AMCase and transcription factors NF-kB p50/p65 and STAT3/6 in HPAEC

Human endothelial cells are capable of expressing a variety of bioactive molecules, including cytokines and growth factors, critical to inflammation. Vascular endothelial cells are known to produce inflammatory chemokines like IL-8. Previously TNF-α has been shown to induce IL-8 in HPAEC [10]; however, the mechanism of IL-8 regulated expression by pro-inflammatory cytokines in HPAEC remains largely unknown. To test if exposure to cytokines IL-13 and TNF-α affected IL-8 expression in HPAEC, cells were treated with increasing dose of IL-13 and TNF-α, alone or in combination. Control and treated cells were harvested and changes in IL-8 mRNA were measured by RT-PCR. IL-8 mRNA was detected in untreated HPAEC, and was copiously up-regulated by TNF-α at 10 ng/ml (Figure 1A). By contrast, IL-8 levels were dose-dependently reduced by IL-13 (Figure 1A); interestingly, the combination of IL-13 with TNF-α did not reduce IL-8 expression compared with cells treated with TNF-α alone (Figure 1A). Previously, we demonstrated that eotaxin-1 was increased in HPAEC by treatment with IL-13 or TNF-α, added as a single cytokine or combined [22]. Other reports showed that IL-13 induced the expression of AMCase in airway epithelial cells and macrophages and that AMCase may exert control on the

production of eotaxin-1 [14, 19]. Thus we tested whether IL-13 also induced changes in AMCase in HPAEC, by assaying the level of AMCase mRNA using RT-PCR. We observed a modest dose-dependent increase in AMCase by IL-13 or the combination of IL-13 and TNF-α, but not TNF-α added alone (Figure 1A). To understand the role of transcriptional factors contributing to altered expression of IL-8 or AMCase by pro-inflammatory cytokines in cultured HPAEC, we determined changes in transcription factors STAT3/6 and NF-κB p50/p65. Results in Figure 1B showed that treatment by TNF-a, which markedly elevated mRNA levels in IL-8, also caused sustained increase (40%) in NF-kB p50 or STAT6. A transient increase in NF-kB p65 or STAT3 occurred at 3 h (Figure 1B), while the elevation of NF-kB p50 or STAT6 continued to 6 h (data not shown). IL-13-mediated changes in AMCase were correlated with 10~20% elevation in STAT3 and STAT6 at 3 h but did not correspond to changes in NF-kB p50 or p65 (Figure 1B).

Figure 1: Induction of IL-8 and AMCase expression by IL-13 and TNF-α. Cultured HPAEC were treated with different concentrations of IL-13 and TNF-α, individually or combined.

(A) Total RNA was isolated from control and treated HPAEC at 6 h following stimulation and IL-8 and AMCase 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). IL-8 or AMCase mRNA levels following treatments were quantified and normalized against GAPDH shown in the bottom panel. Statistically significant difference between cytokine-treated groups compared with vehicle controls was indicated by asterisks (*).

Figure 1: Induction of IL-8 and AMCase expression by IL-13 and TNF-α. Cultured HPAEC were treated with different concentrations of IL-13 and TNF-α, individually or combined.

(B) The expression of transcription factors, NF-κB subunits and STAT3 and STAT6 mRNA in control and treated HPAEC using the indicated cytokines, for 3 h was also assayed by RT-PCR. The respective PCR signals were quantified and normalized against the value of GAPDH for each treatment condition. Asterisks (*) indicated statistically significant difference between cytokine-treated groups compared with vehicle controls at 0 h.

Resveratrol attenuates IL-13 or TNF-α-mediated induction of IL-8 or AMCase mRNA

Since giving individuals who do not or rarely drink a diet supplemented with 250 ml/day of red wine for 4 weeks showed a decrease in levels of inflammation and oxidative-stress-related biomarkers [24], we tested in HPAEC whether IL-8 expression was modulated by red wine polyphenol resveratrol or its metabolite piceatannol, with or without challenge by proinflammatory cytokines IL-13 or TNF-α. RT-PCR was performed on RNA isolated from control and treated cells. The results showed the marked increase in IL-8 mRNA expression in cells exposed to TNF-α or combined cytokine stimulation were significantly reduced by treatment with 25 μM resveratrol (Figure 2A). It is noteworthy that HPAEC treated with 25 μM piceatannol alone induced IL-8 mRNA while paradoxically attenuated IL-8 expression in IL-13-treated HPAEC, with (from ~45-fold to 31-fold) and without (from ~22-fold to 19-fold) supplementation by TNF-α (Figure 2A). Control of IL-8 expression mediated by IL-13 or TNF-α was also studied using biotransformed resveratrol metabolites, respectively, piceid (glycosylated resveratrol) [25] and glucuronidated metabolites (3-O- and 4'-O-glucuronidated resveratrol) [26-29]. The significant stimulation in IL-8 mRNA mediated by IL-13/TNF was increased by an additional 20-25% by 25 μM piceid, and 3-O-glucuronidated resveratrol and by 10-15% using 4'-O-glucuronidated resveratrol; of note, 25 μM piceid alone noticeably reduced IL-8 mRNA level in unstimulated cells (Figure 2B). The AMCase mRNA level was much less conspicuously affected by proinflammatory cytokines; only modest induction of AMCase resulted from exposure to 100 ng/ml IL-13 or to combined IL-13 (50 ng/ml)/TNF-α (100 ng/ml) (Figure 1A). The effects of resveratrol on AMCase were tested with or without addition of IL-13/TNF-α, at 100 ng/ml and a 13% and 60% decrease in the AMCase expression by 10 and 25 μM resveratrol was observed (Figure 2C).

Modulation of IL-13 and TNF-α-mediated STAT3/6 and NF-κB changes by resveratrol and its metabolites

Since resveratrol and piceatannol inhibited TNF-α-induced chemokine expression and attenuated NF-κB expression and function [30,31], we next tested the effects of resveratrol and its metabolites on mRNA expression of transcription factors STAT3/6, and NF-κB p65/p50 in proinflammatory cytokine-treated HPAEC. The NF-κB p50 subunit mRNA was inhibited, to varying degrees ranging from 40% to 65%, by resveratrol, piceatannol, 4'-O- and 3-O-resveratrol-glucuronide following IL-13 and TNF-α combined treatment (Figure 3A), while NF-κB p65 subunit mRNA expression was only inhib-

ited (45%) by 3-O-resveratrol-glucuronide (Figure 3A). No changes in STAT3/6 mRNA by resveratrol or its metabolites were observed, at all conditions tested (Figure 3A).

Figure 2: Modulation of IL-13 and TNF-α induced IL-8 or AMCase expression by resveratrol and/or its metabolites.

(A) HPAEC was pretreated by 5 or 25 μM resveratrol or piceatannol for 1 h and stimulated with IL-13 (50 ng/ml), TNF-α (10 ng/ml), alone or in combination, for 3 h. IL-8 mRNA levels were assayed by RT-PCR, quantified by densitometry, and normalization against GAPDH.

(B) Effect of the metabolites of resveratrol on IL-13 and TNF-α induced IL-8 expression. HPAEC was pretreated with 25 μM piceid, 4'-O-D-glucuronide, or 3-O-D-glucuronide for 1 h and stimulated with IL-13 (50 ng/ml) and TNF-α (10 ng/ml) for 3 h. Changes in mRNA of IL-8 were assayed by RT-PCR (left panel) and quantified by ImageJ and normalized against GAPDH (right panel). Values are expressed as mean±SEM for three experiments.

Figure 2: Modulation of IL-13 and TNF-α induced IL-8 or AMCase expression by resveratrol and/or its metabolites.

(C) Effects of resveratrol on AMCase expression. HPAEC was pretreated by 10 or 25 μM resveratrol for 1 h and stimulated with IL-13 (100 ng/ml), TNF-α (100 ng/ml), alone or in combination, for 6 h. AMCase mRNA levels were assayed by RT-PCR, quantified by densitometry, and normalization against GAPDH.

Figure 3: Effects of the resveratrol and its metabolites on IL-13 and TNF-α induced change of STAT3, STAT6, NF-kB p50 and NF-kB p65 expression.

(A) HPAEC was pretreated with 50 μM resveratrol, piceatannol, piceid, 4'-O-D-glucuronide, or 3-O-D-glucuronide for 1 h and stimulated with IL-13 (50 ng/ml) and TNF-α (10 ng/ml) for 3 h. Changes in mRNA of STAT3/6 and NF-kB p50/p65 were assayed by RT-PCR and quantified and expressed as a fold difference against GAPDH.

(B) HPAEC were pretreated with 50 μM resveratrol then stimulated with IL-13 (50 ng/ml) and TNF-α (10 ng/ml) for 3 h. Western blot analysis was performed to measure the subcellular changes in protein expression of JAK-1, p-STAT6, STAT6 and two subunit of NF-κB on cells treated with 50 ng/ml IL-13 and 10 ng/ml TNF-α. Intensity of specific bands from control and treated cells in panel B was each normalized to actin and expressed as a fold of control.

To better understand the disparate changes in IL-8 or AMCase in response to exposure to proinflammatory cytokines, with and without pre-treatment by resveratrol, we determined changes in subcellular distribution of transcription factors and their upstream activator by western blot analysis. Control and treated cell extracts were fractionated into cytosol and nuclear compartments. Treatment by proinflammatory cytokines increased the nuclear presence of JAK-1, pSTAT6 and NF-κB p65; the induced changes were all down regulated by resveratrol to barely detectable levels. For comparison, combined IL-13/TNF-α induced nuclear translocation of NF-κB p50; prior exposure to resveratrol resulted in decrease nuclear NF-κB p50 (~50%) in both control and cytokine-treated cells (Figure 3B).

Effects of resveratrol or its metabolites on HPAEC NQO1 expression and cell morphology

NQO1 is a phase II enzyme that participates in cardioprotection [32] by regulating the generation of ROS capable of damaging the genetic integrity of endothelial cells. To explore NQO1 gene modulatory effects of resveratrol or its metabolites, we analyzed changes in NQO1 mRNA and protein expression by RT-PCR and immunoblot analysis using samples prepared from control and 48 h resveratrol-treated HPAEC. Treatment of HPAEC increased protein expression of NQO1 (Figure 4B) without affecting its mRNA levels(Figure 4A); in contrast, treatment by piceid, or 3-O- and 4'-O-glucuronidated resveratrol inhibited NQO1 mRNA level (Figure 4A) without changing its protein expression (Figure 4B). In addition, the effects of resveratrol or its metabolites on the cell morphology of cultured HPAEC were also examined. Cells were treated with 50 μM resveratrol or metabolites for 48 h and visualized under a microscope (Figure 4C). Growth as well as morphology of HPAEC was most pronouncedly inhibited by piceatannol, to a lesser extent by resveratrol, and essentially unchanged by other resveratrol metabolites (Figure 4C).

Discussion

The notion that AS is an inflammatory disease characterized by the accumulation of lipid in large and medium elastic arteries is widely accepted. Equally well established is a complex pattern of signaling events, involving participation by cytokines and chemokines functioning as underlying mediators, driving the progression of AS lesions [4]. A dysfunctional endothelium and the plethora of accompanying phenotypic changes are apical events in the pathogenesis of AS. Contributing to a dysfunctional endothelium is the expression of AS-relevant regulatory events encompassing individual as well as gene clusters.

Notably, studies have shown that regulation of AS-relevant genes can be modulated using dietary agents. For example, grape derived polyphenol resveratrol and its metabolites may exert cardioprotective effects either by direct suppression of AS-relevant gene expression or by upregulating vasoprotective genes, with anti-inflammation, anti-thrombotic, and anti-oxidant functions in the endothelium.

Figure 4: Effects of resveratrol and its metabolites on NQO1 mRNA and protein expression and cell morphology in HPAEC.

(A) HPAEC was treated with 50 μM piceid, 4'-O-D-glucuronide, or 3-O-D-glucuronide for 4 h. Changes in mRNA of NQO1 were assayed by RT-PCR and quantified by ImageJ and expressed as a fold difference against GAPDH.

(B) Effects of resveratrol and its metabolites on NQO1 protein expression. Western blot analysis were performed on the HPAEC cells treated with 50 μM resveratrol and/or its metabolites for 48 h. Changes in protein expression of NQO1 were further quantified the intensity of NQO1 bands from control and treated cells and each normalized against actin and expressed as a fold of control.

(C) Cell morphological change of HPAEC induced by exposure to 50 μM resveratrol or its metabolites for 48 h.

In this study, we have used cultured HPAEC to test the cardioprotective/anti-inflammation hypothesis, by focusing on IL-8 and AMCase as markers for endothelial response to proinflammatory cytokines, respectively, IL-13 and TNF-α, and their transcriptional control as being linked to changes in STAT3/6, and NF-κB. We also examined regulation of IL-8 and AMCase by pre-exposure to resveratrol or its metabolites, piceatannol, as well as piceid, resveratrol-4'-O-D-glucuronide, and reveratrol-3-O-D-glucuronide. Our results show that expression of changes in mRNA for IL-8 and AMCase can indeed be considered as markers for response to proinflammatory cytokines. Moreover, altered levels of AMCase and IL-8 are well correlated with changes in transcription factors STAT6 and NF-κB. Further, resveratrol and its metabolites were found to exert varying effects on the expression of these molecular targets, apparently both by transcription and post-transcriptional mechanisms.

In addition, we also observed that quinone reductase NQO1, which is expressed in many tissues including vascular endothelium that require a high level of antioxidant protection [33], can be regulated by complex mechanisms upon exposure to resveratrol or its metabolites. Specifically, treatment of HPAEC by resveratrol increased protein expression of NQO1 without affecting its mRNA level; in striking contrast, treatment by piceid, or 3-O- and 4'-O-glucuronidated resveratrol inhibited NQO1 mRNA level without changing its protein expression. These results suggest that NQO1 is regulated in HPAEC by resveratrol or its metabolites by complex post-transcriptional/translational mechanisms. NQO1 is known to catalyze two-electron conversion of plant/vegetable byproduct quinones to hydroquinones, thereby circumventing the one-electron redox cycling of quinones and suppressing the generation of potentially harmful ROS, as well as preserving cellular glutathione concentrations [32]. Control of NQO1 by resveratrol or its metabolites may be viewed as complementary defenses to protect cardiac cells/tissues against the damages imposed by free radicals, superoxides, and hydrogen peroxides.

The results presented in this study showing clearly that resveratrol metabolites exert varying effects on the panel of target genes and transcriptional factors assayed in cultured HPAEC have implications directly relevant to the lingering concern whether resveratrol should be considered bioefficacious. For example, resveratrol has been detected transiently in rat plasma after administration of red wine and pure resveratrol [34,35]; moreover, the blood concentration of resveratrol peaked at 10 min after ingestion [36,37]. When resveratrol is consumed by humans in the form of resveratrol-containing beverages, native resveratrol is excreted in higher amounts over a 24-h period than either catechin or quercetin [38]. These results are taken as evidence showing that resveratrol has limited bioavailability; furthermore, enzymes involved in the biotransformation of resveratrol to its metabolites remain incompletely known or characterized. However, absorption studies of resveratrol using isolated rat intestine sections or in human feeding experiments with resveratrol clearly show that resveratrol is converted to its glucuronidated and sulfated metabolites [26-28, 39-41], and that only glucuronidated resveratrol passes into the blood stream [27].

In summary, the differential control of IL-8 and AMCase by proinflammatory cytokines in HPAEC may be considered as a salient in vitro model for screening and identifying dietary agents, including grape-derived polyphenols, with anti-inflammatory activities and cardioprotective potentials. The proinflammatory cytokines TNF-α and IL-13 induce IL-8 or AMCase mRNA expression in HPAEC, in coordination with transcriptional control mediated by JAK-1/STAT3/6 and NF-κB expression targeting transcription of IL-8 or AMCase, all of which are effectively suppressed by resveratrol or piceatannol. Figure 5 presents a mechanism linking endothelial damage and inflammation with IL-8, AMCase, and NQO1 expression in relation to AS and CVD and protection by resveratrol and its metabolites.

Figure 5: A mechanism showing modulation by resveratrol on induction of IL-8 and AMCase gene transcription by IL-13 and TNF-α. Resveratrol is postulated to suppress IL-8 or AMCase transcription and expression induced by IL-13 and TNF-α, and that its effects are partially mediated through the inhibition of STAT3/6 and NF-κB p50/p65 expression using signaling events whose details remain to be elucidated. The scheme also shows effects of resveratrol and its metabolites on NQO1, in part contributing to cardioprotection.

Acknowledgements

Results herein 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.

References

1) Heron M, Hoyert DL, Murphy SL, Xu J, Kochanek KD, et al. (2009) Deaths: final data for 2006. Natl Vital Stat Rep 57: 1-134.

2) Beckman JA, Creager MA, Libby P (2002) Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. JAMA 287: 2570-2581.

3) Hink U, Li H, Mollnau H, Oelze M, Matheis E, et al., (2001) Mechanisms underlying endothelial dysfunction in diabetes mellitus. Circ Res 88: 14-22.

4) Hansson GK (2005) Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352: 1685-1695.

5) Stoll G, Bendszus M (2006) Inflammation and atherosclerosis: novel insights into plaque formation and destabilization. Stroke 37: 1923-1932.

6) Mayer K, Merfels M, Muhly-Reinholz M, Gokorsch S, Rosseau S, et al. (2002) Omega-3 fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation. Am J Physiol Heart Circ Physiol 283: 811-818.

7) Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, et al. (2003) Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. Arterioscler Thromb Vasc Biol 23: 622-629.

8) Vlahopoulos S, Boldogh I, Casola A, Brasier AR (1999) Nuclear factor-kappaB-dependent induction of interleukin-8 gene expression by tumor necrosis factor alpha: evidence for an antioxidant sensitive activating pathway distinct from nuclear translocation. Blood 94: 1878-1889.

9) Huber AR, Kunkel SL, Todd RF , Weiss SJ (1991) Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science 254: 99-102.

10) Hashimoto S, Gon Y, Matsumoto K, Takeshita I, Horie T (2001) N-acetylcysteine attenuates TNF-alpha-induced p38 MAP kinase activation and p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells. Br J Pharmacol 132: 270-276.

11) DeForge LE, Preston AM, Takeuchi E, Kenney J, Boxer LA, et al. (1993) Regulation of interleukin 8 gene expression by oxidant stress. J Biol Chem 268: 25568-25576.

12) Krykbaev, R., Krykbaev R, Fitz LJ, Reddy PS, Winkler A, Xuan D, et al. (2010) Evolutionary and biochemical differences between human and monkey acidic mammalian chitinases. Gene 452: 63-71.

13) Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, et al. (2011) Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. Annu Rev Physiol 2011: 479-501.

14) Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, et al. (2004) Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science 304: 1678-1682.

15) Homer RJ, Zhu Z, Cohn L, Lee CG, White WI, et al. (2006) Differential expression of chitinases identify subsets of murine airway epithelial cells in allergic inflammation. Am J Physiol Lung Cell Mol Physiol 291: 502-511.

16) Bierbaum S, Nickel R, Koch A, Lau S, Deichmann KA, et al. (2005) Polymorphisms and haplotypes of acid mammalian chitinase are associated with bronchial asthma. Am J Respir Crit Care Med 172: 1505-1509.

17) Chupp GL, Lee CG, Jarjour N, Shim YM, Holm CT, et al. (2007) A chitinase-like protein in the lung and circulation of patients with severe asthma. N Engl J Med 357: 2016-2027.

18) Yang CJ, Liu YK, Liu CL, Shen CN, Kuo ML, et al. (2009) Inhibition of acidic mammalian chitinase by RNA interference suppresses ovalbumin-sensitized allergic asthma. Hum Gene Ther 20: 1597-1606.

19) Kawada M, Hachiya Y, Arihiro A, Mizoguchi E (2007) Role of mammalian chitinases in inflammatory conditions. Keio J Med 56: 21-7.

20) Floreani M., Napoli E, Palatini, (2000) Protective action of cardiac DT-diaphorase against menadione toxicity in guinea pig isolated atria. Biochem Pharmacol 60: 601-605.

21) Cao Z, Li Y (2004) Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury. Eur J Pharmacol 489: 39-48.

22) Yang CJ, Lin CY, Hsieh TC, Olson SC, Wu JM, et al. (2011) Control of eotaxin-1 expression and release by resveratrol and its metabolites in culture human pulmonary artery endothelial cells. Am J Cardiovasc Dis 201: 16-30.

23) Hsieh TC, Huang YC, Wu JM, (2011) Control of prostate cell growth, DNA damage and repair and gene expression by resveratrol analogues, in vitro. Carcinogenesis 32: 93-101.

24) Avellone G, Di Garbo V, Campisi D, De Simone R, Raneli G, et al. (2006) Effects of moderate Sicilian red wine consumption on inflammatory biomarkers of atherosclerosis. Eur J Clin Nutr 60: 41-47.

25) Romero-Pérez AI, Ibern-Gómez M, Lamuela-Raventós RM, de La Torre-Boronat MC, et al. (1999) Piceid, the major resveratrol derivative in grape juices. J Agric Food Chem 47: 1533-1536.

26) Walle T, Hsieh F, DeLegge MH, Oatis JE Jr, Walle UK, et al. (2004) High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos 32: 1377-1382.

27) Kuhnle G, Spencer JP, Chowrimootoo G, Schroeter H, Debnam ES, et al. (2000) Resveratrol is absorbed in the small intestine as resveratrol glucuronide. Biochem Biophys Res Commun 272: 212-217.

28) Boocock DJ, Faust GE, Patel KR, Schinas AM, Brown VA, et al. (2007) Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. Cancer Epidemiol Biomarkers Prev 16: 1246-1252.

29) Patel KR, Brown VA, Jones DJ, Britton RG, Hemingway D, et al. (2010) Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients. Cancer Res 70: 7392-7399.

30) Csiszar A, Smith K, Labinskyy N, Orosz Z, Rivera A, et al. (2006) Resveratrol attenuates TNF-alpha-induced activation of coronary arterial endothelial cells: role of NF-kappaB inhibition. Am J Physiol Heart Circ Physiol 291: 1694-1699.

31) Ashikawa K, Majumdar S, Banerjee S, Bharti AC, Shishodia S, et al. (2002) Piceatannol inhibits TNF-induced NF-kappaB activation and NF-kappaB-mediated gene expression through suppression of IkappaBalpha kinase and p65 phosphorylation. J Immunol 169: 6490-6497.

32) Zhu H, Li Y (2012) NAD(P)H: quinone oxidoreductase 1 and its potential protective role in cardiovascular diseases and related conditions. Cardiovasc Toxicol 12: 39-45.

33) Siegel D, Ross D, (2000) Immunodetection of NAD(P)H:quinone oxidoreductase 1 (NQO1) in human tissues. Free Radic Biol Med 29: 246-53.

34) Bertelli AA, Giovannini L, Stradi R, Urien S, Tillement JP, et al. (1996) Kinetics of trans- and cis-resveratrol (3,4',5-trihydroxystilbene) after red wine oral administration in rats. Int J Clin Pharmacol Res. 16: 77-81.

35) Juan ME, Lamuela-Raventós RM, de la Torre-Boronat MC, Planas JM (1999) Determination of trans-resveratrol in plasma by HPLC. Anal Chem 71: 747-750.

36) Juan ME, Planas JM, (2002) Trans-resveratrol oral administration does not affect the enzymatic activities in rat small intestine. J Physiol Biochem,. 58: 59-60.

37) Juan ME, Vinardell MP, Planas JM, (2002) The daily oral administration of high doses of trans-resveratrol to rats for 28 days is not harmful. J Nutr 132: 257-260.

38) Goldberg DM, Yan J, Soleas GJ (2003) Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem 36: 79-87.

39) Patel KR, Brown VA, Jones DJ, Britton RG, Hemingway D, et al. (2010) Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients. Cancer Res 70: 7392-7399.

40) Wenzel E, Soldo T, Erbersdobler H, Somoza V (2005) Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. Mol Nutr Food Res 49: 482-494.

41) Wenzel E, Somoza V (2005) Metabolism and bioavailability of trans-resveratrol. Mol Nutr Food Res 49: 472-481.