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Synergy between Ethanol and Grape Polyphenols, Quercetin, and Resveratrol, in the Inhibition of the Inducible Nitric Oxide Synthase Pathway

Marion Man-Ying Chan,†‡§ John A. Mattiacci,† Hyon S. Hwang,‡ Amit Shah† and Dunne Fong‡*

*Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, PA 19140; †Temple University School of Podiatric Medicine, Philadelphia, PA 19107; and ‡Department of Cell Biology and Neuroscience, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, U.S.A.

ABSTRACT. In atherosclerosis and tumor initiation, inducible nitric oxide synthase (iNOS) has been implicated in the damage of vascular walls and DNA, respectively. Moderate consumption of red wine has been ascribed as a preventive for coronary heart disease; however, there has been much debate over whether the beneficial effect is from grape polyphenolic components or ethanol. We studied the interaction of grape compounds on nitric oxide (NO) production by macrophages, mediators of blood vessel damage in atherosclerosis. For the murine macrophage cell line RAW 264.7, stimulation with lipopolysaccharide and interferon-γ led to expression of the iNOS gene and production of NO. The polyphenols quercetin and resveratrol at a micromolar range suppressed iNOS gene expression and NO production, as determined by reverse transcription–polymerase chain reaction and nitrite assay. The polyphenols were also found to be scavengers of NO in an acellular system using sodium nitroprusside under physiological conditions. Ethanol, at a moderate level, did not produce any appreciable level of reduction of iNOS or NO activity. However, its presence at 0.1 to 0.75% enhanced the effect of grape polyphenols concentration-dependently. Thus, the interaction between these components plays a significant role in the health effects of red wine, especially with respect to their effect on the NO pathway. BIOCHEM PHARMACOL **60**;10:1539–1548, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. macrophages; inducible nitric oxide synthase; nitric oxide; quercetin; resveratrol; ethanol

NO‖, an active radical gas molecule, is produced by the enzyme NOS, which exists in at least three isoforms in mammals. The constitutively expressed, Ca^{2+}-dependent NOS-I and NOS-III (eNOS) are present in neuronal cells and endothelial cells; they produce NO at a low level for neurotransmission and vascular homeostasis, respectively. The inducible, Ca^{2+}-independent NOS-II, also known as iNOS, is present in many cell types, including monocytes and macrophages. Upon stimulation by inflammatory agents and in the presence of cytokines (including IFNγ), iNOS gene expression is up-regulated and NO production is sustained at a high level [1, 2].

During inflammation, NO and its metabolites, such as peroxynitrite, are potentially cytotoxic. These molecules are capable of injuring the invading pathogens and eliminating the altered cells and tissues. However, indiscriminate destruction of cells and tissues by NO and its reactive nitrogen intermediates plays a significant role in the pathology of many inflammatory conditions, including atherosclerosis, sepsis, arthritis, and diabetes. NO and its metabolites also may contribute to tumor formation as they have been shown to cause DNA damage and mutations [3]. Thus, selective inhibition of the iNOS pathway is a popular target for the attenuation of many inflammatory diseases.

NO is active in the vascular system. Under normal conditions, endothelial cells produce NO (eNOS-mediated) at a low level to control vessel dilation. Diminished release of NO may lead to hypertension and constriction of coronary arteries. However, in atherosclerosis, a high level of NO has been found within early lesions and advanced atheroma, even though expression of eNOS in endothelial cells is reduced. At the lesions, intima of the vascular wall is infiltrated with T lymphocytes and macrophages that express iNOS. These macrophages are one of the major producers of NO that causes damage to blood vessels [4]. Furthermore, an inverse relation between iNOS and eNOS has been shown. In endothelial cells, inflammatory agents (e.g. LPS and cytokines) induce expression of iNOS but reduce the expression of eNOS [5].

§ Corresponding author: Dr. M. M. Chan, Department of Microbiology and Immunology, Temple University School of Medicine, 3400 North Broad St., Philadelphia, PA 19140. Tel. (215) 625-5270; FAX (215) 629-1622; E-mail: marionc@astro.temple.edu

‖ *Abbreviations*: eNOS, endothelial nitric oxide synthase; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; NOS, nitric oxide synthase; RT–PCR, reverse transcription–polymerase chain reaction; and SNP, sodium nitroprusside.

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The French Paradox suggests that moderate wine consumption is beneficial with respect to coronary heart disease and some types of cancer [6]. Similarly, the Mediterranean diet, with consumption of wine with meals as one of the salient characteristics, has been attributed the same health benefits [7]. Epidemiological studies have shown that although alcohol by itself may be effective, wine, especially red wine, confers additional protection [8, 9]. Experimental studies have shown that alcohol-free red wine solids delay tumor onset in transgenic mice [10].

The protective components in red wine and grapes include the polyphenolic compounds quercetin, a flavonoid, and resveratrol, a stilbene. Molecular mechanisms of these compounds are current topics of interest [11–18]. With respect to NOS enzymes, quercetin and resveratrol inhibit LPS-stimulated NO production and iNOS gene expression in rat astrocytes, rat Kupffer cells, and murine macrophages [19–23], but these compounds also enhance endothelium-dependent relaxation of pre-contracted rabbit and rat aortic rings through an increase in NO production via eNOS [24–28]. Thus, grape components may contribute towards the balance between iNOS and eNOS.

Considering the protective effect of wine on coronary heart disease and the importance of NO in the cardiovascular system, we asked whether ethanol, and two polyphenols of grapes, quercetin and resveratrol, affect the production of NO in monocytes/tissue macrophages. Here, we confirm that quercetin and resveratrol reduce NO production of macrophages through scavenging of NO and reduction of iNOS gene expression. In addition, we extend the knowledge by conclusively demonstrating the synergistic effect between alcohol and grape polyphenols.

MATERIALS AND METHODS

Materials

Quercetin, resveratrol, MTT, and SNP were purchased from the Sigma Chemical Co. The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection. The macrophages were cultured in RPMI-1640 medium containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, 2 mM glutamine, 0.01 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.05 mM 2-mercaptoethanol, and 10% fetal bovine serum. RPMI-1640 and other tissue culture reagents were from Life Technologies, except for the serum, which was obtained from HyClone. LPS of *Salmonella typhosi* was from Difco. Recombinant murine IFNγ was provided by Dr. Sidney Pestka of the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School. The Purescript RNA isolation kit was from Gentra. Reverse transcriptase, RNase inhibitor, and oligo(dT_{16}) were from Promega. Primers for PCR were from Clontech. SYBR Green I dye was from Molecular Probes.

Cell Culture

The murine macrophage cell line RAW 264.7 was used to study the inhibition of NO production, based on previous reports with curcumin [29] and *Ginkgo biloba* extract EGb 761 [30]. Cells were cultured at 10^6/mL in 24-well plates in the presence of 10 U/mL of IFNγ and 0.2 μg/mL of LPS (i.e. stimulated macrophages) to induce NO production [30–32]. For concentration-dependent analysis of quercetin and resveratrol, various concentrations of the polyphenols were dissolved to the appropriate concentration in absolute ethanol. Immediately before the addition of LPS, 10 μL of preparation was added to 1 mL of culture medium, thus forming a final ethanol concentration of 1%. To study the synergism between ethanol and polyphenols, the polyphenol was first dissolved to the appropriate concentration in absolute ethanol, and then 1 μL was added to 1 mL of culture medium, thus forming an ethanol concentration of 0.1%. A final concentration of 0.25, 0.5, or 0.75% ethanol was attained by further addition of absolute ethanol. Cells were cultured at 37° in a CO_2 incubator. For analysis of iNOS mRNA, cell samples were harvested for RNA isolation after 4 hr of incubation. For analysis of NO production, supernatants from cell samples were collected for nitrite assay after 20 hr of incubation.

Chemical NO Production

The procedures for NO production by SNP followed those previously reported for testing NO scavenging capacities of the phytochemicals curcumin [33] and *Ginkgo biloba* extract EGb 761 [34]. SNP was freshly prepared as a 25 mM stock solution in PBS. Ethanol, quercetin, or resveratrol was added to the solution. The polyphenols were first dissolved in ethanol and then added at 10 or 15 μL, so as to obtain the desired concentration in 3 mL of SNP–PBS. The preparations were incubated at 25° for 180 min. Aliquots of samples were quantified for nitrite content at the stated time or time intervals.

Nitrite Assay

NO was quantified by the detection of nitrite, a metabolite of NO and oxygen, using Griess reagent [35, 36]. To each 50-μL sample, an equal volume of Griess reagent (1% sulfanilamide in water and 0.1% naphthylethylenediamide in 2.5% phosphoric acid) was added, and the absorbance was read at 546 nm.

MTT Assay

The experiments were set up in a manner similar to that for the nitrite assay. After 24 hr of incubation, the cells were washed three times to remove the polyphenols. Then the MTT assay was performed as described previously [35, 37].

RT–PCR

Total RNA was isolated from harvested RAW 264.7 cells using a Purescript RNA isolation kit. RT–PCR was performed according to published procedures [31, 32]. For first strand cDNA synthesis, 1 μg of RNA from each sample was reverse transcribed using 100 U MMLV reverse transcriptase, 20 U RNase inhibitor, 0.6 mM dNTP, and 0.4 μM random hexamers. PCR analyses were performed on aliquots of the cDNA preparations for detection of iNOS and β-actin gene expression. For iNOS, the reactions occurred in a 30 μL volume for 27 cycles, with 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 2 U of *Taq* DNA polymerase, and 50 pmol of 5′ and 3′ primers. After an initial denaturation for 4 min at 94°, the amplification cycle was 94° for 45 sec, 65° for 45 sec, and 72° for 2 min. PCR conditions for the control housekeeping gene β-actin were those recommended by the manufacturer (Clontech). The PCR products were separated on 1.6% agarose gel and stained with SYBR Green I dye. The relative amount of mRNA in the samples was determined from the intensity of the bands using a gel documentation and analysis system (Storm, Molecular Dynamics). The degree of inhibition was calculated as a percent of the LPS + IFNγ control by the following formula: percent of change = 100 × [1 − (polyphenol and ethanol-treated)/(LPS + IFNγ control)].

Statistical Analysis

For each experiment, Student's *t*-test was used to determine statistical significance between samples. A P value of < 0.05 was taken to indicate statistical significance. Each experiment was repeated three times.

RESULTS

Synergy Between Ethanol and Quercetin or Resveratrol in Inhibiting NO production of Stimulated Macrophages

In the presence of 1% ethanol, quercetin, from 3 to 100 μM, reduced NO production in a concentration-dependent manner by 21.8 to 99% (Fig. 1). The IC_{50} value was 7.6 μM. To examine the synergism between ethanol and quercetin, ethanol was decreased further, titrating from 0.75 to 0.1%. A concentration of 30 μM was at the log phase of the quercetin inhibition curve when 0.1% ethanol was added, and thus was selected to study whether ethanol increases the efficacy of polyphenol (data not shown). When ethanol was at 0.1% and quercetin at 30 μM, nitrite production was reduced to 58% of the control (cells stimulated with LPS and IFNγ only) (Fig. 2). When the ethanol content was increased to 0.25, 0.50, and 0.75% in the culture, in a concentration-dependent manner, the degree of nitrite production was reduced further to 38, 30.7, and 20.7%, respectively (Fig. 2).

Resveratrol, similar to quercetin, also inhibited nitrite production by macrophages, and ethanol potentiated this action in a concentration-dependent manner. In the presence of 1% ethanol, resveratrol (3–100 μM) reduced NO production by 15.6 to 94.7% (Fig. 3). It had an IC_{50} value of 23.4 μM. A higher concentration of resveratrol was needed to achieve the same level of inhibition as quercetin. For examining the synergism between ethanol and resveratrol, similar to the quercetin experiment, ethanol concentration was decreased, from 0.75 to 0.1%. When 0.1% ethanol was added, a concentration of 60 μM was at the log phase of the resveratrol inhibition curve, and thus this concentration was selected for determining the effect of ethanol. Whereas 60 μM reduced nitrite production to 74% of the control (LPS- and IFNγ-stimulated cells), when ethanol were increased to 0.25, 0.50, and 0.75%, nitrite production was reduced further to 55, 45, and 31%, respectively (Fig. 4).

Without the polyphenols, ethanol at 0.1, 0.25, 0.5, and 0.75% only reduced the level of nitrite in the supernatants of stimulated macrophages to 98, 89, 84, and 67%, respectively (Fig. 5). Thus, although not very effective by itself, ethanol enhanced bioactivity of the grape polyphenols, quercetin and resveratrol, significantly and in a concentration-dependent manner. Furthermore, a viability assay, using MTT reagent, showed that quercetin, resveratrol, and ethanol at the experimental concentrations did not affect cellular metabolism, thus verifying that the decrease in NO production was not due to general toxicity. As shown in Table 1, all values obtained using LPS- and IFNγ-stimulated cells treated with ethanol and the grape polyphenols were similar. (The higher value for the unstimulated sample indicated cell growth.)

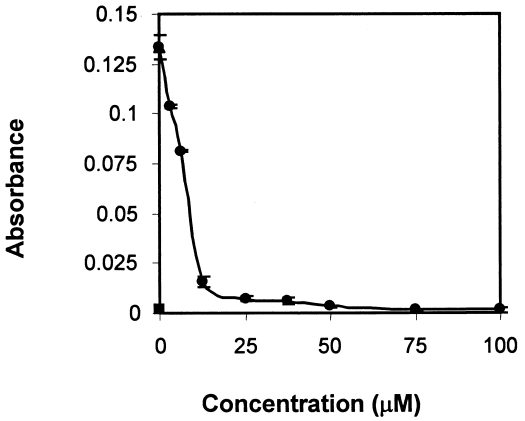

FIG. 1. Concentration-dependent effect of quercetin on nitrite production in LPS- and IFNγ-stimulated macrophages. Quercetin (circles) was prepared in ethanol and added to the cell culture medium to achieve the final concentrations as indicated (0–100 μM). The final ethanol concentration in all samples was 1%. Controls included both stimulated (triangle) and unstimulated (square) samples without ethanol or quercetin. Ethanol alone had no effect on stimulation (triangle hidden behind circle at 0 μM quercetin). The amounts of NO were determined as nitrite production using Griess reagent. From sodium nitrite standards, an absorbance of 0.1 U was determined to correspond to 30 μM nitrite (data not shown). Values are means ± SD, N = 3.

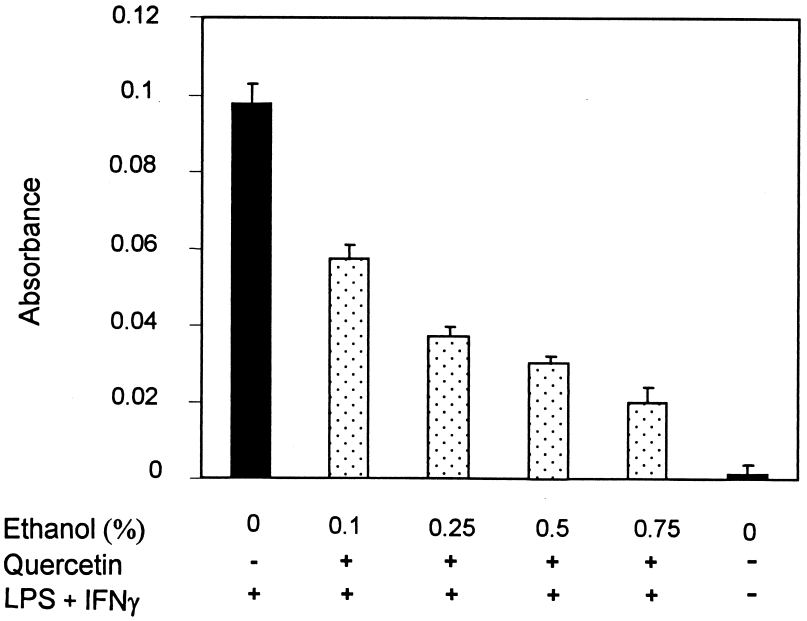

FIG. 2. Effect of quercetin and ethanol on nitrite production in stimulated macrophages. Quercetin stock solution was prepared in 0.1% ethanol, and the solution was added at 1 μL to LPS and IFNγ-stimulated macrophages cultured in 1 mL medium to yield a final concentration of 30 μM quercetin. Additional ethanol was used to achieve the final 0.25 to 0.75% ethanol. All samples were incubated for 20 hr, and aliquots were taken, in triplicate, for detecting the amounts of NO as nitrite production with Griess reagent. Values are means ± SD, N = 3.

Inhibition of iNOS Gene Expression in Stimulated Macrophages by Quercetin and Resveratrol

To deduce the mechanism of inhibition, the effects of ethanol, quercetin, and resveratrol on iNOS gene expression were examined. As shown by RT–PCR, the basal level of iNOS gene expression was increased by stimulation with LPS and IFNγ (Fig. 6, lanes A1 and A6, B1 and B5, C1 and C4). Quercetin (Fig. 6A), at 30 μM, did not reduce iNOS expression when added with 0.1 or 0.25% ethanol. However, inhibition was obtained by increasing the ethanol content to 0.5 and 0.75%. INOS mRNA expression was sharply suppressed to 23 and 18%, even though ethanol by itself did not affect the level of iNOS gene expression at these concentrations (Fig. 6C, lanes C2 and C3). As for resveratrol, at 0.1% ethanol, a 60 μM concentration of the polyphenol reduced iNOS mRNA expression only slightly, to 87%. Maintaining resveratrol at 60 μM and increasing the ethanol content to 0.25 and 0.5%, iNOS mRNA expression was decreased further to 82 and 72%. [Student's *t*-test showed that the difference was significant ($P < 0.05$) only at 0.5% ethanol.] Generally, the overall effect of ethanol on the action of resveratrol was modest, gradual, and concentration-dependent (Fig. 6B).

As shown, reduction in gene expressions of the cytoskeletal protein β-actin was not observed in any of the treatments (Fig. 6). Thus, for all three of the red wine components, the effects on inhibition of NO production and iNOS gene expression were specific to the NO synthesis pathway and not due to a decrease in cell viability.

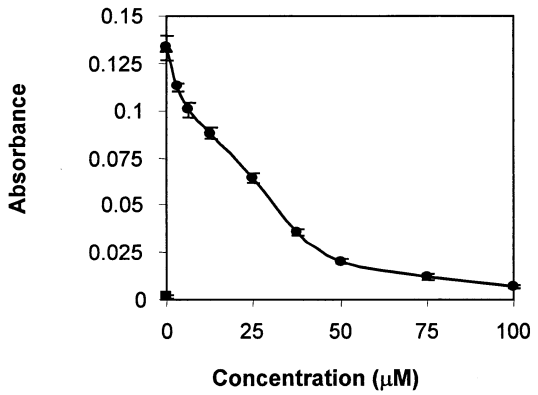

FIG. 3. Concentration-dependent effect of resveratrol on nitrite production in stimulated macrophages. Cultures were set up and nitrite production was determined with Griess reagent as described in the legend to Fig. 1 except that resveratrol was used instead of quercetin. Values are means ± SD, N = 3.

Scavenging of Chemically Generated NO by Quercetin and Resveratrol

In addition to iNOS gene expression, we studied the effect of ethanol and grape compounds on chemically generated NO using SNP. SNP is an NO donor that spontaneously generates NO in aqueous solution at physiological pH. Addition of SNP to PBS, in a course of 180 min, resulted in the production of NO in a time-dependent manner (Fig. 7A). Addition of ethanol, by itself, reduced the amount of nitrite only slightly. However, the addition of quercetin (30 μM) or resveratrol (30 μM) in ethanol reduced nitrite output from SNP significantly ($P < 0.05$). For example, at 180 min, ethanol alone reduced NO generation by 9.4%,

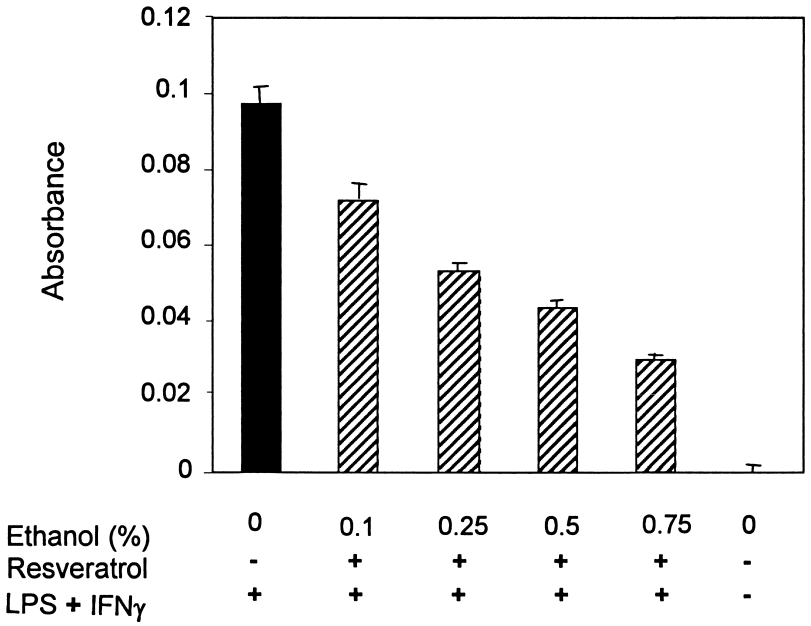

FIG. 4. Effect of resveratrol and ethanol on nitrite production in stimulated macrophages. Cultures were set up and nitrite production was determined with Griess reagent as described in the legend to Fig. 2 except that resveratrol (at 60 μM) was used instead of quercetin. Values are means ± SD, N = 3.

whereas 30 μM quercetin and 30 μM resveratrol reduced the nitrite level by 41.9 and 32.1%, respectively. The polyphenols, at concentrations between 5 and 50 μM (Fig. 7B), showed a significant ($P < 0.05$) reduction of 47.2 to 57.5% for quercetin and 31.4 to 46.2% for resveratrol.

DISCUSSION

Alcohol is the major component of wine. Whether ethanol or grape polyphenols confer the health benefits of red wine has been a subject of much debate. Many favor one component over another; however, few have looked for interactions between the components. In this report, we have shown that ethanol acts in synergy with quercetin and resveratrol. We have found that the grape polyphenols inhibit iNOS-mediated NO production of stimulated murine macrophages more effectively when given in increasing concentrations of ethanol.

High-dose exposure to ethanol, resembling that in alcohol abusers, has been shown to inhibit the expression of many genes associated with inflammation and immune functions [38–41]. An average wine has an alcohol content

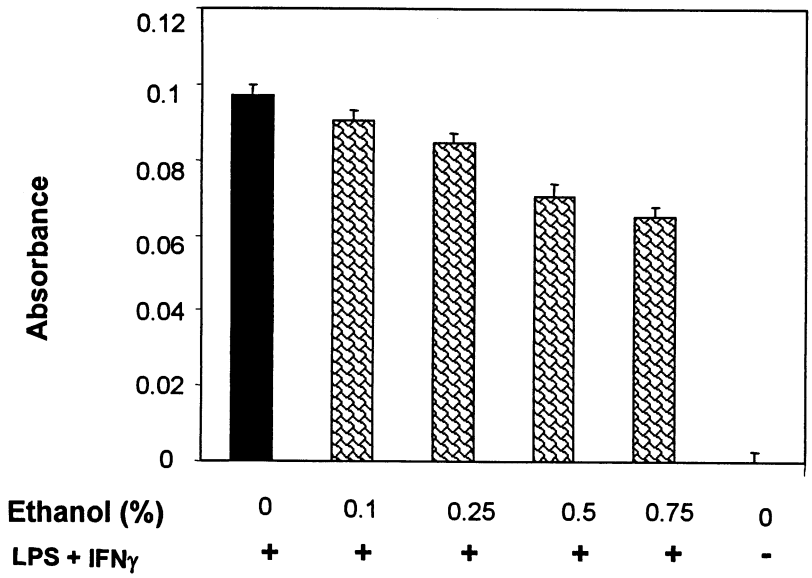

FIG. 5. Effect of ethanol on nitrite production in stimulated macrophages. Ethanol was added to RAW 264.7 cells at final concentrations as indicated and compared with stimulated and unstimulated controls. Samples were incubated and aliquots were taken, in triplicate, for the detection of nitrite with Griess reagent. Values are means ± SD, N = 3.

TABLE 1. Effects of ethanol, quercetin, and resveratrol on cell viability

	Polyphenols	Ethanol (%)	Absorbance (540 nm)	SD
Unstimulated	—	0	0.096	0.006
LPS + IFNγ	—	0	0.037	0.004
LPS + IFNγ	—	0.1	0.046	0.002
LPS + IFNγ	—	0.25	0.044	0.002
LPS + IFNγ	—	0.5	0.045	0.003
LPS + IFNγ	—	0.75	0.041	0.002
LPS + IFNγ	Quercetin (30 μM)	0.1	0.045	0.001
LPS + IFNγ	Quercetin (30 μM)	0.25	0.042	0.003
LPS + IFNγ	Quercetin (30 μM)	0.5	0.044	0.003
LPS + IFNγ	Quercetin (30 μM)	0.75	0.041	0.001
LPS + IFNγ	Resveratrol (60 μM)	0.1	0.041	0.002
LPS + IFNγ	Resveratrol (60 μM)	0.25	0.038	0.002
LPS + IFNγ	Resveratrol (60 μM)	0.5	0.038	0.002
LPS + IFNγ	Resveratrol (60 μM)	0.75	0.036	0.006

Along with an unstimulated sample, RAW 264.7 cells were stimulated with LPS and IFNγ in the presence of different amounts of ethanol (0 to 0.75%), with or without the grape polyphenol (30 μM quercetin or 60 μM resveratrol). After 24 hr of incubation, cells were washed and then assayed for viability using MTT tetrazolium salt with measurement at 540 nm. Absorbance in the MTT assay is proportional to the number of viable cells. The data were representative of three similar experiments.

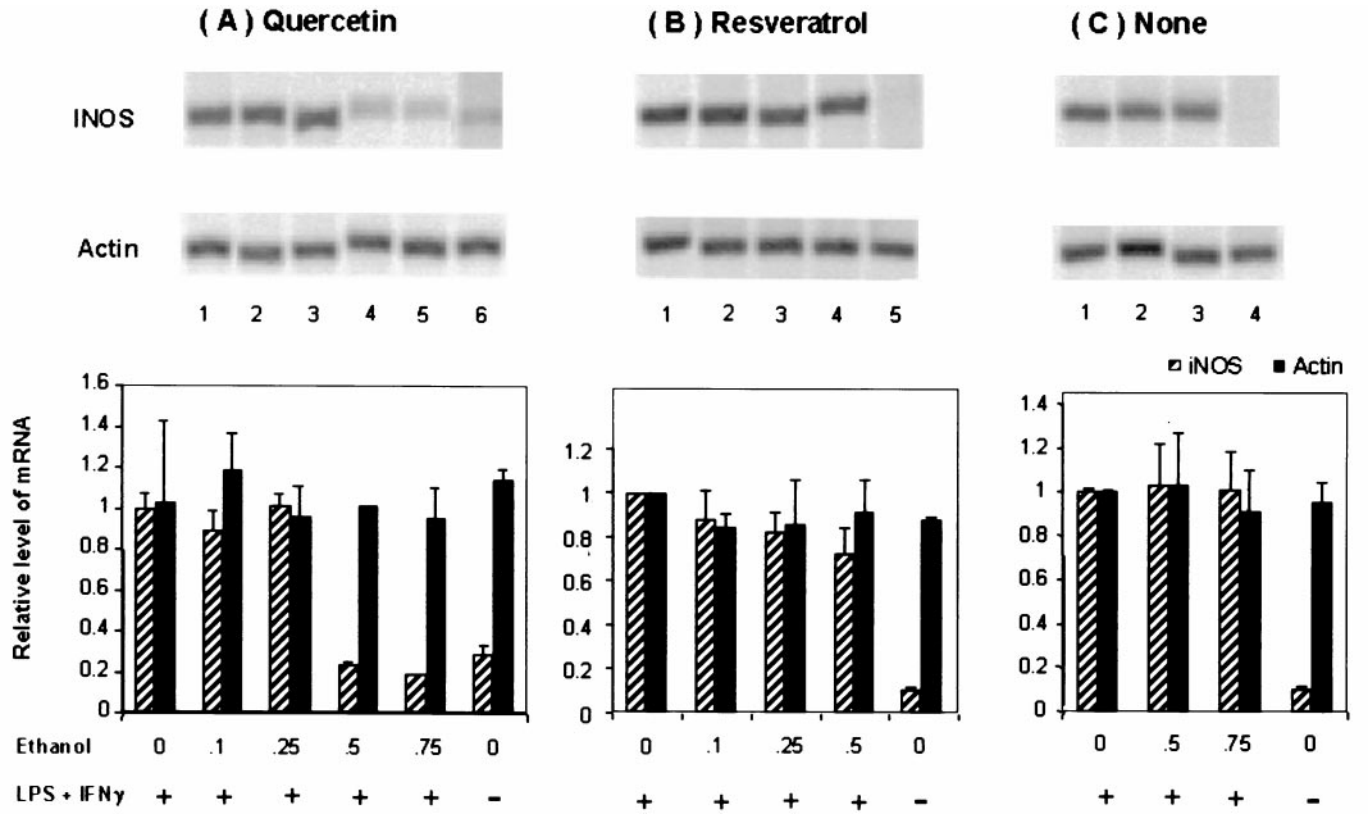

FIG. 6. Effects of combining ethanol with quercetin or resveratrol on macrophage iNOS mRNA expression. Cultures were set up as described in the legend to Fig. 2. The stimulated culture received LPS and γ-IFN (lanes A1–5, B1–4, and C1–3). The unstimulated culture did not receive LPS or γ-IFN (lanes A6, B5, and C4). In panel A, quercetin was added at 30 μM, and ethanol was at final concentrations of 0.1, 0.25, 0.5, and 0.75%. In panel B, resveratrol was added at 60 μM and ethanol was at final concentrations of 0.1, 0.25, and 0.5%. In panel C, ethanol was added at 0.5 and 0.75%. After 4 hr of incubation, the cells were harvested, RNA was extracted, and then RT–PCR was performed for iNOS (experimental) and β-actin (control). The sizes were 496 bp for the NOS band and 540 bp for the β-actin band. From agarose gels, relative levels of mRNA were determined by scanning the PCR products and comparing the intensity of stained DNA bands. The error bars represent SD in optical density of the bands from triplicates of RT–PCR reactions.

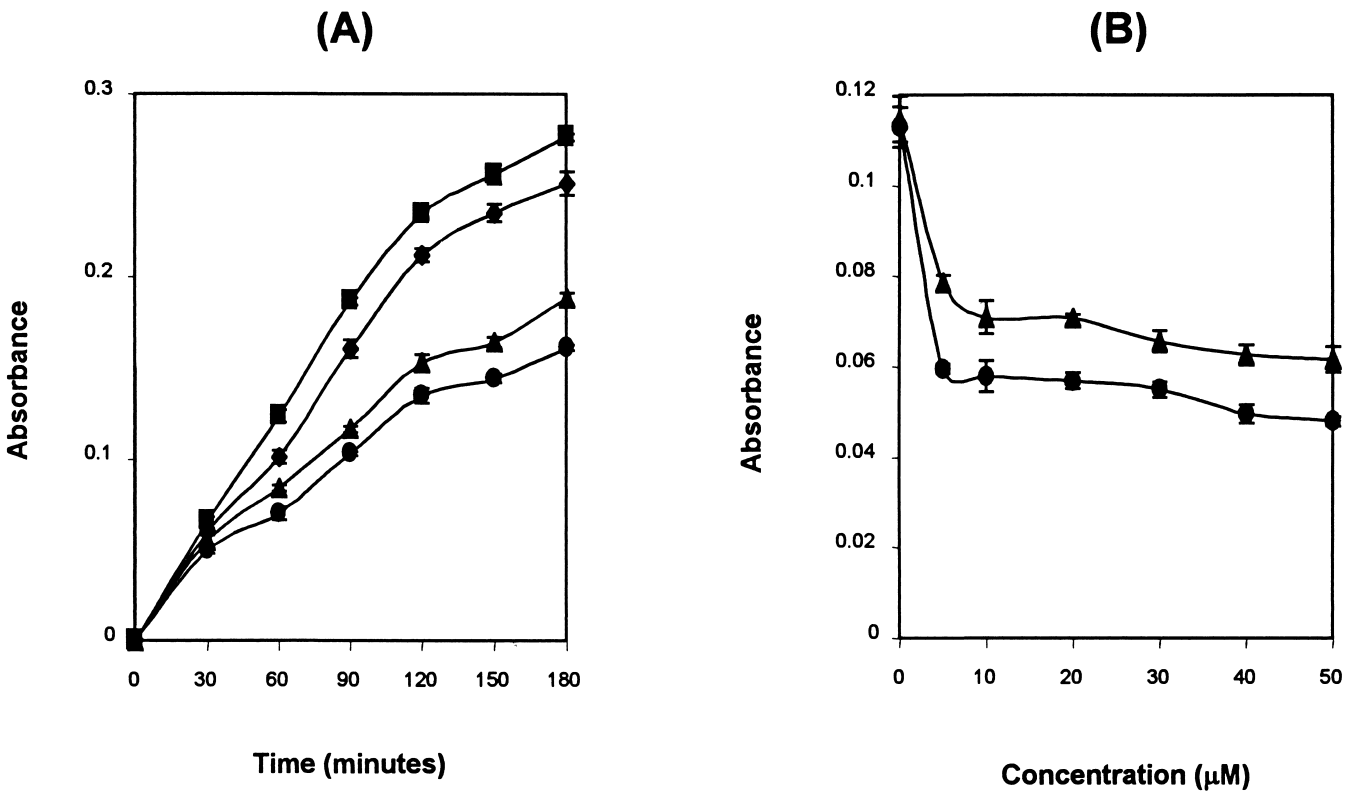

FIG. 7. Scavenging of NO generated from SNP by quercetin and resveratrol. (A) A 10 μL volume of ethanol alone (diamonds), ethanol with 30 μM quercetin (circles), or ethanol with 30 μM resveratrol (triangles) was added to 3 mL of PBS containing 25 mM SNP (final ethanol concentration at 0.33%). The control was SNP in PBS without ethanol (squares). Samples were incubated and aliquots were taken, in triplicate, at a 30-min interval for the detection of nitrite. (B) A 15 μL volume of ethanol with quercetin (0–50 μM, circles) or ethanol with resveratrol (0–50 μM, triangles) was added to 3 mL of PBS containing 5 mM SNP (final ethanol concentration at 0.5%). Samples were incubated for 180 min and then assayed for nitrite content using Griess reagent. Each point represents the mean ± SD of the triplicates.

of 10–13% [42]. Beneficial health effects have been attributed to moderate wine consumption at 2–3 glasses per day. Here, we show that, under our experimental conditions, the effect of ethanol by itself on NO production and iNOS gene expression, is minimal.

Ethanol, however, synergistically increased the efficacy of quercetin and resveratrol in reducing NO production by both scavenging NO and reducing iNOS gene expression. In a cell- and protein-free system, 0.3% ethanol was sufficient for the polyphenols to scavenge over 50% of the NO molecules. Interactions between quercetin and ethanol were implicated with intact cells: quercetin needed $> 0.5\%$ ethanol to inhibit iNOS mRNA expression; efficacy was not detected when the ethanol content was less than 0.5%. One possible explanation is that, as quercetin is insoluble in water, ethanol may act as a solvent to increase its solubility. This abrupt increase, however, was not observed when measuring the production of NO. Hence, most likely, the effect is not due merely to an increase in solubility. Besides, the same pattern of enhancement did not occur with resveratrol. A gradual and moderate enhancement by approximately 10% was observed instead, with statistically significant reduction at 0.5% ethanol. For both compounds, the mechanisms of synergy remain to be investigated, and there are many possibilities. Ethanol may facilitate cellular uptake of the polyphenols. It has been suggested that ethanol inhibits the binding of tannins, another polyphenolic component of red wine, to protein and therefore increases tannin bioavailability [43]. Moreover, these wine components may produce independent effects on different regulatory pathways that in combination result in a higher efficacy. For example, ethanol and quercetin, both scavengers of free radicals, may reduce iNOS mRNA expression by preventing oxidative stress-induced action on the transcription factor NFκB.

Whereas we have used ethanol with quercetin and resveratrol, DMSO was the solvent in other studies. Using the same murine macrophage cell line RAW 264.7, Tsai *et al.* [21], Kim *et al.* [23], and Wadsworth and Koop [22] dissolved quercetin and resveratrol in DMSO (to final concentrations of 0.03, 0.1, and 0.2%, respectively). By itself, DMSO is an inhibitor of NO production and a scavenger of free radicals [44]. Thus, similar to alcohol in wine, it may augment the actions of the grape polyphenols. Extending from these studies, we may speculate that a combination of the antioxidative compounds and molecules in the wine may have additive effects.

Comparing the data among the studies, we found that

the level of efficacy correlated with the amount and nature of the stimulants, implying that clinical efficacy may vary with the severity of inflammation. The relative amount of LPS used may account for the difference in efficacy. Tsai *et al.* [21], using 50 ng/mL of LPS, were able to obtain more effective inhibition of iNOS expression at the mRNA and protein levels with resveratrol than in our study (200 ng/mL of LPS). Wadsworth and Koop [22] used 100 ng/mL of LPS and obtained inhibition of NO production: 24% by 100 μM quercetin, and 16% by 50 μM resveratrol. These values are similar to ours, although we had better inhibition with quercetin. For quercetin inhibition of macrophage NO production, Kim *et al.* [23] used 1 μg/mL of LPS and obtained an IC_{50} value of 107 μM, a much higher value than our 7.6 μM.

Another contributing factor may be the presence of T lymphocyte-derived cytokine, IFNγ. We used both IFNγ (10 U/mL) and LPS to induce macrophage NO production. The others used LPS only [21–23]. Kawada *et al.* [19] examined the effect of quercetin and resveratrol with rat Kupffer cells, and, similar to our findings, they observed inhibition of NO production but not iNOS mRNA expression [19]. It is possible that the freshly isolated cells might have retained cellular sources of IFNγ, although it is also possible that the regulatory controls, such as cytokine requirements for iNOS induction in murine macrophages and rat Kupffer cells, may differ. They detected synthesis of iNOS at the protein level, implicating post-transcriptional control. Nonetheless, we cannot rule out the possibility that the variation in efficacy may be due to batch differences in the polyphenols. Moreover, unfortunately, we were unable to evaluate the methods and solvents used to prepare the compounds in this report.

Independent of ethanol, the mechanisms of action of quercetin and resveratrol may act more by scavenging of NO radicals than by inhibition of iNOS gene expression. The rationale for this deduction is that, without a sufficient concentration of alcohol, these compounds were not very effective in reducing iNOS mRNA under our experimental conditions (in 0.1% ethanol with 10 U/mL of IFNγ and 200 ng/mL of LPS). We detected scavenging of SNP-generated NO by quercetin and resveratrol. Wadsworth and Koop [22], however, reported enhancement. It is not uncommon for antioxidants to be biphasic. The difference may be due to experimental conditions and polyphenol concentrations [45, 46].

Other naturally occurring, antioxidative, phenolic compounds have been shown to scavenge NO and inhibit NO production [47, 48]. For example, genistein, another flavonoid, reduces nitrite levels of stimulated macrophages [49]. Moreover, because quercetin is one of the flavonoids contained in *Ginkgo* extract, we speculate that it may contribute to the NO scavenging and macrophage NO inhibitory activities that have been observed with *Ginkgo biloba* extract EGb 761 [30, 34]. At the level of gene expression, many phenolic compounds that scavenge free radicals, such as curcumin, are inhibitors of iNOS gene expression in murine macrophages [29, 32, 47]. The effect of alcohol on the bioefficacy or bioavailability of these compounds remains to be determined.

The synergism between ethanol and the grape components quercetin and resveratrol may have potential clinical implications. It has been reported that quercetin enhances eNOS-mediated relaxation of rat aortic rings [24, 26], although quercetin inhibits the activity of eNOS enzyme in the form of extracts from bovine aortic endothelial cells [50]. It is interesting to note that both ethanol and resveratrol increase eNOS expression in intact cells [51, 52]. When endothelial cells are stressed, reactive oxygen intermediates, including superoxides, are generated coordinately with NO. Superoxide anion can react with NO to form another highly reactive intermediate, peroxynitirite, which destroys the endothelium. Ethanol and the polyphenols may scavenge NO and superoxide anion, thus preventing the destruction of eNOS-producing endothelial cells in the endothelium of the aortic rings. Enhancement of eNOS has been correlated with suppression of NO production by iNOS, probably due to a decrease of peroxynitrite formation and an attenuation of endothelium destruction [53]. In addition to scavenging NO and inhibiting iNOS gene expression in stimulated macrophages within the inflammation cascade, resveratrol has been shown to inhibit the gene expression of cyclooxygenase-2 through its blocking of protein kinase C and transcription factor AP-1 [18, 54, 55]. Both iNOS and cyclooxygenase-2 are therapeutic targets for inflammatory disorders. Thus, an intake of the grape compounds with alcohol, as in wine, may potentiate their anti-inflammatory actions.

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References

1. Nathan C, Inducible nitric oxide synthase: What difference does it make? *J Clin Invest* **100:** 2417–2423, 1997.
2. Moncada S, Palmer RM and Higgs EA, Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43:** 109–142, 1991.
3. Burney S, Caulfield JL, Niles JC, Wishnok JS and Tannenbaum SR, The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutat Res* **424:** 37–49, 1999.
4. Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG and Marsden PA, Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol* **17:** 2479–2488, 1997.
5. MacNaul KL and Hutchinson NI, Differential expression of iNOS and cNOS mRNA in human vascular smooth muscle cells and endothelial cells under normal and inflammatory conditions. *Biochem Biophys Res Commun* **196:** 1330–1334, 1993.
6. Renaud S and de Lorgeril M, Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **339:** 1523–1526, 1992.

7. Trichopoulou A and Lagiou P, Healthy traditional Mediterranean diet, An expression of culture, history, and lifestyle. *Nutr Rev* **55:** 383–389, 1997.
8. Gronbaek M, Deis A, Sorensen TI, Becker U, Schnohr P and Jensen G, Mortality associated with moderate intakes of wine, beer, or spirits. *BMJ* **310:** 1165–1169, 1995.
9. Rimm EB, Klatsky A, Grobbee D and Stampfer MJ, Review of moderate alcohol consumption and reduced risk of coronary heart disease: Is the effect due to beer, wine, or spirits? *BMJ* **312:** 731–736, 1996.
10. Clifford AJ, Ebeler SE, Ebeler JD, Bills ND, Hinrichs SH, Teissedre PL and Waterhouse AL, Delayed tumor onset in transgenic mice fed an amino acid-based diet supplemented with red wine solids. *Am J Clin Nutr* **64:** 748–756, 1996.
11. Hertog MG and Hollman PC, Potential health effects of the dietary flavonol quercetin. *Eur J Clin Nutr* **50:** 63–71, 1996.
12. Soleas GJ, Diamandis EP and Goldberg DM, Resveratrol: A molecule whose time has come? And gone? *Clin Biochem* **30:** 91–113, 1997.
13. Formica JV and Regelson W, Review of the biology of quercetin and related bioflavonoids. *Food Chem Toxicol* **33:** 1061–1080, 1995.
14. Kuo SM, Dietary flavonoid and cancer prevention: Evidence and potential mechanism. *Crit Rev Oncog* **8:** 47–69, 1997.
15. Brouillard R, George F and Fougerousse A, Polyphenols produced during red wine ageing. *Biofactors* **6:** 403–410, 1997.
16. Rice-Evans CA and Packer L, *Flavonoids in Health and Disease*. Marcel Dekker, New York, 1998.
17. Anonymous, Safety assessment and potential health benefits of food components based on selected scientific criteria. ILSI North America Technical Committee on Food Components for Health Promotion. *Crit Rev Food Sci Nutr* **39:** 203–316, 1999.
18. Lin JK and Tsai SH, Chemoprevention of cancer and cardiovascular disease by resveratrol. *Proc Natl Sci Counc Repub China B* **23:** 99–106, 1999.
19. Kawada N, Seki S, Inoue M and Kuroki T, Effect of antioxidants, resveratrol, quercetin, and *N*-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. *Hepatology* **27:** 1265–1274, 1998.
20. Soliman KF and Mazzio EA, *In vitro* attenuation of nitric oxide production in C6 astrocyte cell culture by various dietary compounds. *Proc Soc Exp Biol Med* **218:** 390–397, 1998.
21. Tsai SH, Lin-Shiau SY and Lin JK, Suppression of nitric oxide synthase and the down-regulation of the activation of NFκB in macrophages by resveratrol. *Br J Pharmacol* **126:** 673–680, 1999.
22. Wadsworth TL and Koop DR, Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochem Pharmacol* **57:** 941–949, 1999.
23. Kim HK, Cheon BS, Kim YH, Kim SY and Kim HP, Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochem Pharmacol* **58:** 759–765, 1999.
24. Fitzpatrick DF, Hirschfield SL and Coffey RG, Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am J Physiol* **265:** H774–H778, 1993.
25. Andriambeloson E, Kleschyov AL, Muller B, Beretz A, Stoclet JC and Andriantsitohaina R, Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *Br J Pharmacol* **120:** 1053–1058, 1997.
26. Chen CK and Pace-Asciak CR, Vasorelaxing activity of resveratrol and quercetin in isolated rat aorta. *Gen Pharmacol* **27:** 363–366, 1996.
27. Cishek MB, Galloway MT, Karim M, German JB and Kappagoda CT, Effect of red wine on endothelium-dependent relaxation in rabbits. *Clin Sci* **93:** 507–511, 1997.
28. Flesch M, Schwarz A and Bohm M, Effects of red and white wine on endothelium-dependent vasorelaxation of rat aorta and human coronary arteries. *Am J Physiol* **275:** H1183–H1190, 1998.
29. Brouet I and Ohshima H, Curcumin, an anti-tumour promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthase in activated macrophages. *Biochem Biophys Res Commun* **206:** 533–540, 1995.
30. Kobuchi H, Droy-Lefaix MT, Christen Y and Packer L, *Ginkgo biloba* extract (EGb 761): Inhibitory effect on nitric oxide production in the macrophage cell line RAW 264.7. *Biochem Pharmacol* **53:** 897–903, 1997.
31. Chan MM, Fong D, Ho CT and Huang HI, Inhibition of inducible nitric oxide synthase gene expression and enzyme activity by epigallocatechin gallate, a natural product from green tea. *Biochem Pharmacol* **54:** 1281–1286, 1997.
32. Chan MM, Huang HI, Fenton MR and Fong D, *In vivo* inhibition of nitric oxide synthase gene expression by curcumin, a cancer preventive natural product with anti-inflammatory properties. *Biochem Pharmacol* **55:** 1955–1962, 1998.
33. Sreejayan and Rao MN, Nitric oxide scavenging by curcuminoids. *J Pharm Pharmacol* **49:** 105–107, 1997.
34. Marcocci L, Maguire JJ, Droy-Lefaix MT and Packer L, The nitric oxide-scavenging properties of *Ginkgo biloba* extract EGb 761. *Biochem Biophys Res Commun* **201:** 748–755, 1994.
35. Chan MM, Ho CT and Huang HI, Effects of three dietary phytochemicals from tea, rosemary and turmeric on inflammation-induced nitrite production. *Cancer Lett* **96:** 23–29, 1995.
36. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS and Tannenbaum SR, Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal Biochem* **126:** 131–138, 1982.
37. Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:** 55–63, 1983.
38. Deaciuc IV, Alcohol and cytokine networks. *Alcohol* **14:** 421–430, 1997.
39. Zhao X, Jie O, Li H, Xie J, Giles TD and Greenberg SS, Ethanol inhibits inducible nitric oxide synthase transcription and post-transcriptional processes *in vivo*. *Alcohol Clin Exp Res* **21:** 1246–1256, 1997.
40. Syapin PJ, Alcohol and nitric oxide production by cells of the brain. *Alcohol* **16:** 159–165, 1998.
41. Greenberg SS, Jie O, Zhao X, Wang JF and Giles TD, The potential mechanism of induction of inducible nitric oxide synthase mRNA in alveolar macrophages by lipopolysaccharide and its suppression by ethanol, *in vivo*. *Alcohol Clin Exp Res* **22:** 260S–265S, 1998.
42. Soleas GJ, Diamandis EP and Goldberg DM, Wine as a biological fluid: History, production, and role in disease prevention. *J Clin Lab Anal* **11:** 287–313, 1997.
43. Serafini M, Maiani G and Ferroluzzi A, Effect of ethanol on red wine tannin-protein (BSA) interactions. *J Agric Food Chem* **45:** 3148–3151, 1997.
44. Liang JF and Akaike T, Inhibition of lipopolysaccharide and cytokine mixture-mediated hepatocyte nitric oxide synthesis by dimethyl sulfoxide. *Biochem Biophys Res Commun* **239:** 517–521, 1997.
45. Decker EA, Phenolics: Prooxidants or antioxidants? *Nutr Rev* **55:** 396–398, 1997.
46. Ohshima H, Yoshie Y, Auriol S and Gilibert I, Antioxidant and pro-oxidant actions of flavonoids: Effects on DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion. *Free Radic Biol Med* **25:** 1057–1065, 1998.

47. Chan MM and Fong D, Modulation of the nitric oxide pathway by natural products. In: *Cellular and Molecular Biology of Nitric Oxide* (Eds. Laskin J and Laskin D), pp. 333–351. Marcel Dekker, New York, 1999.
48. van Acker SA, Tromp MN, Haenen GR, van der Vijgh WJ and Bast A, Flavonoids as scavengers of nitric oxide radical. *Biochem Biophys Res Commun* **214:** 755–759, 1995.
49. Król W, Czuba ZP, Threadgill MD, Cunningham BDM and Pietsz G, Inhibition of nitric oxide ($NO^{\cdot-}$) production in murine macrophages by flavones. *Biochem Pharmacol* **50:** 1031–1035, 1995.
50. Chiesi M and Schwaller R, Inhibition of constitutive endothelial NO-synthase activity by tannin and quercetin. *Biochem Pharmacol* **49:** 495–501, 1995.
51. Hsieh TC, Juan G, Darzynkiewicz Z and Wu JM, Resveratrol increases nitric oxide synthase, induces accumulation of p53 and $p21^{WAF1/CIP1}$, and suppresses cultured bovine pulmonary artery endothelial cell proliferation by perturbing progression through S and G_2. *Cancer Res* **59:** 2596–2601, 1999.
52. Venkov CD, Myers PR, Tanner MA, Su M and Vaughan DE, Ethanol increases endothelial nitric oxide production through modulation of nitric oxide synthase expression. *Thromb Haemost* **81:** 638–642, 1999.
53. Wattanapitayakul SK, Weinstein DM, Holycross BJ and Bauer JA, Endothelial dysfunction and peroxynitrite formation are early events in angiotensin-induced cardiovascular disorders. *FASEB J* **14:** 271–278, 2000.
54. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC and Pezzuto JM, Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275:** 218–220, 1997.
55. Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, Jang M, Pezzuto JM and Dannenberg AJ, Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J Biol Chem* **273:** 21875–21882, 1998.