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Natural Compounds Derived from Foods Modulate Nitric Oxide Production and Oxidative Status in Epithelial Lung Cells

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The effects of natural antioxidants on nitric oxide (NO) modulation and oxidative status were determined in rat epithelial lung cells (L-2). Cells were stimulated with cytokines and treated with one of the following: resveratrol, soybean saponin group B (SSB), quercetin, genistein, olive leaf polyphenol concentrate (OLPC), or *N*-acetyl-L-cystein (NAC). NAC had no effect on NO levels, whereas resveratrol and OLPC were found to be effective in reducing nitrite levels, modifying iNOS mRNA, and decreasing free radical production. OLPC affected the levels of MnSOD while resveratrol did not, indicating that they act via different pathways. Quercetin and genistein reduced nitrite levels without affecting iNOS levels, presumably by scavenging NO. SSB did not affect nitrite levels, but exposure did reduce iNOS mRNA expression and protein levels, possibly due to antioxidant activity. Naturally occurring antioxidants, in particular resveratrol and OLPC, may have therapeutic potential in the treatment of inflammatory diseases.

KEYWORDS: Nitric oxide; inducible nitric oxide synthase; lung epithelial cells; cytokines; resveratrol; polyphenols

INTRODUCTION

Nitric oxide (NO) plays a key role in many physiological processes, including modulation of vascular tone, neurotransmission, immune defense, and proinflammatory activities (*1*). NO is synthesized from the amino acid arginine by nitric oxide synthase (NOS). Under normal physiological conditions, NO production is closely regulated by the calcium-dependent constitutive isoforms of NOS (cNOS), which include the neuronal and endothelial subtypes. It has been shown that NO can induce specific responses via second messenger cGMP. NO binds to the heme group on the soluble isoform of guanylase cyclase (sGC) and activates the enzyme to produce cGMP. Most of the physiological activities induced by NO, such as vasodilatation, are modulated by cGMP (*5*−*7*).

Under pathological conditions, NO production is increased by the inducible isoform of NOS (iNOS). This isoform produces NO in large potentially damaging amounts in a calcium-independent manner (*1*). iNOS is activated by proinflammatory cytokines such as tumor necrosis factor-α (TNFα) and interferon-γ (INFγ) and also by oxidative stress (*2*). Factors such as polyphenols and other antioxidants that influence redox balance in cells may have a direct impact on iNOS activity and expression.

The lung represents a unique tissue in regard to oxidative stress because, as opposed to other organs, it is directly exposed to higher oxygen tensions. A typical characteristic of lung diseases is inflammation and activation of inflammatory cells that cause reactive oxygen species (ROS) production. In lung diseases such as asthma, iNOS expression is often elevated accompanied by increased NO production. While ROS are essential in many physiological processes, when airway cells and tissues are exposed to oxidative stress, high levels of ROS have deleterious effects. Therefore, when the redox balance in cells or tissues is disrupted, it is important to prevent or inhibit oxidative damage. Both enzymatic systems and small antioxidant molecules serve to protect the body against oxidative damage.

One of the most important enzymes involved in antioxidant defense in the lungs is superoxide dismutase (SOD), which turns superoxide radicals into the less harmful peroxide. Superoxide is known to react with NO to produce reactive nitrogen species (RNS), such as peroxynitrite. Thus, SODs have multiple functions in regulating intracellular and extracellular levels of superoxide, peroxide, and RNS (*3*, *4*).

Recently, much attention has focused on natural antioxidants in foods. Diets rich in fruits and vegetables were found to reduce cardiovascular diseases, cancer, and inflammation. It is thought

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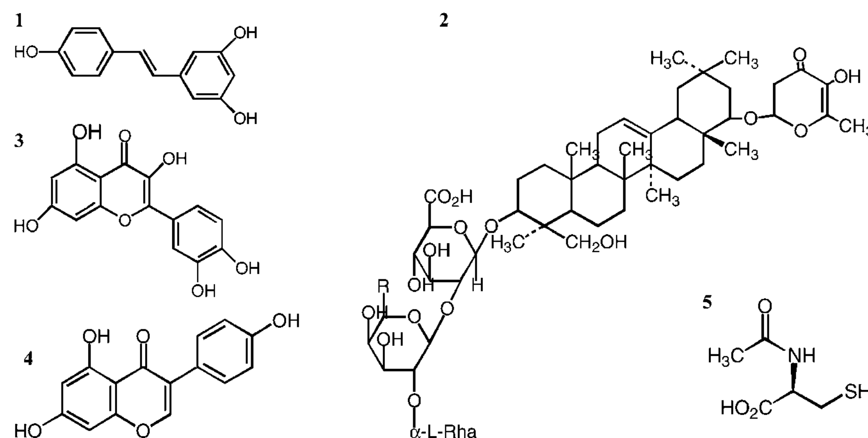

Figure 1. Chemical structures of the compounds used in the study. Resveratrol (1); saponin group B (SSB) with a conjugated 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (R = H or CH_2OH) (2); quercetin (3); genistein (4); and NAC (5).

that the high polyphenol content of these foods is responsible for their biological activity (*8*, *9*). These compounds may also have a beneficial role in preventing the cytotoxic effects of oxidative stress in lung tissue. The mechanisms may involve scavenging of free radicals including NO and modification of gene transcription via induction or inhibition of specific transcription factors.

In the present study, the effect of naturally occurring antioxidants found in food on NO modulation in epithelial lung cells was examined. It was hypothesized that the polyphenols genistein, quercetin, and resveratrol, concentrated olive leaf (*Olea europea*) polyphenols, and group B soybean saponins (SSB), which are conjugated with the reducing moiety 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), would have a beneficial effect on the redox balance in cytokine-stimulated L-2 cells (**Figure 1**).

MATERIALS AND METHODS

Cell Culture. L-2 cells (ATCC CCL-149) were acquired from the American Type Culture Collection. Cells were maintained in culture flasks with F-12 nutrient media supplemented with 1% L-glutamine, 1% penicillin−streptomycin (100 U/mL to 100 μg/mL), and 10% fetal bovine serum (Sigma, Rehovot, Israel) in a humidified atmosphere of 5% CO_2.

Induction with Cytokines. Cells were seeded at 5×10^5 per well onto six well plates. At confluence, the cells were incubated in serum-free media for 24 h. Cells were exposed for 24 h to the following cytokines: murine recombinant TNFα (500 U/mL) and lipopolysaccharide (10 μg/mL) (Sigma), rat recombinant INFγ (50 U/mL; Chemicon International, CA). Cells that were not exposed to cytokines were used for determination of basal NO production.

Cell Treatment with Antioxidants. All experiments were carried out in cytokine-stimulated L-2 cells and cotreated with various antioxidants in increasing concentrations: *N*-acetyl-L-cystein (NAC, 10−35 mM), resveratrol (50−350 μM), quercetin (10−100 μM), and genistein (10−75 μM) (all purchased from Sigma-Aldrich). In addition, the cells were treated with OLPC (0.05−0.35 gallic acid mequiv/mL) or SSB (20−80 μg/mL). OLPC was prepared from olive leaves as follows: The leaves were randomly picked from the Barnea cultivar in the Jezreel Valley region of Israel and immediately freeze-dried on dry ice. After the leaves were thoroughly rinsed with sterile distilled water to remove dust, insecticides, and contaminating material, the leaves were ground and successively Soxhlet extracted with hexane for 3 h and 80% aqueous ethanol for 6 h. The alcoholic extract was concentrated under reduced pressure at 25 °C, reconstituted with 30% ethanol in water, and fractionated by solid phase extraction (SPE) using prepacked Extract-Clean C18 SPE cartridges (10 g; Alltech, Deerfield, IL). The amount of total polyphenols in OLPC was determined using Folin−Ciocalteu reagent and calibrated against gallic acid (*10*). The agents were administrated to cell media concurrently with cytokines. SSB was prepared as described previously (*11*): Dried, finely ground soybean (*Glycine max*) powder was extracted with 1 L of 70% aqueous ethanol with stirring for 3 h at room temperature. The extract was condensed to 100 mL with a rotary evaporator (Büchner, Brinkman, R-114, Switzerland) at <30 °C and loaded on a C-18 extract-clean column (High Capacity C18, Alltech) equilibrated with 10:90 (v/v) methanol/water and then fractionated with a linear gradient of aqueous methanol from 30 to 100%. Saponin-rich fractions were visualized on thin-layer chromatography plates (*13*) and pooled. The fractions containing the SSB were collected and evaporated to dryness at <30 °C. The residue was redissolved in water to obtain a crude SSB solution. High-performance liquid chromatography showing the characteristic absorbance of the DDMP moiety at 292 and 205 nm (*11*) served to ascertain the presence of the conjugated SSB-DDMP as the major compound in these preparations.

Determination of Cell Viability. The cell membrane integrity was detected by flow cytometry (FACSort, BD, San Jose, CA) as a measurement of cell viability. For this assay, the nonpermeant DNA interchelating dye PI that is excluded by viable cells was used. The fluorescence setting was excitation at 488 nm and emission at 575 nm. Cell viability was also measured using trypan blue. Cells were harvested from plates, centrifuged for 5 min at 1100 rpm, and diluted 1:4 with trypan blue. Cells were counted under a microscope.

Medium Collection and Lysates. After treatments with antioxidants, the medium was collected and kept at −80 °C for the nitrite assay. Cells were incubated for 20 min in lysis buffer (150 mM NaCl, 50 mM tris base, 5 mM EDTA, 0.5% NP-40, and protease inhibitors). Lysates were collected and kept at −20 °C for future assays.

Nitrites Assay. Nitrite in culture media was measured by the Griess reaction (*12*) and used as an index of NO production by L-2 cells. The measurement of nitrite was performed by mixing 100 μL of culture media with 100 μL of Griess solution. The absorbance was measured at 540 nm, using an enzyme-linked immunosorbent assay reader. The values obtained were compared with standards of sodium nitrite dissolved in F-12 nutrient media. Nitrite release was calculated and expressed in micromolar.

iNOS/SOD Protein Expression (Western Blot Analysis). Proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. Polyclonal antibodies against iNOS and MnSOD (Transduction Laboratories, Lexington, KY) or against CuZnSOD (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were diluted 1:20000, 1:5000, and 1:2000, respectively. The immune reaction was detected by enhanced chemiluminescence. Bands were quantified by scanning densitometry and expressed as arbitrary units.

Total RNA Isolation and Reverse-Transcribed Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was isolated by the procedure of Chomczynski and Sacchi (*14*). Analyses of mRNA levels of iNOS and GapdH were performed using RT-PCR. For the synthesis of cDNA, 3 μg of total RNA was incubated with 0.5 μg of Oligo dT

at 100 °C for 5 min. After 5 min of incubation in ice, 5 μL of 5× PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl, 1.25 μL of 10 mM (each) dNTP Mix (Nucleotide mix) and 0.5 μL of M-MLV RT (H−) were added. The reaction mixture was left to equilibrate at 25 °C for 15 min before synthesis was allowed to proceed at 40−55 °C for 50 min. Finally, the reaction was terminated by incubation at 70 °C for 15 min. PCR amplification was performed over 32 cycles in the Biometra T personal cycler instrument. Cycles were performed at 94 °C for 30 s followed by 57 °C for 1 min and 72 °C for another min. cDNA was incubated with gene specific PCR primers, designed using Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_ www.cgi). The primers were synthesized by MBC-Israel. iNOS: forward primer, 5′-CAGCACAGAGGGCTCAAAGG-3′; reverse primer, 5′-TCGTCGGCCAGCTCTTTCT-3′. As a loading control, RNA was hybridized with a probe of the housekeeping gene, β-actin: forward primer, 5′-GCCGGGACCTGACAGACTAC-3′; reverse primer, 5′-TGTACCCAGGCATTGCTGAC-3′. PCR products were electrophoresed on 1% agarose gel containing 30 μL of ethidium bromide and the gel image was quantified using Doc-it gel image analysis program (UVP, United Kingdom).

Gel Assay for SOD Activity. Cell lysates were diluted 4:1 with glycerol. Samples were subjected to (10%) PAGE. The in-gel assay was preformed under nondenaturizing conditions, as previously described (*14*). Briefly, the gel was incubated in a staining solution containing 0.01% riboflavin, 0.02% EDTA, and 50 mM tris buffer (pH 7.6) for 10 min. The gel was then incubated in a solution containing 0.025% nitrioblue tetrazolium (NBT) and 0.02% EDTA in 50 mM tris buffer (pH 7.6) for an additional 10 min. Next, the gel was exposed to UV light for 1 min, washed with water to remove excess NBT, and then dried without heating for 1 h, and scanned. The densitometric analysis was preformed using the Gel-pro 32 program.

cGMP Measurement. cGMP levels were detected by radioimmunoassay. Fifty microliters from each sample was incubated with 50 μL of antibody against cGMP (Sigma Chemicals, St. Louis, MO) for 4 h on ice. A 200 μL amount of cGMP-I^{125} was added to each test tube, and samples were incubated for 20−24 h at 4 °C. The reaction was stopped by adding 200 μL of 10% bovine serum albumin solution, and samples were centrifuged for 15 min at 1400*g*. The supernatant was vacuumed, and the pellet was read in a γ-counter.

Intracellular ROS Production. Intracellular ROS were detected using H_2DCFH-DA. After the different treatments, the cells were washed three times with PBS. Cells were centrifuged (1100*g* rpm, 5 min), resuspended in PBS, and incubated with dichlorodihydrofluorescein diacetate (H_2DCFH-DA) (25 μM) for 30 min at 37 °C. To detect intracellular fluorescence, the fluorochrome-loaded cells were excited using a 488 nm argon-ion laser in a flow cytometer (FACSort, BD). The dichlorofluorescein emission was recorded at 530 nm. Data were collected from at least 10000 cells.

Statistics. A two-way analysis of variance test was used for statistical analysis. Differences were examined by the Tukey−Kramer test and found to be significant at $p < 0.05$.

RESULTS

Dose−Response Measurements. To determine appropriate working concentrations for each of the compounds used in this study, dose−response experiments were preformed. To this end, L-2 cells were incubated with a cytokine mixture (10 μg/mL lipopolysaccharide, 100 U/mL INFγ, and 500 U/mL TNFα) and specific compounds in increasing concentrations. All experiments in this study were carried out using cotreatment of cytokine stimulators and antioxidants because in the presence of antioxidants alone no detectable impact on NO levels was observed. The working concentration of each compound was selected as the maximum concentration that affected nitrite levels without impairing cell viability. Concentrations chosen for each compound were as follows: NAC, 10 mM; resveratrol, 100 μM; SSB, 60 μg/mL; OLPC, 0.05 gallic acid mequiv/mL; quercetin, 75 μM; and genistein, 50 μM. Cell viability was not affected by any of the compounds at the selected concentration.

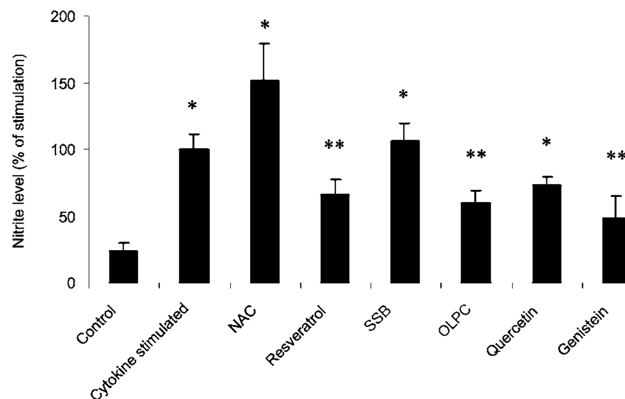

Figure 2. Effect of different compounds on nitrite levels in the cell culture medium. Results are shown as percent of stimulated group. *$p < 0.05$, significant from control group. **$p < 0.05$, significant from control and stimulated.

Nitrite Levels in the Medium. Nitrite levels in the cell medium following incubation with different compounds were determined. L-2 cells were incubated with acytokine mixture and treated with specific compound (**Figure 2**). Exposure to cytokines significantly elevated medium nitrite levels in comparison to controls. The addition of resveratrol, genistein, and OLPC to cell cultures cotreated with cytokines significantly reduced nitrite levels by approximately 40%. Other compounds tested had no significant effect on NO levels.

mRNA and Protein Levels of iNOS. The above results show that nitrite levels are affected by the application of selected compounds. To determine whether the change of nitrite levels was mediated by iNOS expression, mRNA and protein levels were analyzed. Cells were treated as described above, and cell lysate or total RNA was collected 24 h after treatment. Protein expression was determined by western blot analysis (**Figure 3**). iNOS expression was significantly higher after stimulation with cytokine mixture as compared to control. Enzyme expression decreased by 60 and 50% following incubation with resveratrol and SSB, respectively. Inducible NOS mRNA levels in untreated cells (control) were very low (**Figure 4**) and increased in induced cells. Resveratrol, SSB, and OLPC decreased iNOS mRNA levels by 40%. Significant results were only achieved using OLPC.

ROS Production. To determine whether oxidative stress is involved in NO/iNOS modulation, ROS production by L-2 cells was tested after incubation with the cytokine mixture, resveratrol and OLPC. Twenty-four hours after treatments, cells were harvested and incubated with DCFH for 30 min. ROS production levels were determined using FACS (**Figure 5**). Incubation with the cytokine mixture led to increased ROS levels. Incubation with resveratrol or OLPC reduced ROS levels similar to those of nontreated cells.

SOD Isoform Expression and Total SOD Activity. The total SOD activity and SOD isoform expression following incubation with different compounds were determined, because SOD plays an important role in antioxidant defense in the lungs. The total SOD activity was measured using the in-gel activity assay, and differential isoform expression was determined by western blot analysis. The total SOD activity and CuZnSOD expression were not affected by cytokine stimulation or by application of any of the compounds (data not shown). MnSOD expression was significantly higher after incubation with cytokines as compared to control and moderately reduced after incubation with SSB. MnSOD expression increased after incubation with OLPC, quercetin, and genistein (**Figure 6**).

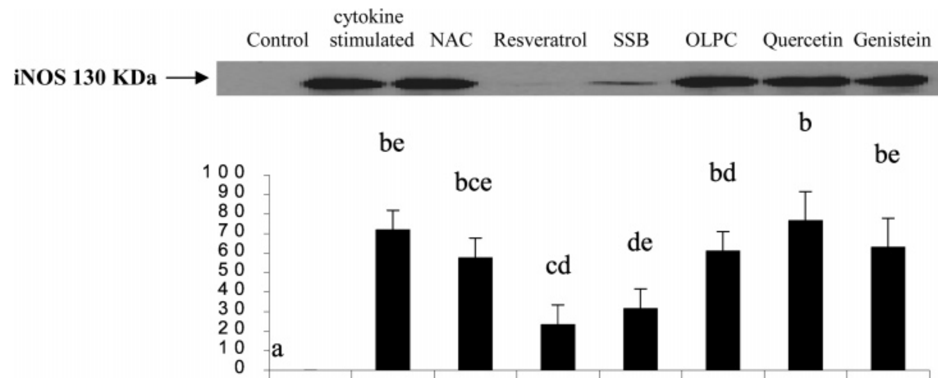

Figure 3. iNOS protein levels in cytokine-stimulated L-2 cells exposed to natural compounds. Protein levels are expressed as arbitrary units. Values are means ± SE; means with different letters differ, $p < 0.05$.

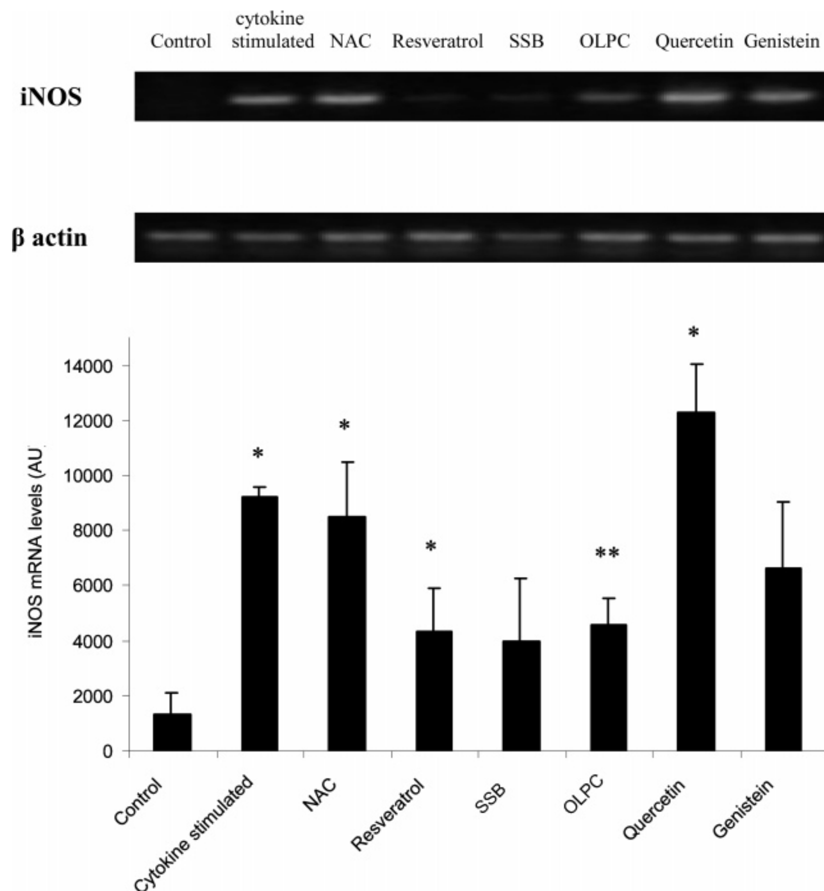

Figure 4. Effect of natural compounds on iNOS mRNA levels in cytokine-stimulated L-2 cells. iNOS mRNA levels are expressed as arbitrary units. *$p < 0.05$, significant from control group. **$p < 0.05$, significant from control and stimulated.

cGMP Levels. The modulation of several of the physiological activities of NO by the second messenger cGMP is well-known. Therefore, the effect of the selected compounds on cGMP levels was measured, using RIA, 24 h after incubation of the L-2 cells with cytokines. However, no measurable differences were found in cGMP levels following stimulation with cytokines or incubation with the studied compounds.

DISCUSSION

The present study examined the effect of natural dietary products on nitrite production and NO levels in rat lung epithelial cells (L-2). It is well-established that iNOS is a key enzyme involved in mediating inflammatory processes in the lung. Therefore, enzyme expression following induction of

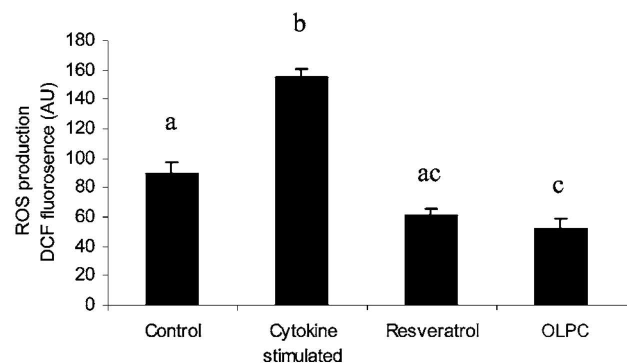

Figure 5. ROS production in cytokine-stimulated L-2 cells treated with resveratrol and OLPC. Results are shown as arbitrary units. Values are means ± SE; means with different letters differ, $p < 0.05$.

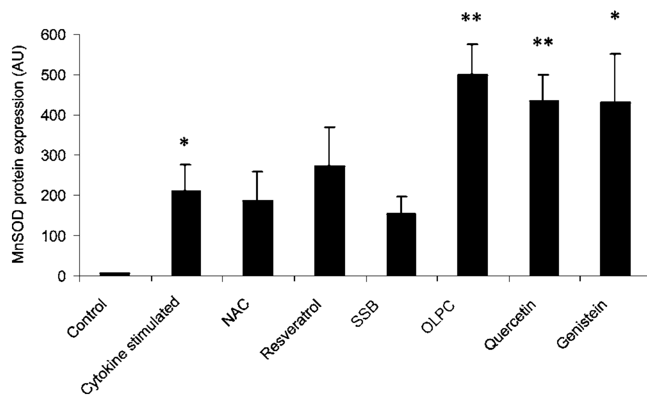

Figure 6. Effect of natural compounds on MnSOD expression in cytokine-stimulated L-2 cells. MnSOD protein levels are expressed in arbitrary units. *$p < 0.05$, significant from control group. **$p < 0.05$, significant from control and stimulated.

inflammation and treatment with various natural products was determined.

Cytokine stimulation led to a significant increase in NO levels in the L-2 cell model. However, elevated NO levels were significantly reduced following treatment with either resveratrol or OLPC (**Figure 2**). A similar but somewhat smaller inhibitory effect on NO production was also observed using quercetin. Resveratrol is a polyphenol found in red wine and grapes and was selected for its recognized antioxidative properties and health-promoting potential. In this work, it reduced iNOS mRNA and enzyme levels (**Figures 3** and **4**). Several studies have previously demonstrated resveratrol's ability to reduce oxidative agents and modulate iNOS expression in cell lines (*16*, *17*). Tsai et al. (*18*) found that resveratrol inhibited NO production in activated macrophages and significantly reduced iNOS levels (both mRNA and protein levels). An additional antioxidative mechanism for resveratrol was proposed by Chan et al. (*19*) suggesting that resveratrol has the ability to chelate NO molecules, thereby lowering cellular NO levels. The effects of resveratrol have been shown to be tissue specific. Work in cardiac tissue, for example, indicates that exposure to resveratrol increases NO production via both eNOS and iNOS (*20*, *21*).

Unlike resveratrol, quercetin was also able to reduce NO levels in stimulated lung cells but without modifying iNOS levels (**Figures 2**−**4**). This observation suggests that quercetin does not impact protein synthesis; rather, it acts as an antioxidant and is capable of chelating NO molecules. Banerjee et al. (*22*) showed that quercetin is able to lower iNOS levels with no apparent change in its mRNA levels. This discrepancy may be attributed to the different cell lines used in the experiments. Banerjee et al. used a human lung carcinoma cell line, while the cells used in the present study were healthy rat lung cells.

In the present experimental model, exposure to OLPC resulted in a dramatic decrease in NO levels, suggesting that OLPC may be beneficial for use in treatment of lung inflammations. To the best of our knowledge, this is the first evidence that polyphenols extracted from olive leaves have antiinflammatory properties.

Genistein, a principal polyphenol in soy, was also tested in the present study. Genistein alone did not change iNOS levels. However, it was capable of reducing NO levels (**Figures 2**−**4**). Soy isoflavones are thought to be potent chelators of nitrite radicals (*23*). Although several studies have previously demonstrated genistein's ability to lower NO levels (*24*), our study is the first to show that genistein is a potent agent in reducing NO levels in inflammatory lung cells.

SSB, a group of reducing saponins abundant in soybean (*13*), had a little impact on NO levels in this model (**Figure 2**). However, SSB reduced mRNA and protein levels of iNOS (**Figures 3** and **4**). NO levels were not influenced by SSB application, thus suggesting that SSB may affect other isoforms of NOS (i.e., eNOS). The antioxidant agent NAC also showed no effect on NO or iNOS levels in lung epithelial cells (**Figures 2**−**4**). However, it has been reported that in hepatic and endothelial cells treated with NAC, a precursor of glutathione, which traps free radicals, iNOS expression was elevated (*25*, *26*). Differences in tissue specificity for NAC action would account for these seemingly conflicting results.

Additional experiments were carried out to examine the relationship between the NO levels and the oxidative state. The data presented here demonstrate that resveratrol and OLPC had the most notable effect on NO and iNOS levels. Therefore, ROS concentrations were measured following the application of these two compounds. Indeed, radical scavenging capacities of these compounds (*27*, *28*) were demonstrated by the reduction of cytokine-elevated ROS levels (**Figure 5**).

cGMP, a known second messenager of NO action, was not affected by cytokines alone or by the various treatments in this experimental model. Although NO levels differed among treatments, the possibility exists that in lung epithelial cells, NO has a greater impact on peroxynitrate levels or act via tyrosine kinases while other factors determine cGMP levels.

Altering the expression or the activity of antioxidant enzymes such as SOD is another cell defense mechanism against oxidizing agents. SOD is known to play a key role in antioxidative processes in lung tissues (*29*, *30*). Thus, the effects of the natural products on the expression of two isoforms of SOD, MnSOD, and CuZnSOD were determined along with total SOD activity. Surprisingly, the overall activity was not affected by the stimulatory treatment with cytokines and/or the natural products (**Figure 6**). Although CuZnSOD is the most abundant isoform in the cytosol (*31*), in this study, its levels were not modified following various treatments, while MnSOD levels were considerably elevated. These results are confirmed by previous studies demonstrating that CuZnSOD levels are not affected by cytokine-induced inflammation (*3*, *32*−*34*). The elevation of MnSOD levels in L-2 cells detected following treatment with quercetin, OLPC, or genistein may have contributed to the decreased levels of oxidizing agents, leading to reduction of NO levels in the cells. To the best of our knowledge, this effect has not been reported before for these compounds, although polyphenols from other sources have been shown to affect SOD enzymes (*35*, *36*). Resveratrol, NAC, and SSB showed no effect on SOD activity; thus, their mode of action presumably does not involve SOD modulation.

To conclude, the effects of dietary antioxidants on NO and iNOS levels were studied following cytokine-induced inflammation in lung epithelial cells. Several antiinflammatory mechanisms were induced in the tested cells by the different natural products. While some compounds act as NO scavengers, others modify iNOS levels. Additional mechanisms of control via SOD were also assessed. This work demonstrates the feasibility of using dietary antioxidants as possible "alternative treatments" for lung inflammation.

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