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Original Scientific Paper

EVALUATION OF RESVERATROL AND PICEATANNOL CYTOTOXICITY IN MACROPHAGES, T CELLS, AND SKIN CELLS*

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The cytotoxicity of resveratrol and of piceatannol, a structural analog of resveratrol, was examined in cultured cells. Using a MTT-based assay, which measures the conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to a colored formazan product in living cells, resveratrol was found to inhibit the viability of transformed mouse macrophages, tumor-derived human T cells and human epidermoid carcinoma cells in a concentration-dependent manner, with the effect decreasing in the order: T cells (LC_{50} ~27 μmol L^{-1}, 24 h; ~9 μmol L^{-1}; 48h) > macrophages (LC_{50} ~29 μmol L^{-1}, 24 h; 39 μmol L^{-1}, 48 h) > skin cells (LC_{50} ~91 μmol L^{-1}, 24 h ; ~66 μmol L^{-1}, 48 h). Paradoxically, a high concentration of resveratrol (50 μmol L^{-1}) inhibited the proliferation of all three cell types, and a low concentration (5 μmol L^{-1}) stimulated the proliferation of macrophages. The viability of macrophages was also decreased by piceatannol in a concentration-dependent manner. The stimulation of macrophages with zymosan lowered the cytotoxicity of both resveratrol and piceatannol. Scanning electron microscopy of cells treated with resveratrol revealed changes in cellular morphology that were consistent with toxicity. In macrophages and skin cells, resveratrol (50 μmol L^{-1}) induced a time-dependent increase in reduced glutathione levels but did not alter the background levels of thiobarbituric acid-reactive substances. Taken together, the present data indicate that resveratrol is toxic to cultured macrophages, T cells and skin cells at concentrations ≥25 μmol L^{-1}, and that the cytotoxicity occurs via a mechanism that does not involve oxidative stress. Furthermore, the degree of toxicity of both resveratrol and piceatannol towards macrophages depends on the activation status of these cells, with zymosan-activated cells appearing more resistant than nonstimulated cells.

KEY WORDS: *A-431 cells, CEM T cells, glutathione, RAW264.7 cells, TBARS, viability, zymosan*

Resveratrol is an antioxidant compound naturally found in peanuts, pistachio nuts, red grape seeds and skins, red wine, and other plant-derived food products (1-5). From a chemical standpoint, the structure of this polyphenol (Figure 1) bears a close similarity to that of the synthetic estrogen diethylstilbestrol (6), a feature that may account for its reported estrogenic activity (7, 8). In plants, the synthesis of resveratrol is under the control of stilbene synthase, an enzyme that can undergo up-regulation in response to environmental stressors, such as exposure to ultraviolet light or infection (9, 10).

Numerous *in vitro* and *in vivo* studies have determined resveratrol to possess both cytoprotective and cytotoxic properties. Thus, in addition to functioning as an antioxidant and demonstrating cardioprotective, chemopreventive, and neuronal-sparing effects (11-14), this compound is also reported to be tumoricidal, hemolytic, and toxic to the genitourinary tract (11, 15). The mechanism by

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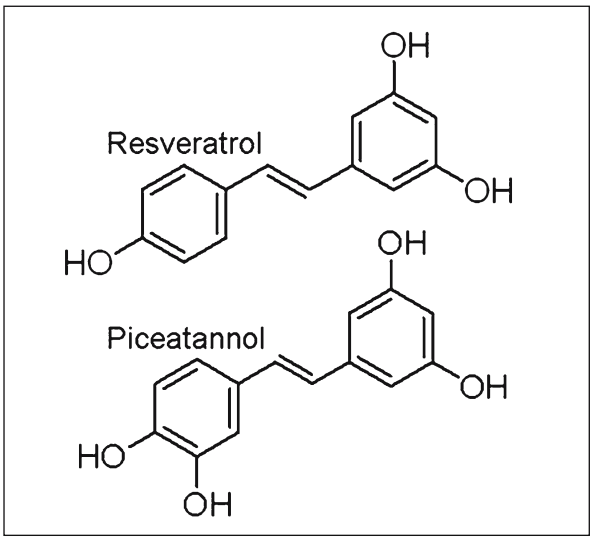

Figure 1 *Chemical structures of resveratrol (5-[2-(4-hydroxyphenyl) vinyl]benzene-1,3-diol), and piceatannol (4-[2-(3,5-dihydroxyphenyl)vinyl]benzene-1,2-diol)*

which resveratrol can act both as a cytoprotectant and as cytotoxicant may stem from the wide range of biological actions that it can exert in mammalian cells, including the regulation of cell proliferation, the induction of apoptosis, the promotion of cell differentiation, the inhibition of pro-inflammatory mediator production, and the suppression of reactive oxygen and nitrogen species formation (14, 16-18).

With respect to its effects on the immune system, resveratrol exhibits antiproliferative activity and appears to be a potent inducer of T cell apoptosis (19-23). Though the anti-proliferative effects of resveratrol on macrophages have not been fully studied and defined, its potent anti-inflammatory properties toward cells participating in nonspecific immunity has been observed. To this end, resveratrol is a potent inhibitor of reactive oxygen species produced by zymosan-stimulated macrophages, monocytes, and neutrophils (24). Resveratrol and its analogs have also been found to inhibit the production of proinflammatory mediators such as prostaglandins and nitric oxide from stimulated macrophages exposed to endotoxin (25). Whereas these two studies demonstrate how macrophages stimulated with immunogens such as zymosan or endotoxins respond to resveratrol, the response of nonstimulated macrophages to a challenge with this polyphenol remains poorly defined. Similarly, the cytotoxic potential of resveratrol towards skin cells is at present unclear. However, while evidence exists to suggest an antiproliferative activity of this compound on certain types of skin cells *in vitro* (26-28), data generated from *in vivo* studies have not been as conclusive (29).

Therefore, the present study was undertaken with three specific aims: (a) to evaluate the cytotoxic properties of resveratrol *in vitro* by using RAW264.7 macrophages, CEM T cells, and A-431 skin cells; (b) to determine if resveratrol has any effect on the intracellular antioxidant status; and (c) to compare the cytotoxicity of resveratrol with that of its metabolite piceatannol, a hydroxylated analog of resveratrol, in RAW264.7 macrophages, with and without stimulation by zymosan.

MATERIALS AND METHODS

Chemicals

Resveratrol (5-[2-(4-hydroxyphenyl)vinyl]benzene-1,3-diol), piceatannol (4-[2-(3,5-dihydroxyphenyl)vinyl]benzene-1,2-diol), zymosan (from *Saccharomyces cerevisiae)*, and all other chemicals, unless otherwise indicated, were obtained from Sigma Chemical Company (St Louis, MO). The declared purity of both resveratrol and piceatannol was >99 %.

Cells and cell culturing conditions

RAW264.7 mouse macrophages and A-431 human epidermoid carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). The former cells were established from a tumor induced by Abelson murine leukemia virus (30), and the latter ones were skin cells established from a solid tumor in an elderly female (31). CEM cells are human T lymphoblasts originally isolated from the peripheral blood of a child with acute lymphocytic leukemia (32) and were kindly provided by Dr. Jason Chen (St. John's University, Jamaica, NY). In all experiments, cells were grown to 90 % confluence in Dulbecco's Modified Eagle's Medium (DMEM) containing glutamine, 4.5 g L^{-1} glucose, 0.1 mg mL^{-1} gentamicin (Invitrogen), and 10 % heat-inactivated fetal bovine serum (complete DMEM) on 24-well plates.

Cell viability studies

Macrophages and A-431 cells. All media was removed from the wells and replaced with 0.5 mL of serum-free and phenol red-free growth medium containing increasing concentrations of resveratrol (0 μmol L^{-1} to 50 μmol L^{-1}). In studies evaluating the effect of immunostimulation of macrophages with zymosan, the medium was replaced with serum-

and phenol red-free growth medium containing increasing concentrations of resveratrol or piceatannol (0 μmol L^{-1} to 50 μmol L^{-1}) plus zymosan (0 μmol L^{-1} to 30 μg mL^{-1}). All cells were cultured for either 24 h or 48 h, after which the MTT viability assay was performed as described by Mosmann (33). In separate experiments, macrophages were similarly treated with piceatannol (0 μmol L^{-1} to 50 μmol L^{-1}).

T cells. Cells were grown to 90 % confluence in complete DMEM in cell culture plates, after which they were divided into four equal portions, centrifuged, and re-fed with serum-free and phenol red-free growth medium containing increasing concentrations of resveratrol (0 μmol $L^{-1}$to 50 μmol L^{-1}). Cells were then cultured for 24 h or 48 h, after which the MTT assay was performed.

Cell proliferation studies

Macrophages, T cells, and human epidermoid carcinoma cells were inoculated into 6- well plates ($1x10^4$ cells/well), and cultured in complete DMEM in the absence or presence of resveratrol (0 μmol L^{-1} to 50 μmol L^{-1}). Cells were harvested at specified intervals, and the number of cells per well was determined with the aid of a hemocytometer. The cells were washed twice with phosphate buffered saline (PBS) prior to harvesting, and only those cells able to exclude trypan blue were counted.

Scanning electron microscopy

Nonadherent T cells were grown in culture solution and adherent skin cells were grown on cover slips. Both cell types were seeded in 6-well plates and exposed to increasing concentrations of resveratrol (0 μmol L^{-1} to 100 μmol L^{-1}) for 24 h or 48 h. Prior to fixation, T cells were isolated by filtration onto filter paper with the aid of suction. All samples were washed with PBS, and fixed in 1.5 % glutaraldehyde in phosphate buffer (pH 7.4) for 1 h at 0 °C to 4 °C. Dehydration was accomplished through a series of water to acetone steps. Cells were critically point dried (CPD) in a Polaron E 3000 using bone dry carbon dioxide as a transition fluid, and sputter-coated with 15 nm of platinum for 90 s using a Polaron E 5100 series II coater set at 2.5 kV. The specimens were viewed on a Hitachi S-530 scanning electron microscope at 25 kV with a eucentric stage.

Glutathione assay

The intracellular levels of reduced glutathione (GSH) in both macrophages and epidermoid carcinoma cells were measured by an adaptation of the method described by Tietze (34). Briefly, cells were first incubated in the presence (50 μmol L^{-1}) or absence of resveratrol for either 24 h or 48 h, then washed twice with PBS, scraped, and collected into microfuge tubes and followed by a brief centrifugation. After discarding the supernatant, the cell pellet was resuspended in 100 μL of PBS, freeze/thawed three times, mixed with 100 μL of 5 % metaphosphoric acid, and centrifuged at 14000 rpm for 5 min. A 100 μL of volume of supernatant was withdrawn, transferred to a new microfuge tube, and mixed with a reagent solution containing triethanolamine, EDTA, PBS, and 5,5'-dithiobis (2-nitrobenzoic acid). The absorbance of the solution was read on a spectrophotometer at 405 nm , and used to calculate the GSH content by comparison to a GSH standard curve prepared from graded dilutions of a GSH solution (1 mmol L^{-1} in water).

Lipid peroxidation

Evidence of lipid peroxidation in both macrophages and epidermoid carcinoma cells was obtained by measuring the levels of malondialdehyde (MDA) present as thiobarbituric acid reactive substances (TBARS) according to Fraga (35). Briefly, cells were treated with increasing concentrations of resveratrol (0 μmol L^{-1} to 50 μmol L^{-1}), incubated for 48 hr, collected into microfuge tubes and then centrifuged at 4000 rpm for 10 min. Separate groups of cells were also incubated for 48 hr in the presence of varying concentrations of the nitrogen mustard mechlorethamine (0 μmol $L^{-1}$to 50 μmol L^{-1}), a known inducer of TBARS formation, to serve as a positive control. From each sample, 400 μL of supernatant was removed, transferred to a test tube, mixed with 800 μL of TBARS reagent (15 g of trichloroacetic acid, 0.375 g of thiobarbituric acid, 4.16 mL of 6 mol L^{-1} HCl in sufficient water to make 100 mL), and incubated at 95 °C for 1 h. After cooling the tube under running water, the absorbance of the solution was read at 532 nm. The concentration of TBARS was calculated by comparison to a MDA standard curve prepared from graded dilutions of 1,1,3,3-tetraethoxypropane (10 ng mL^{-1} in water) and treated as the samples.

Statistical analysis of data

Unless otherwise indicated, all experiments were carried out in triplicate, and their results are reported

as the mean ± standard error of the mean from at least three representative experiments. Statistical comparisons were made using GraphPad Prism 4.0® software (GraphPad Software, Inc., San Diego, CA) by Student's *t*-test, followed by one-way ANOVA and Newman-Keuls *post hoc* test. Differences were considered to be significant at $p<0.05$.

RESULTS AND DISCUSSION

The effect of resveratrol on cell viability was ascertained by a standard MTT assay after culturing A-431 skin cells, RAW264.7 macrophages, and CEM T cells for 24 h and 48 h in serum-free DMEM containing 0 μmol L^{-1} to 75 μmol L^{-1} of resveratrol. Resveratrol reduced the viability of all three cell types in a concentration-dependent manner (Table 1). Among these three cell types, skin cells were the most resistant (LC_{50} ~91 μmol L^{-1} at 24 h and ~66 μmol L^{-1} after 48 h), T cells the least resistant (LC_{50} ~27 μmol L^{-1} at 24 h and ~9 μmol L^{-1} after 48 h), and macrophages exhibited an intermediate sensitivity (LC_{50} ~29 μmol L^{-1} at 24 h and ~39 μmol L^{-1} after 48 h) to resveratrol. Finding that the LC_{50} value for macrophages incubated with resveratrol for 48 h to be higher than that of cells treated for only 24 h was unexpected and deserving of a future explanation. It is possible that the macrophages mobilize defense mechanisms that will protect them from the treatment agent and which only become fully active after 24 h. Alternately, these cells might metabolize the compound over time to a form that is less toxic. It is likely that the decreased viability of all three cell types in the presence of resveratrol is occurring, at least in part, through a mechanism ending in apoptosis. This assumption is based on reports from other laboratories describing the occurrence of DNA fragmentation in cells exposed to this natural polyphenol *in vitro* (36-38).

Finding that relatively higher concentrations of resveratrol are required to kill skin cells than either macrophages or T cells is taken as a suggestion of a higher innate resistance to resveratrol by cells derived from solid tumors than blood-derived tumor cells and, at the same time, may explain why resveratrol failed to prevent the spread of melanoma cells, another type of tumor-derived skin cell, in a mouse model (29). Differences in cell sensitivity to resveratrol may also be related to the presence of p53, a protein known to play a role in a cell response to DNA damage by xenobiotics, including resveratrol (39-41). In particular, human epidermoid cells have been found to express a mutated form of p53 that codes for a missense variant (Arg to His at codon 273, R273H) (42,43). Expression of the p53-R273H variant in skin cells has been found to increase cellular resistance towards other toxic compounds such as methotrexate and doxorubicin and may, in part, contribute to the greater resistance of skin cells to resveratrol than either macrophages or T cells (43).

Scanning electron microscopy (SEM) was used to determine if cytotoxicity by resveratrol was also accompanied by changes in cell morphology. As shown in Figure 2, left panel, untreated T cells demonstrated a normal morphology and a smooth surface. However, the treatment of these cells with resveratrol (50 μmol L^{-1}; 24 h) led to the morphological changes seen in Figure 2, center panel. Deterioration of the cell membrane is evident, resulting in a rough appearance of the cells. Doubling the concentration of resveratrol (100 μmol L^{-1}; 24 h), caused the T cells to become crumpled and to acquire a deflated appearance (Figure 2, right panel), changes which paralleled their decrease in viability. The effects of resveratrol on skin cells differed somewhat from those observed with T cells. For example, at a lower concentration of resveratrol (5 μmol L^{-1}; 48 h) the morphology of these cells (Figure 3, center panel) was no different from that of untreated cells (Figure 3, left panel). However, a high concentration of resveratrol (100 μmol L^{-1}; 48 h) resulted in obvious morphological alterations (Figure 3, right panel). Blebbing of the membrane

Table 1 *Effect of resveratrol on cell viability*

Cell type	LC_{50} value (μmol L^{-1}) [a,b]	
	24 h	48 h
RAW264.7 macrophages	29.1±2.53 (N=13)	39.1±2.81 (N=17)
CEM T cells	27.3±6.71 (N=3)	8.88±1.04 (N=4)**
A-431 epidermoid cells	91.0±6.66 (N=3)	66.0±8.72 (N=3)**

[a] *LC_{50} values represent the mean ± the standard error of the mean*
[b] *Significantly different from cells treated with resveratrol for 24 h by Student's t-test and ANOVA at **p<0.01*

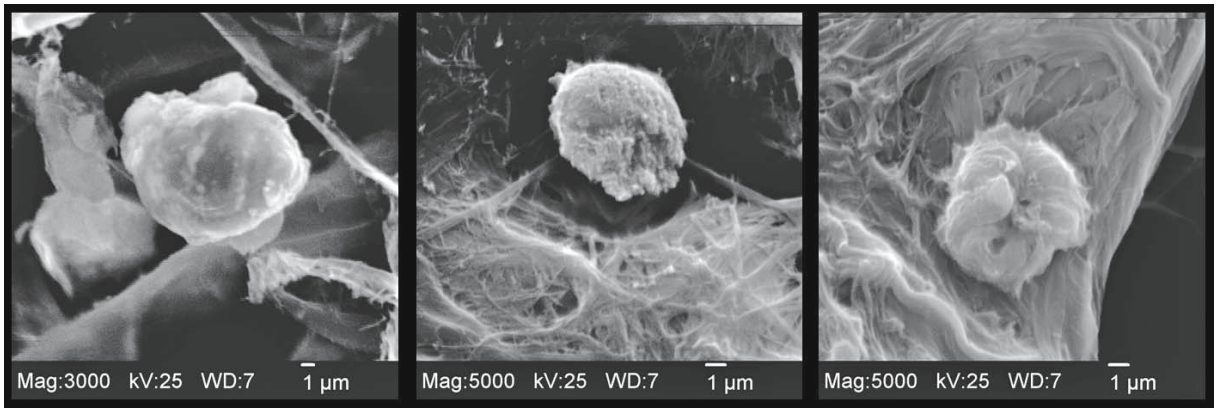

Figure 2 *Effect of resveratrol on T cell morphology (24 h treatment). Left panel, vehicle control; center panel, cells treated with 50 µmol L^{-1} resveratrol; right panel, cells treated with 100 µmol L^{-1} of resveratrol. In these panels, the cells are observed as round structures and the grayish-fibrous material on which the cells are resting is the filter paper onto which the nonadherent T cells were suction-filtered during preparation of the samples for viewing. It is apparent that treating the cells with 50 µmol L^{-1} and 100 µmol L^{-1} of resveratrol led to significant membrane damage*

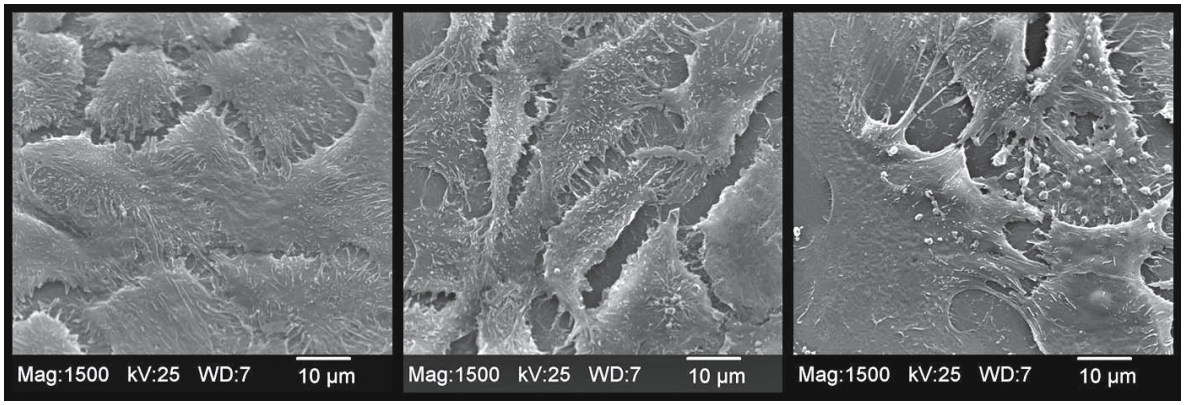

Figure 3 *Effect of resveratrol on skin cell morphology (48 h treatment). Left panel, vehicle control; center panel, cells treated with 5 µmol L^{-1} of resveratrol; right panel, cells treated with 100 µmol L^{-1} of resveratrol. Cells treated with 100 µmol L^{-1} of resveratrol exhibited significant membrane blebbing compared to controls*

was observed and was accompanied by alterations of cellular projections consistent with toxicity and lack of cell viability. The possibility that cell blebbing is due to the disruptive action of resveratrol on tubulin is suggested by a recent study in which the resveratrol analog 3,4',5-trimethoxystilbene was shown to display a potent anti-mitotic action in Caco-2 cells through inhibition of tubulin polymerization (44, 45).

To study the effect of resveratrol on the proliferation of skin cells, macrophages, and T cells, these cells were separately seeded at a low density on culture dishes, and grown in serum-containing DMEM plus 0 μmol L^{-1} to 75 μmol L^{-1} of resveratrol. Periodically, the viability of the growing cells was checked using the trypan blue exclusion method. The effects of resveratrol varied according to the cell type. Thus, it was unexpectedly found to exert a dual effect on the growth of macrophages (Figure 4), with a low concentration (5 μmol L^{-1}) stimulating cell proliferation and higher concentrations (25 μmol L^{-1} and 50 μmol L^{-1}) inhibiting it. In contrast, resveratrol was found to inhibit the growth of skin cells (25 μmol L^{-1} and 50 μmol L^{-1}) and T cells (50 μmol L^{-1}) with no significant growth-stimulatory effects at lower concentrations. The present findings for macrophages, although somewhat surprising, are not untenable. Thus, whereas a number of studies looking at the influence of resveratrol on cell growth find this compound to only exert an antiproliferative effect (20-24), there is at least one report in support of a stimulatory effect in osteoblasts *in vitro* (46). Furthermore, the dual effect of resveratrol on the growth of macrophages is comparable to the effect described for quercetin (47, 48), a plant flavonoid which shares a number of other bioactivities with resveratrol.

To determine if resveratrol could influence the intracellular antioxidant status, the cellular levels of GSH were measured in macrophages and skin cells

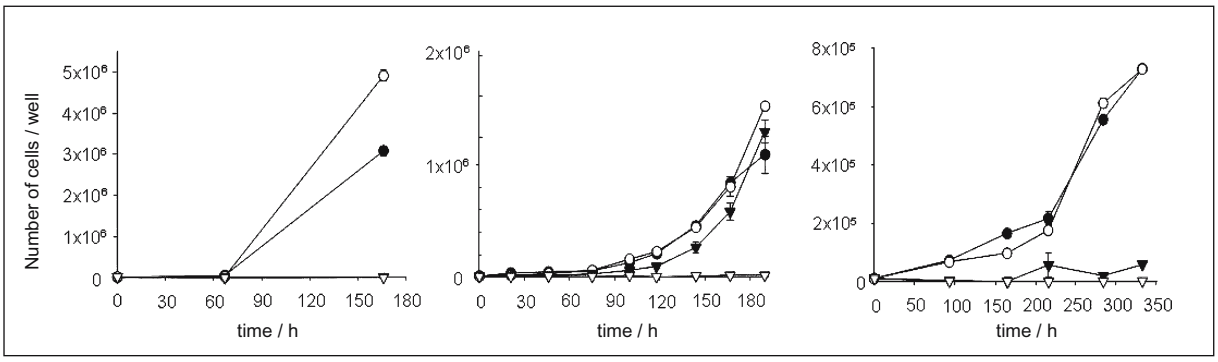

Figure 4 *Effect of resveratrol on cell proliferation. Left panel, transformed mouse macrophages (RAW264.7); center panel, transformed human T cells (CEM); right panel, tumor-derived skin cells (A-431). Values are given as the mean ± the standard error of the mean for three samples selected from representative experiments. For the entire duration of the experiment, cells were incubated in the absence (0.1 % DMSO, filled circles), or presence of 5 μmol L^{-1} (open circles), 25 μmol L^{-1} (filled triangles) or 50 μmol L^{-1} (open triangles) resveratrol.*

in the absence and presence of this compound. Regardless of the cell type, an exposure to resveratrol (50 μmol L^{-1}) was found to significantly elevate the intracellular GSH above the levels of untreated cells (Table 2). In macrophages, elevations in GSH levels amounted to about 2-fold and 7.7-fold after 24 h and 48 h, respectively. In skin cells, on the other hand, the GSH level was about 3.7-fold higher after 24 h than that of untreated cells. Furthermore, treatment of T cells with resveratrol has been previously found in this laboratory to increase the cellular GSH content (data not shown). A similar trend of results has been observed in resveratrol-treated endothelial (49) and cardiac (50) cells. In turn, the stimulatory effect for resveratrol could be centered on two key enzymes for GSH synthesis, glutathione synthetase (GS) and γ-glutamyl-cysteinyl ligase (GCL). Expression of these enzymes is controlled by the antioxidant/electrophilic response element (ARE/EpRE) which is activated by bZIP transcription factors such as Nrf2 (51). Hence, it is conceivable that resveratrol could be increasing intracellular pools of GSH in macrophages, skin cells, and T cells by activating Nrf2. This view is supported by a recent study which found resveratrol to activate Nrf2-dependent transcription *in vitro* (52). Although definitive proof of this effect will necessitate a future investigation, the results derived from our experiments clearly rule out a mechanism involving the reduction of cellular antioxidants as being part of the antiproliferative effect of resveratrol.

The potential for resveratrol to foster lipid peroxidation and, in this manner, to contribute to cytotoxicity, was examined in the macrophages and skin cells. Although the majority of published studies find resveratrol to have potent antioxidant properties, there are also reports indicating that this polyphenol

Table 2 *Effect of resveratrol on cellular levels of reduced glutathione (GSH)*

Cell type	GSH (nmol x 10^6 cells)[a]	
	24 h[b]	48 h[c]
RAW264.7 macrophages		
Control	9.27±1.28	9.03±0.83
Resveratrol (50 μmol L^{-1})	19.40±4.05[*]	69.1±27.3[††]
A-431 epidermoid cells		
Control	873.9±116.3	ND[d]
Resveratrol (50 μmol L^{-1})	3193.8±212.6[*]	ND[d]

[a]*Values represent the mean±the standard error of the mean from 3 independent experiments*
[b]*Significantly different from control cells at 24 h by Student's t-test at* [*]$p<0.05$
[c]*Significantly different from control cells at 48 h by Student's t-test at* [††]$p<0.01$
[d]*ND: not determined*

can act as a prooxidant (53-55). In the present study, a treatment with resveratrol (0 μmol L^{-1} to 50 μmol L^{-1}) did not result in levels of TBARS that were significantly different from those in untreated cells (Table 3). Although decreases in viability occurred at these concentrations of resveratrol in macrophages and skin cells (see Table 1, 48 h), and microscopic alterations consistent with toxicity were observed in skin cells at a higher (100 μmol L^{-1}) resveratrol concentration (see Figure 3), the decrease in viability was not dependent on the accumulation of lipid peroxidation products.

To determine whether resveratrol can influence nonstimulated macrophages in the same manner and extent as stimulated cells, RAW264.7 cells were treated with resveratrol or the related compound piceatannol, (0 μmol L^{-1} to 50 μmol L^{-1}; 48 h), in the presence or absence of zymosan. Zymosan, from the cell wall of *Saccharomyces cerevisiae*, is composed of polysaccharides that are complexed with proteins and lipids (56), and acts as a potent stimulator of the Toll-like receptors TLR2 and TLR6 (57). Activation of these receptors by zymosan in macrophages triggers several signaling pathways, culminating in the release of cytotoxic products such as reactive oxygen and nitrogen-containing species, including the very reactive chemical species nitric oxide ($NO^{\bullet}$), superoxide anion ($O_2^{\bullet -}$), and peroxynitrite ($ONOO^-$) (58-60). In addition, zymosan can bind to and activate other receptors on macrophages that promote the phagocytosis of foreign particles (61, 62). On these bases, it was reasoned that the cytotoxic properties of resveratrol on macrophages would be exacerbated following cell stimulation with zymosan. Unexpectedly, however, macrophages stimulated with zymosan were found to be significantly more resistant to the toxicity of resveratrol than nonstimulated ones (Table 4). A similar trend was observed for zymosan-stimulated macrophages treated with piceatannol (Table 4). Although an explanation accounting for these results is not apparent at the present time, future work should explore the role of TLR2 and TLR6. At the concentrations used here to activate macrophages (20 μg mL^{-1} to 30 μg mL^{-1}), zymosan was previously found in this laboratory to stimulate the release of $NO^{\bullet}$ and $O_2^{\bullet -}$ from these cells to a significant extent and in a time-dependent manner (data not shown). Under this circumstance, and in light of two recent studies demonstrating the nitration of resveratrol and piceatannol under acidic conditions (63,64), we hypothesize that the reason why both of these structurally-related compounds are found to be less toxic in stimulated than in unstimulated macrophages may be the consequence of nitration by peroxynitrite which forms when $NO^{\bullet}$ and $O_2^{\bullet -}$ are released from activated cells. Nitration of resveratrol and piceatannol by peroxynitrite would significantly alter the trans-stereochemistry of these agents (63,64) and could also potentially change their respective hydrophobic

Table 3 *TBARS production in RAW264.7 macrophages and A-431 skin cells*

Cell type	TBARS (nmol x 10^6 cells)[a,b,c,d]
RAW264.7 macrophages	
Control	0.0013±0.0002
Resveratrol (25 μmol L^{-1})	0.0014±0.0002
Resveratrol (50 μmol L^{-1})	0.0013±0.0002
Mechlorethamine (25 μmol L^{-1})	0.0049±0.0005††
Mechlorethamine (50 μmol L^{-1})	0.0667±0.0148††
A-431 epidermoid cells	
Control	0.0003±0.0002
Resveratrol (25 μmol L^{-1})	0.0003±0.0001
Resveratrol (50 μmol L^{-1})	0.0002±0.0001
Mechlorethamine (25 μmol L^{-1})	0.0094±0.0010††
Mechlorethamine (50 μmol L^{-1})	0.0144±0.0027††

[a] *Values represent the mean±the standard error of the mean from 3 independent experiments*
[b] *Cells were cultured for 48 h in the absence or presence of each compound*
[c] *Mechlorethamine was used as a positive control for lipid peroxidation (65)*
[d] *Significantly different from control cells by Student's t-test at* †† $p<0.01$

Table 4 *Effect of zymosan on the cytotoxic effects of resveratrol and piceatannol in macrophages*

Treatment [a]	**Number of cells per well (1 x 10^6 cells)**[b]	
Resveratrol	Unstimulated[c]	Zymosan-stimulated[d,e]
Control	1.6174±0.0216	1.7484±0.0121
Resveratrol (5 μmol L^{-1})	1.6517±0.0594	1.6294±0.0254†
Resveratrol (25 μmol L^{-1})	1.4107±0.0301*	1.6017±0.0298†
Resveratrol (50 μmol L^{-1})	0.3504±0.0186**	0.7824±0.0194^{††,o}
Piceatannol		
Control	1.3380±0.0129	1.3637±0.0313
Piceatannol (10 μmol L^{-1})	1.2807±0.0272	1.2534±0.0178†
Piceatannol (30 μmol L^{-1})	0.9007±0.0201*	0.9937±0.0030$^{\dagger,\}}$
Piceatannol (50 μmol L^{-1})	0.5644±0.0112**	0.6940±0.0127$^{\dagger\dagger,\}}$

[a]*All cells were cultured for 48 h in the absence or presence of each compound*
[b]*Values represent the mean±the standard error of the mean from 3 independent experiments*
[c]*Significantly different from unstimulated control cells at $^{*}p<0.05$ or $^{**}p<0.01$ by Student's t-test and ANOVA*
[d]*Significantly different from zymosan-stimulated control cells at $^{\dagger}p<0.05$ or $^{\dagger\dagger}p<0.01$ by Student's t-test and ANOVA*
[e]*Significantly different from unstimulated cells treated with 50 μmol L^{-1} resveratrol at $^{o}p<0.05$ or 50 μmol L^{-1} piceatannol at $^{\}}p<0.05$ by Student's t-test and ANOVA*

properties, thus, decreasing their ability to accumulate within the cells and bring about toxicity. A future study examining the response of macrophages to nitrated and non-nitrated forms of resveratrol will help to settle this issue.

CONCLUSIONS

The effect of resveratrol on cell growth, studied with macrophages, T cells and skin cells, varied according to the cell type and the concentration of compound added. In macrophages, a low concentration of this compound (5 μmol L^{-1}) stimulated and a high concentration (50 μmol L^{-1}) inhibited cell proliferation. At the higher concentration it also inhibited the growth of T cells and skin cells. However, growth-stimulatory effects were not observed in skin cells and T cells treated with the lower concentration of resveratrol.

The MTT viability assay found resveratrol to be cytotoxic to all three cell types when present in concentrations ≥25 μmol L^{-1} at 24 h and ≥9 μmol L^{-1} at 48 h.

Scanning electron microscopy revealed resveratrol to alter the cell morphology at concentrations ≥50 μmol L^{-1}.

Cytotoxicity by resveratrol was accompanied by an increase in the intracellular levels of GSH, but not by alterations of the base line levels of TBARS.

Piceatannol, the hydroxylated metabolite of resveratrol, was cytotoxic to macrophages in the MTT viability test. However, this effect was attenuated to a significant extent by a cotreatment with zymosan.

Resveratrol was toxic to macrophages, T cells and skin cells. In the case of macrophages, the degree of toxicity appears to be related to the activation status of the cells since zymosan-activated macrophages were more resistant than nonstimulated cells.

The cytotoxicity that resveratrol manifested toward all cells studied appears not to involve oxidative stress since it led neither to glutathione depletion nor to the accumulation of TBARS.

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Sažetak

EVALUACIJA CITOTOKSIČNOSTI RESVERATROLA I PICEATANOLA U MAKROFAZIMA, T-STANICAMA I STANICAMA KOŽE

Citotoksičnost resveratrola i piceatanola, strukturnog analoga resveratrola, ispitivana je u uzgojenim stanicama. Primjenom MTT-testa koji mjeri pretvorbu 3-[4,5-dimetiltiazol-2-il]2,5-difenil-tetrazolijeva bromida (MTT) u obojeni formazan produkt u živim stanicama, nađeno je da resveratrol inhibira preživljavanje transformiranih makrofaga miša, ljudskih tumorskih T-stanica i humanih stanica epidermoidnog karcinoma u ovisnosti o koncentraciji, a učinak opada redom: T-stanice (LC_{50} ~27 μmol L^{-1}, 24 h; ~9 μmol L^{-1}; 48 h) > makrofazi (LC_{50} ~29 μmol L^{-1}, 24 h; 39 μmol L^{-1}, 48 h) >stanice kože (LC_{50} ~91 μmol L^{-1}, 24 h; ~66 μmol L^{-1}, 48 h). Paradoksalno, pri visokoj koncentraciji resveratrola (50 μmol L^{-1}) inhibirana je proliferacija svih triju tipova stanica, a pri niskim koncentracijma (5 μmol L^{-1}) stimulirana je proliferacija makrofaga. Preživljavanje makrofaga bilo je također smanjeno primjenom piceatanola ovisno o koncentraciji. Stimulacija makrofaga zimosanom smanjila je citotoksičnost i resveratrola i piceatanola. Skenirajuća elektronska mikroskopija stanica tretiranih resveratrolom pokazala je promjene u morfologiji stanica, što je bilo u skladu s toksičnosti. U makrofazima i stanicama kože resveratrol (50 μmol L^{-1}) inducirao je porast smanjenja razina glutationa ovisan o vremenu, ali nije mijenjao osnovne razine reaktivnih spojeva tiobarbiturne kiseline. Gledajući skupno, prikazani rezultati indiciraju da je resveratrol toksičan za uzgojene makrofage, T-stanice i stanice kože pri koncentracijama ≥25 μmol L^{-1} i da se citotoksičnost zbiva mehanizmom koji ne uključuje oksidativni stres. Nadalje, stupanj toksičnosti resveratrola i piceatanola prema makrofagima ovisi o aktivacijskom statusu tih stanica, pri čemu su stanice aktivirane zimosanom rezistentnije od nestimuliranih stanica.

KLJUČNE RIJEČI: *A-431-stanice, CEM T-stanice, preživljavanje, RAW264.7-stanice, glutation, zimosan, TBARS*

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