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Resveratrol Inhibits the Proliferation of Normal Human Keratinocytes In Vitro

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Abstract Resveratrol, a phytoalexin found in grapes and other plants, is a potent antioxidant, anti-inflammatory, and antiproliferative agent that is thought to have chemopreventive properties with respect to carcinogenesis. However, the antiproliferative effects of resveratrol have been described primarily for cultured tumor cells and its effects on the proliferation of normal cells are not clear. We evaluated the viability and proliferation of cultured normal human keratinocytes (KCs) exposed to resveratrol (0.25–100 μM) for different lengths of time (5–72 h) by means of ^{3}H-thymidine incorporation, direct cell counts, and a tetrazolium-based formazan reaction. The first two methods indicated that resveratrol, even at low concentrations, induced a time-and concentration-dependent inhibition of KC proliferation. However, formazan production was actually increased at moderate resveratrol concentrations (10 μM) and diminished only at higher concentrations. Even brief exposure (5 h) of KCs to resveratrol resulted in a concentration-dependent elevation in formazan production. This was blocked by ionomycin but was not dependent on Ca^{2+}. We conclude that resveratrol, even at submicromolar concentrations, inhibits the proliferation of normal human KCs in vitro and, at higher concentrations (40–100 μM), is cytotoxic to these cells. J. Cell. Biochem. Suppl. 36:55–62, 2001.

Key words: resveratrol; keratinocytes; tetrazolium; cell proliferation; phytoalexin; MTS; NADPH oxidase

Resveratrol, a stilbene phytochemical constituent of grapes and wine, has emerged as a candidate chemopreventive agent that has been shown to inhibit experimental carcinogenesis at the stages of initiation, promotion, and progression [Jang et al., 1997]. The actions of resveratrol have been investigated predominantly using malignant cells proliferating in culture where it has usually been found to inhibit cell proliferation. For instance, resveratrol inhibited ^{3}H-thymidine incorporation into murine lymphoblastic leukemia cells [Fontecave et al., 1998], into HL-60 human promyelocytic leukemia cells [Ragione et al., 1998; Surh et al., 1999], and human prostate cancer cells [Hsieh and Wu, 1999] and also arrested the growth of MCF-7 human breast cancer cells in both a concentration- and time-dependent manner [Mgbonyebi et al., 1998]. Resveratrol also possesses antioxidant and antiinflammatory activities that may be related to these effects on cell transformation and proliferation [Soleas et al., 1997]. At present, it is not known whether the chemopreventive potential of resveratrol is dependent on its antiproliferative action or whether its antioxidant or antiinflammatory activities retard the promotion and progression of experimental carcinogenesis.

Antitumor or chemopreventive compounds must be selective either qualitatively or quantitatively for transformed or malignant cells and should ideally have little or no effect on the viability and proliferation of normal cells. The few studies that have examined the effects of resveratrol on the proliferation of normal cells show that it inhibits the proliferation of cultured smooth muscle cells [Zou et al., 1999] but does not affect the viability of normal or phytohemagglutinin-activated human peripheral lymphocytes (72 h, 32 μM resveratrol) [Clement et al., 1998]. Resveratrol inhibited

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^{3}H-thymidine incorporation into primary rat hepatic stellate and Kupffer cells [Kawada et al., 1998], but stimulated its incorporation into normal mouse osteoblastic cells [Mizutani et al., 1998]. Thus, it remains unclear whether resveratrol, in the micromolar concentration range, is a broad spectrum inhibitor of cell proliferation or if it differentially affects normal and malignant cells.

There are several methods by which proliferation of cultured cells, as induced by extracellular mitogens or inhibitors, is commonly measured but each has its own advantages and disadvantages. Direct cell counts obtained using a hemocytometer provide a reliable indication of viable cell numbers but the method is tedious and error-prone, especially when studying substrate-adherent cells. On the other hand, ^{3}H-thymidine incorporation measures DNA synthesis that has occurred during the incorporation period but does not distinguish between synthesis preparative to cell division and synthesis that is the result of DNA repair activity. Thus this method may not accurately reflect changes in cell number in cases where DNA repair levels are altered or cell cycle blockade occurs during or after S phase. Finally, cell proliferation is commonly measured based on the NAD(P)H-dependent bioreduction of tetrazolium salts to colored formazan products [Berridge and Tan, 1993]. In theory, the extent of formazan production should be proportional to the number of viable cultured cells [Mosmann, 1983], but studies of genistein-treated tumor cells including MCF-7 breast tumor, L929 transformed mouse fibroblasts, and Jurkat T leukemia cells [Pagliacci et al., 1993]; transformed KCs treated with cyclosporine or interferon [Marionnet et al., 1997]; or deoxyspergualin-treated Raji cells, J774 macrophages, and NIH-3T3 cells [Odaka et al., 1999] show that this is not necessarily the case. The aforementioned studies have shown that results from the tetrazolium–formazan assay are sometimes inconsistent with those of other proliferation assays such that this method often overestimates the cell number in drug-treated cultures. Reduction of tetrazolium compounds such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] or MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] is dependent on cell type, media pH, glucose concentration, cell culture age, stage of the cell cycle, and ultimately upon intracellular NADH and NADPH levels [Vistica et al., 1991]. As a result of the foregoing considerations, it is prudent to employ more than one method to accurately determine the response of cell lines to agents that may alter their rates of DNA repair, cell cycle kinetics, substrate adherence, or redox states.

The primary purpose of the present investigation was to determine the effects of resveratrol on the proliferation of normal human KCs in culture. Discrepancies seen among the results of experiments in which direct cell counts, ^{3}H-thymidine incorporation, and formazan production were determined led us to further examine the mechanism of resveratrol's effects and gave further insight into the published effects of resveratrol.

MATERIALS AND METHODS

Cells, Cell Culture, and Resveratrol Preparation

Human foreskin KCs, in primary culture at passage 2 or 3, were maintained in Keratinocyte Growth Medium (KGM) containing 0.15 mM Ca^{2+} and supplemented with bovine pituitary extract (Clonetics, Temecula, CA) at 37°C in a 5% CO_2/95% air atmosphere. Cells were released from flasks by brief exposure to 0.025% trypsin with 0.01% EDTA after which trypsin was neutralized and suspended KCs were centrifuged to remove the trypsin and EDTA. KCs were resuspended in KGM and counted using a hemocytometer. Resveratrol (Sigma Chemical Co., St. Louis, MO) was dissolved in 95% ethanol and added to cells such that the final concentration of ethanol in resveratrol-treated cultures was 0.1%. Ethanol vehicle was added to untreated control cultures to this same concentration. Resveratrol stock solutions and all cell cultures were protected from light.

Cell Counts and Viability Determinations

KCs were plated into 6-well plates at 20,000 cells per 2 ml media per well, and allowed to equilibrate for 2 h, at which time resveratrol was added to final concentrations of 0.25, 0.50, 1, 2, 10, 20, 40, and 100 μM. Cells were incubated in KGM for 24, 48, or 72 h, at which time the media were removed from the wells and the cells released by treatment with 0.025% trypsin containing 0.01% EDTA. Cells were centrifuged (250 × g for 5 min), the trypsin

solution removed, and the cells were resuspended in PBS with trypan blue and counted using a hemocytometer. Total cell number and the number of viable cells were determined.

^{3}H-Thymidine Incorporation

DNA synthesis was measured as the amount of incorporation of ^{3}H-thymidine (New England Nuclear, 0.5 μCi/well) into human keratinocyte DNA occurring in the 24 h prior to cell harvest. Keratinocytes (25,000 cells/2 ml of media/well) were plated in duplicate wells in 6-well plates and cells were allowed to equilibrate for approximately 3 h after which time resveratrol (2, 10, or 100 μM) was added. After 24, 48, and 72 h of resveratrol treatment the media were removed, cells were washed with 5 ml of cold PBS and the experiment terminated by the addition of 2 ml of ice-cold 10% trichloroacetic acid (TCA). The plates were centrifuged, the TCA removed, the insoluble residue resuspended in 0.5 ml of 0.1 N NaOH, 10 ml of scintillation cocktail was added, and samples were counted in a scintillation counter (Tm Analytic, Elk Grove Village, IL). Results are expressed as percent of ^{3}H-thymidine incorporated at each concentration of resveratrol relative to its incorporation into untreated cells.

Tetrazolium-Based Cell Proliferation Assay

KCs were plated into 96-well plates (2,000 cells/100 μl KGM/well). Resveratrol was added into quadruplicate wells to final concentrations of 0.25, 0.5, 1, 2, 10, 40, and 100 μM. Vehicle control wells containing 0.1% ethanol (final concentration) were also included. Cultures were incubated for 24, 48, or 72 h, at which times the KGM was removed from the wells and replaced with 100 μl of HEPES-buffered saline containing 5 mM glucose, 1 mM $MgSO_4$, and 0.2 mM $CaCl_2$, pH 7.4. Because cell metabolic status and the tetrazolium reaction are known to be affected by medium pH and glucose concentration, these factors were closely controlled [Jabbar et al., 1989; Vistica et al., 1991]. Twenty microliters of CellTiter 96® AQ reagent (Promega Corp., Madison, WI) were added to each well using a multichannel micropipette, the solutions in the wells were mixed gently, and the plates were returned to the incubator. Three hours after adding the CellTiter 96® AQ reagent, the amount of reaction product in each well was determined by reading the plates at 490 nm in a multiwell plate reader (Molecular Devices, Menlo Park, CA). The absorbance of the blank, which contained only buffer and CellTiter 96® AQ reagent, was subtracted from each of the other readings. Note that resveratrol itself, at the concentrations used here, had no significant effect on the absorbance readings obtained from this assay.

Alternatively, for experiments in which KCs were exposed to resveratrol for only 5 h, KCs were initially plated into 96-well plates and 24 h later the KGM was replaced with HEPES-buffered saline with or without calcium. Resveratrol (1.0–100 μM) and ionomycin (1 μM final; Sigma) were added, the plates were returned to the incubator, and 2 h later the CellTiter 96® AQ reagent was added. Three hours later, the plates were read as described above.

RESULTS

^{3}H-Thymidine Incorporation

Incorporation of ^{3}H-thymidine into KC DNA was inhibited by resveratrol (Fig. 1). After 24 h of resveratrol treatment, ^{3}H-thymidine incorporation was significantly inhibited ($P < 0.05$; n = 3; paired t-tests) at resveratrol concentrations ≥10 μM. Futhermore, after 48 or 72 h of resveratrol treatment, ^{3}H-thymidine incorporation was significantly inhibited ($P < 0.01$; n = 3; paired t-tests) at resveratrol concentrations ≥2 μM. The IC_{50} for resveratrol (concentration at which 50% inhibition occurred) as determined from ^{3}H-thymidine incorporation ranged between 2 and 8 μM.

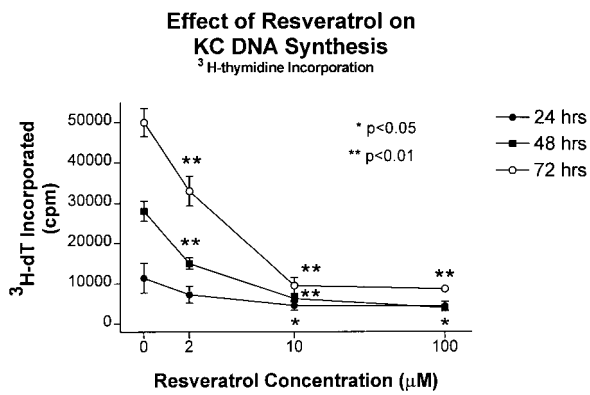

Fig. 1. Effect of resveratrol on the incorporation of ^{3}H-thymidine into KCs. Cells were cultured with resveratrol (2, 10, 100 μM) or vehicle alone for 24, 48, or 72 h. During the final 24 h in culture, ^{3}H-thymidine (0.5 μCi/well) was present and TCA-precipitable radioactivity was used as a measure of DNA synthesis. Each point represents the mean±SEM (n = 3).

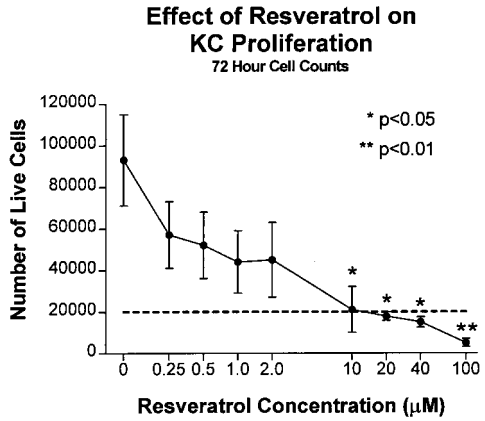

Fig. 2. Effect of resveratrol on the proliferation of human KCs as determined by direct cell counts. Cells were cultured with resveratrol (0.25–100 μM) or vehicle alone for 72 h at which time the numbers of live, trypan-blue excluding cells were determined. The dashed line represents the number of live cells (20,000) added to each well at the beginning of the experiment. Each point represents the mean±SEM (n = 5).

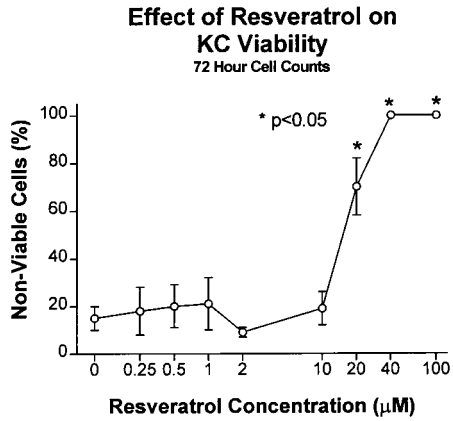

Fig. 3. Effect of resveratrol on human KC viability as determined by direct cell counts of trypan blue stained cells. Cells were cultured with resveratrol (0.25–100 μM) or vehicle alone for 72 h at which time the numbers of non-viable and total cells were determined and the percent non-viable cells calculated. Each point represents the mean±SEM (n = 5).

Hemocytometer Cell Counts

KC proliferation (Fig. 2) was inhibited even at the lowest resveratrol concentrations tested here (0.25 μM) and proliferation was significantly inhibited ($P < 0.05$ or $P < 0.01$; n = 5; paired *t*-tests) at concentrations ≥10 μM. Very similar results were seen in the cultures treated with resveratrol for 24 and 48 h (data not shown). The IC_{50} for resveratrol as determined from cell counts was 0.5 μM. At low concentrations (0.25–10 μM), resveratrol did not affect KC viability which remained at 80–90% of the total cells in culture (Fig. 3). Even at 10 μM resveratrol, a concentration at which KC proliferation was totally suppressed, cell viability was normal. Higher concentrations of resveratrol, however, caused a sharp increase in cell death such that at 40 and 100 μM resveratrol all cells were non-viable.

Tetrazolium-Based Cell Proliferation Assay

KCs were exposed to 0.25–100 μM resveratrol for 24, 48, or 72 h (Fig. 4) and the formation of formazan reaction product was determined. Significantly greater ($P < 0.05$; n = 4) amounts of reaction product were seen in KCs exposed to 10 μM resveratrol for 72 h as compared to untreated KCs at that time. Significantly less ($P < 0.001$; n = 4) reaction product formed in KCs exposed to high concentrations of resveratrol (100 μM) at all three time points studied here (24, 48, and 72 h). The IC_{50} for resveratrol as determined from formazan reaction product formation was > 80 μM at all three time points studied.

Effects of Calcium and Ionomycin

To study the mechanism by which resveratrol affects formazan production from MTS, we exposed KCs to resveratrol for a shorter time (5 h) during which alterations in proliferation were not manifest but other effects might be seen. Ionomycin has a variety of effects on mammalian cells. Prominent among these, it activates NADPH oxidase and is a Ca^{2+} ionophore [Dahlgren et al., 1992]. Ionomycin stimulates NADPH oxidase activity thereby depleting NADPH and glutathione levels and promoting the formation of toxic reactive oxygen species (ROS). It has been shown that ROS may cause the reduction of tetrazolium compounds such as MTT and MTS to formazan [Burdon et al., 1993]. By modulating the intracellular Ca^{2+} concentration, we attempted to distinguish the ionophoretic effects of ionomycin from its other effects.

In the presence of Ca^{2+} without ionomycin, exposure of KCs to resveratrol for 5 h resulted in a resveratrol concentration-dependent increase in formazan levels (Fig. 5) such that significantly increased amounts of formazan reaction product were seen at 2, 10, and 100 μM resveratrol ($P < 0.02$, $P < 0.02$, $P < 0.05$, respectively; n = 4). In the absence of resveratrol, ionomycin had no effect on formazan reaction

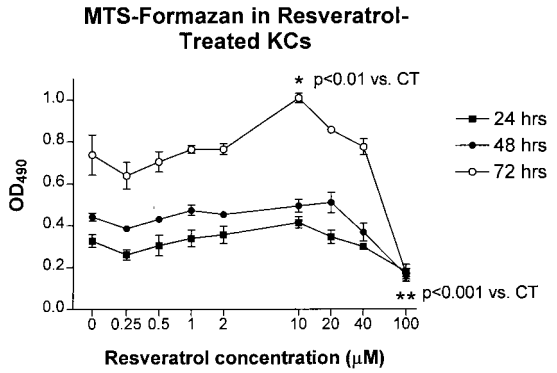

Fig. 4. Effect of resveratrol on MTS reduction to formazan in human KCs cultured with resveratrol (0.25–100 μM) for 24, 48, or 72 h. Formazan production was determined for quadruplicate wells at each resveratrol concentration by measuring the absorbance at 490 nm. Each point represents the mean±SEM from four different experiments (n = 4).

product formation by KCs. In the presence of resveratrol (1–100 μM) and Ca^{2+}, ionomycin prevented the concentration-dependent increases in formazan levels seen with resveratrol alone. As a result, the formazan levels in the KCs exposed to resveratrol in the presence of Ca^{2+} without ionomycin were significantly greater than those seen with ionomycin ($P < 0.03$ at 1 μM; $P < 0.008$ at 2 mM; $P < 0.05$ at 10 μM).

In the absence of Ca^{2+}, the amount of formazan produced was similar to that seen in

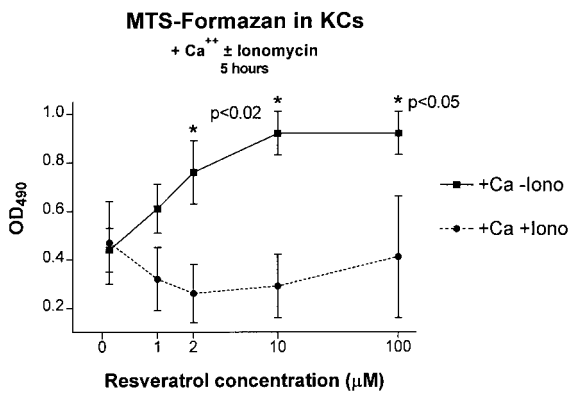

Fig. 5. Effect of resveratrol on MTS reduction to formazan in human KCs cultured with resveratrol (1, 2, 10, 100 μM) for 5 h. Formazan production was determined for quadruplicate wells at each resveratrol concentration by measuring the absorbance at 490 nm. Calcium ion was present in all samples and ionomycin (1 μM) was added to one group. Each point represents the mean±SEM from four different experiments (n = 4). For the groups without ionomycin, the *P*-values compare formazan production at the resveratrol concentrations shown to that of the untreated control (paired *t*-tests).

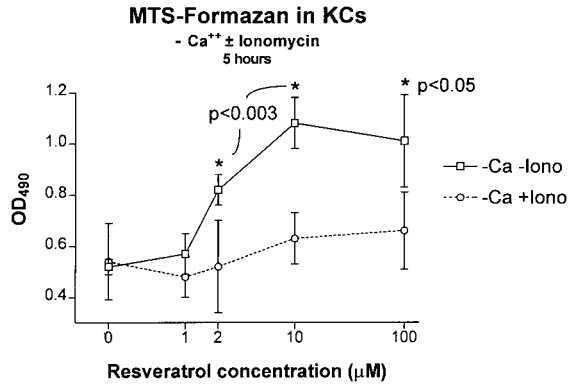

Fig. 6. Effect of resveratrol on MTS reduction to formazan in human KCs cultured with resveratrol (1, 2, 10, 100 μM) for 5 h. Formazan production was determined for quadruplicate wells at each resveratrol concentration by measuring the absorbance at 490 nm. Calcium ion was absent in all samples and ionomycin (1 μM) was added to one group. Each point represents the mean±SEM from four different experiments (n = 4). For the groups without ionomycin, the *P*-values compare formazan production at the resveratrol concentrations shown to be that of the untreated control (paired *t*-tests).

the presence of Ca^{2+} and a resveratrol concentration-dependent increase in formazan levels was also observed (Fig. 6). Significantly increased amounts of formazan reaction product formed in KCs exposed to resveratrol for 5 h in Ca^{2+}-free media without ionomycin ($P < 0.003$ at 2 and 10 μM; $P < 0.05$ at 100 μM resveratrol; n = 4). In the absence of resveratrol and Ca^{2+}, ionomycin had no effect on formazan reaction product formation by KCs. However, in Ca^{2+}-free media with resveratrol (1.0–100 μM), ionomycin prevented the concentration-dependent increases in formazan levels seen with resveratrol alone. As a result, the formazan levels in the KCs exposed to resveratrol in the absence of Ca^{2+} and ionomycin were significantly greater than those seen with ionomycin ($P < 0.04$ at 10 μM).

DISCUSSION

Resveratrol, a phytoalexin present in grapes and other plants, has been found to be a potent antioxidant, antiinflammatory, and antiproliferative agent. It is currently believed that its antiproliferative capacity contributes to its putative chemopreventive action. To establish whether resveratrol targets only transformed cells or also suppresses proliferation of normal cells, we determined the effects of resveratrol on the proliferation of normal human KCs in

culture. When cell proliferation was quantified by incorporation of ^{3}H-thymidine into KC DNA, resveratrol behaved as a potent inhibitor of DNA synthesis such that a significant reduction in ^{3}H-thymidine incorporation was detected at concentrations as low as 2 μM resveratrol. This antiproliferative action toward human KCs was confirmed using direct cell counts where inhibition was seen at concentrations as low as 0.25 μM resveratrol. However, reduction of MTS to its colored formazan reaction product consistently underestimated the antiproliferative potency of resveratrol. Formazan production by KCs exposed to resveratrol (0.25–40 μM) for 24 or 48 h was unchanged as compared to untreated controls and decreased formazan production was noted only with 100 μM resveratrol. Furthermore, exposure of KCs to 10 μM resveratrol for 72 h even resulted in significantly increased formazan production and decreased formazan production was noted only in the presence of 100 μM resveratrol. Similarly, others [Gehm et al., 1997] have found that, after several days of treatment with 10 μM resveratrol, MCF-7 cells appeared to continue to proliferate according to the MTS assay, but manual cell counting showed that proliferation was actually inhibited by resveratrol in a dose- and time-dependent manner [Mgbonyebi et al., 1998; Lu and Serrero, 1999].

There is strong evidence that reduction of tetrazolium compounds to their formazan products: (1) is dependent on cellular NAD(P)H levels [Berridge et al., 1996], (2) requires metabolism of glucose [Vistica et al., 1991], and (3) is coupled with the respiratory chain [Kunimoto et al., 1999]. Although tetrazolium-based assays are widely used to measure cell proliferation, discrepancies have been noted when results obtained from these assays were compared with direct cell counts or with ^{3}H-thymidine incorporation data [Pagliacci et al., 1993; Marionnet et al., 1997; Odaka et al., 1999]. In such studies, drug-treated cells often were growth-arrested in different phases of the cell cycle. Agents that inhibit cell proliferation by inducing cell cycle arrest also seem to promote an increase in cell volume and mitochondrial content which may contribute to altered glucose utilization and increased intracellular NAD(P)H levels. Thus, MCF-7 cells were arrested either in G1 phase (with ursolic acid) or at the G2/M boundary (with genistein) and arrested cells exhibited increased cell volume and mitochondrial numbers. These alterations were thought to contribute to the discrepant cell proliferation results obtained with the tetrazolium-based assays [Es-saady et al., 1996].

Using flow cytometry, we have observed that gastric adenocarcinoma cells treated with resveratrol (10–100 μM) for 4–24 h undergo arrest in G1 phase [Holian, unpublished results]. Resveratrol may also inhibit cell cycle progression of human KCs resulting in increased average cell size with concomitantly increased levels of intracellular NAD(P)H, thus contributing to the increased production of formazan without an actual increase in cell numbers. In support of this, KCs treated with resveratrol for 24–72 h clearly exhibited increased cell size when observed in the inverted phase microscope (data not shown).

We further evaluated the relationship between cell numbers and formazan production by treating KCs with resveratrol for shorter periods of time (5 h) and found that cell numbers and cell size underwent little change, but formazan production nonetheless increased proportionate with the amount of resveratrol added. This suggested that resveratrol also altered formazan production by a more direct mechanism than cell cycle blockade. Resveratrol has been shown to suppress oxygen radical production by murine macrophage-like cells [Jang et al., 1999] and by human neutrophils [Rotondo et al., 1998]. The formation of ROS requires an active NADPH oxidase to catalyze the transfer of electrons from NADPH to molecular oxygen. Resveratrol may also inhibit NADPH oxidase in KCs, thereby permitting NADPH to accumulate and be rerouted into the production of formazan from MTS instead. Alternatively, resveratrol may affect formazan production by interacting with other related enzyme systems. Using respiratory chain inhibitors, it has been shown that cellular reduction of tetrazolium compounds is coupled to either an NAD(P)H-dependent cytochrome oxidase [Kunimoto et al., 1999] or succinate dehydrogenase [Berridge and Tan, 1993; Berridge et al., 1996] depending on the specific tetrazolium compound used. Resveratrol may directly interact with these enzyme systems to potentiate the reduction of MTS (see Fig. 7). Thus, the formazan levels seen in KCs treated with resveratrol for 5 h primarily reflect alterations in the intracellular NAD(P)H levels whereas those of KCs exposed for longer time periods

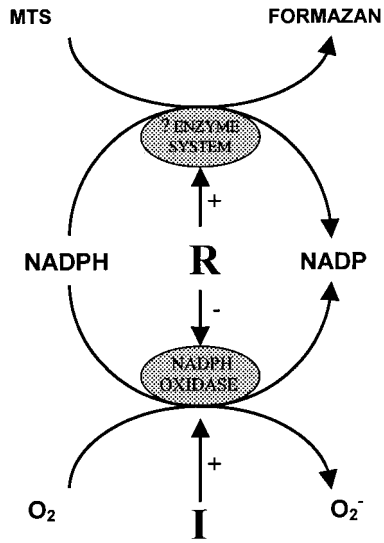

Fig. 7. Possible mechanism for the observed effects of resveratrol (R) and ionomycin (I) on MTS reduction to formazan. Resveratrol suppresses ionomycin-induced generation of free radical oxygen (O_2^-) by inhibiting NADPH oxidase. This results in increased availability of cellular NADPH which could then be redirected into MTS reduction. Alternatively, resveratrol may have a direct stimulatory effect upon the enzyme system responsible for MTS reduction in KCs.

(24–72 h) reflect increases in NAD(P)H levels balanced against decreases in cell proliferation.

Upon addition of ionomycin, the short-term effect whereby resveratrol increased formazan production was abolished (see Figs. 5, 6). The ionomycin-activated NADPH oxidase known to be present in human KCs [Turner et al., 1998] may have depleted any NADPH that accumulated due to the effects of resveratrol, thereby precluding the increased formazan formation that was induced by resveratrol treatment alone. The action of ionomycin was not dependent on the presence of extracellular Ca^{2+} ions since it still occurred in KCs maintained in Ca^{2+}-free medium. However, ionomycin may mobilize Ca^{2+} from intracellular stores elevating free cytoplasmic Ca^{2+} levels somewhat. Such elevations would be very transient, lasting only seconds to a few minutes, due to the rapid diffusion of Ca^{2+} out of the cells through ionomycin channels in the plasma membrane, and subsequent extensive dilution in the extracellular medium [Pillai et al., 1993]. Thus, it seems unlikely that such Ca^{2+} fluxes would have a significant effect on formazan production in KCs exposed to resveratrol for 5 h in Ca^{2+}-free medium suggesting that the observed effects on formazan production were not directly related to the ionophoretic properties of ionomycin.

We conclude that MTS and possibly other tetrazolium compounds give spurious results when used to study cell proliferation in the presence of resveratrol. Using other measures of proliferation it is evident that, even at submicromolar concentrations, resveratrol inhibits the proliferation of normal human KCs in vitro and, at higher concentrations (40–100 μM), is highly cytotoxic to these normal cells. This implies that resveratrol is not selectively antiproliferative or cytotoxic for transformed cells which are growth-arrested or killed at similar resveratrol concentrations. Resveratrol has also been shown to inhibit COX-1 and COX-2 activities [Jang et al., 1997; Subbaramaiah et al., 1998] and to induce alterations in the cellular redox state as indicated by the results of our experiments examining the effects of ionomycin, Ca^{2+}, and resveratrol on formazan production in KCs. Resveratrol-induced suppression of COX-1 and COX-2 may contribute to its chemopreventive potential and its antiproliferative effects may result from its action on the cellular redox state.

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