Can Apple Antioxidants Inhibit Tumor Cell Proliferation? Generation of H$_2$O$_2$ during Interaction of Phenolic Compounds with Cell Culture Media

TAIR LAPIDOT,[†] MICHAEL D. WALKER,[‡] AND JOSEPH KANNER*,[†]

Department of Food Science, ARO Volcani Center, Bet Dagan 50250, Israel, and Department of
Biological Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

It has recently been suggested that the ability of apple extracts to inhibit proliferation of tumor cells in vitro may be due to phenolic/flavonoid antioxidants. Our study demonstrates that this inhibition is caused *indirectly* by H$_2$O$_2$ generated through interaction of the phenolics with the cell culture media. The results indicate that many previously reported effects of flavonoids and phenolic compounds on cultured cells may result from similar artifactual generation of oxidative stress. We suggest that in order to prevent such artifacts, the use of catalase and/or metmyoglobin in the presence of reducing agents should be considered as a method to decompose H$_2$O$_2$ and prevent generation of other reactive oxygen species, which could affect cell proliferation. The use of tumor cells and "nontumor cells" in a bioassay to measure antioxidant activity, in this context, is potentially misleading and should be applied with caution.

KEYWORDS: Apple; cell culture; H$_2$O$_2$; free iron; antioxidants; phenolics

INTRODUCTION

The possible beneficial health effects of diets containing fruits, vegetables, and certain beverages, such as wine and tea, have led to renewed interest in flavonoids and other plant phenolics (*1, 2*). Recently, Eberhardt et al. reported inhibition of tumor cell proliferation in vitro by apple extracts (*3*). They suggested that the inhibition could be due to the presence of phytochemicals (phenolic acids and flavonoids) other than ascorbic acid in whole apples. While it is true that whole apple extracts contain high concentrations of phenolic antioxidants (*4, 5*), the suggestion that these compounds inhibit proliferation of tumor cells through an antioxidant effect on the cells was not examined critically, and could involve other mechanisms, including generation of H$_2$O$_2$ through an interaction of the apple extract with cell culture media. This possibility is consistent with the findings of others that incubation of tea polyphenols and other reducing agents can induce dose-dependent formation of H$_2$O$_2$ (*6, 7*).

Many laboratories are examining the effect of flavonoids and phenolic compounds on cultured cells, working with transformed or other cells. It has been reported that phenolic compounds induce apoptosis in several tumor cell lines (*6, 8, 9*), and modify enzymatic activity and signal transduction (*10, 11*). Although flavonoids and phenolic compounds are strong reducing agents, in the presence of metal ions such as copper or iron they can stimulate prooxidant effects under a variety of in vitro conditions (*12−14*)

The aim of this study was to examine the mechanism whereby apple extract and polyphenols inhibit proliferation of cultured cells such as HepG2 cells: in particular, we wished to determine whether the inhibition is caused by an antioxidant effect or through an indirect generation of oxidative stress, via components of cell culture medium.

MATERIALS AND METHODS

Tissue culture medium and serum were from Biological Industries Ltd. (Bet Haemek, Israel). Chelex-100 resin was from Bio-Rad (Richmond, CA). Other chemicals and enzymes were from Sigma (St. Louis, MO). Apples (Red Delicious) were bought at a commercial store.

Apple extraction was according to Eberhardt et al. (*3*). The phenolics in the extract were determined with Folin−Ciocalteu reagent (*15*) and calculated as quercetin equivalent. The production of H$_2$O$_2$ in HepG2 medium, due to addition of glucose/glucose oxidase (4×10^{-4} Sigma units producing 149 ± 5 μM/h), apple extract, or phenolics, was assayed by the FOX2 method (*16*). The amount of chelatable iron ions in the medium was analyzed according to Carter et al. (*17*). Other metals ions were measured by inductively coupled plasma atomic emission spectrometry (ICP−AES) (Spectro, Kleve, Germany). Samples and blanks were prepared for analysis by microwave-assisted digestion using an MLS 1200 mega microwave digestion unit (Milestone Sorisole (BG), Italy).

Oxygen consumption was analyzed by oxygen monitor (model 53, Yellow Springs Instrument Co., Yellow Springs, OH) with a Clark electrode.

Tissue Culture. HepG2 cells (15,000 cell/well) were plated in 96-well plates (Falcon, Becton-Dickinson, UK), 24 h prior to the treatments. Cultures were maintained in Dulbecco's modified Eagle's medium
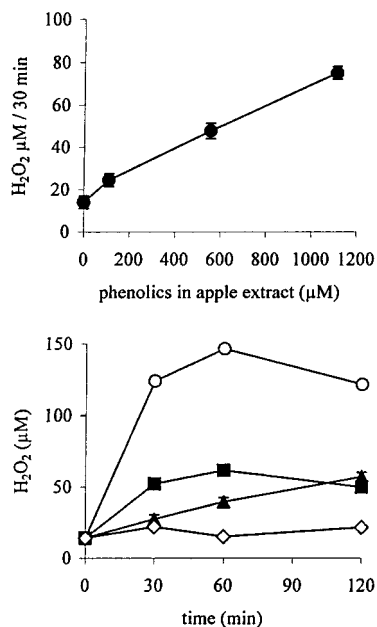
Figure 1. Production of H_2O_2 in HepG2 medium. Medium was incubated at 37 °C in the presence of apple extract and diluted apple extract (phenolics in apple extracts were calculated as quercetin equivalent, 100–1100 μM) (a); and gallic acid (–O–), quercetin (–■–), and caffeic acid (–▲–), 50μM each, compared with medium control (–◇–) (b). Values are mean ± SD. (*n* = 3)

(DMEM)/Ham's F12 medium (1/1), supplemented with 10% (v/v) fetal calf serum, pyruvate (100 μM), Hepes (25 μM, pH 7.2), penicillin (100 IU/mL), and streptomycin (100 μg/mL). The tested compounds (5–10 μL, each) were added to the wells, diluted or dissolved in PBS (except quercetin, which was dissolved in PBS with 0.5% dimethyl sulfoxide (DMSO)). Cell proliferation was determined using the thymidine incorporation method (*18*), 20 h after incubation with the test substance, and 18 h after addition of [^{3}H]thymidine. Growth medium for the cell line βTC1 (*19*) contained DMEM supplemented with 15% (v/v) horse serum, 2.5% (v/v) fetal calf serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL).

RESULTS AND DISCUSSION

Incubation of several flavonoids (including quercetin, gallic acid, and caffeic acid) or phenolics of apple extracts, in the presence of cell growth medium, produces hydrogen peroxide in a time- and dose-dependent manner, which was not effected by light (**Figure 1a,b**). The inclusion of catalase (1 Sigma U/mL) in control samples completely inhibited the FOX assay color, indicating that no hydroperoxides were produced, but only H_2O_2 (not shown). These data are consistent with previous findings that incubation of H661 human cancer cells in cell growth media containing tea polyphenols induced dose-dependent formation of H_2O_2 (*6*). Similar results were recently obtained by Long et al. (*7*) upon incubation of cell culture media with epigallocatechin, epigallocatechin gallate, catechin, and quercetin. Autoxidation of phenolic compounds in physiologic pH and in the presence of transition metals ions (such as iron or copper) was documented. This autoxidation leads to the production of reactive oxygen species including H_2O_2 (*20, 21*). Thus, we examined the possibility that formation of H_2O_2 upon addition of phenolic compound was due to direct reaction between these compounds and iron or copper ions in the culture media. The amount of chelatable iron ions in a few batches of media and sera were determined (**Table 1**). Other transition metals were measured by ICP–AES analysis, but the results were below the detection level (not shown). Although the found

Table 1. Chelateable Iron Level in Several Culture Media and Sera (results are average of measurements from two batches)

medium	chelateable iron (μM)
DMEM/F12 (HepG2 cells)	3.8 ± 1
DMEM	4.2 ± 1
DMEM/F12 + 10% FCS	17.1 ± 6
DMEM + 10% FCS	12.1 ± 4
FCS	103.8 ± 20
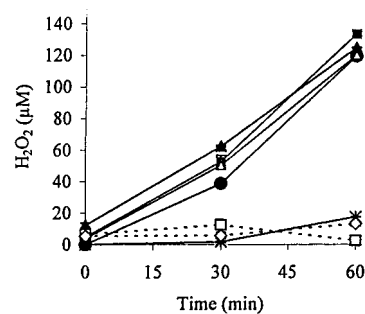
Figure 2. Production of H_2O_2 upon addition of gallic acid (50 μM): in DMEM medium (–■–); in DMEM medium with Fe^{3+} (25 μM) (–▲–); in DMEM medium with desferrioxamine (50 μM) (–●–); in DMEM medium with Fe^{3+} (25 μM) and desferrioxamine (50 μM) (–△–); DMEM control (–✳–); in Chelex 100 treated purified water at pH 7.4 (–□–); in Chelex 100 treated purified water at pH 7.4 with Fe^{3+} (25 μM) (–◇–). Duplicates were incubated at 37 °C. Values are mean ± SD.
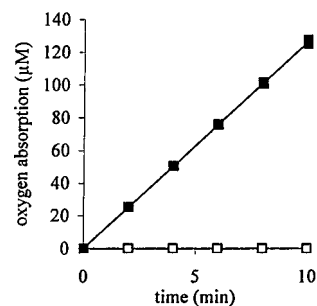

Figure 3. Oxygen absorption upon addition of gallic acid. Gallic acid (200 μM) alone was added to DMEM medium (–■–) or to Chelex 100 treated purified water at pH 7.4 together with Fe^{3+} (25 μM) (–□–). The measurements were preformed in duplicates at 25 °C. Values are mean ± SD.

iron levels are in a range of reacting with phenolic compounds and forming H_2O_2 (*20*), addition of more iron ($FeCl_3$, up to 40 μM) to the culture media together with the phenolics did not cause faster formation of H_2O_2. Moreover, inclusion of the iron chelator, desferrioxamine (50 μM) did not inhibit the H_2O_2 formation. Parallel reactions were performed in purified water treated with Chelex-100 and adjusted to pH 7.4, but the H_2O_2 formation in water was significantly lower (**Figure 2**). We have also measured the oxygen consumption in these reactions by oxygen monitor, which resulted in the same trend (**Figure 3**). These data indicate that the production of H_2O_2 in the culture media may not result from direct interaction between the phenolics and free iron ions, but could occur through involvement of a third component which serves as a metal ion chelator. Hodnick et al. (*21*) reported that the presence of chelator (as EDTA) is necessary to form redox-cycle interaction between flavonoids and iron ions in aqueous solution at physiologic pH. Other redox-cycle active complexes of iron ions, which involve amino acid, were reported by Berlett et al. (*22*). This may also explains the ineffectiveness of desferrioxamine to inhibit the reaction, as desferrioxamine can inhibit redox-cycle reactions
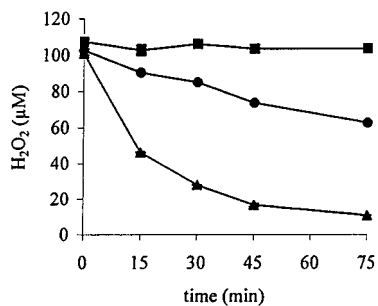
Figure 4. Decomposition of supplemented H_2O_2 (100 μM) in culture media containing 10% FCS. Control of purified water at pH 7.4 (–■–); DMEM medium (–●–); DMEM/F12 medium (–▲–). Duplicates were incubated at 37 °C. Values are mean ± SD.
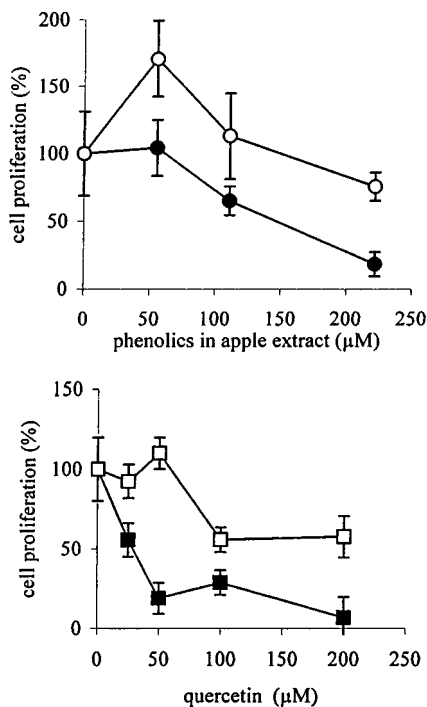

Figure 5. Effect of apple extract and diluted apple extract (a) and quercetin (b) with or without myoglobin on HepG2 cell proliferation. Effects on cell proliferation (mean ± SD, $n = 4$) were determined in the absence (filled symbols) or presence (open symbols) of myoglobin (30 μM). The effects of myoglobin in both (a) and (b) were statistically significant according to Student's t test ($p < 0.006$).

of free metal ions (*23*). A second possible explanation of the data is another mechanism of redox reaction between phenolics and components in the culture media which does not involve iron.

Interestingly, we have found that in culture media containing fetal calf serum (FCS), the H_2O_2 generation upon phenolics addition was less than that in serum-free media. Thus, we have measured the decomposition of supplemented H_2O_2 (100 μM) in serum-containing media (**Figure 4**). The results indicate that H_2O_2 was catalytically decomposed and the catalysis rate differs with the media and the sera batches. This decomposition did not appear in serum-free media and was probably due to residues of enzymatic activity in the serum.

In accordance with the results of Eberhardt et al. (*3*), we observed that apple extracts significantly inhibited cell proliferation, to an extent similar to that of quercetin. However, this inhibition was significantly reduced by inclusion of metmyoglobin (30 μM) in the medium (**Figure 5a,b**). DMSO at the concentration used (0.5%) had no effect (not shown). Further-
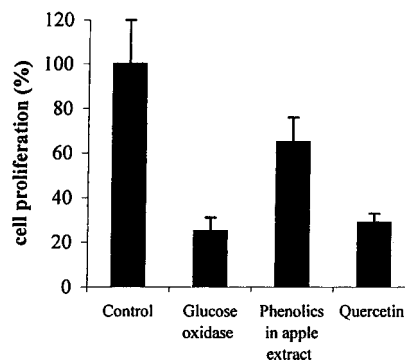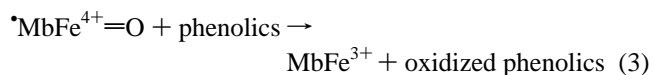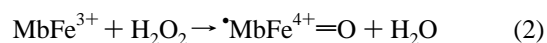

Figure 6. Effect of apple extract, quercetin, and glucose oxidase on HepG2 cell proliferation (mean ± SD, $n = 4$). The phenolics concentration in the apple extract (111 μM) was calculated as quercetin equivalent. Quercetin concentration was 100 μM. H_2O_2 production by glucose oxidase (2.5 μg/mL) in the culture medium was 149 ± 5 μM/h^{-1} as determined by the FOX2 method.

more, this effect was mimicked by exposure of parallel cultures to comparable concentrations of H_2O_2 generated by glucose oxidase (**Figure 6**). No cytotoxicity of the apple extract was observed at any of the concentrations tested as indicated by the trypan blue staining method (not shown).

The paper published by Eberhardt et al. (*3*) claims the antioxidant character of the apple extract was responsible for the inhibition of the tumor cell proliferation. This antioxidative character of polyphenols is well-known to be connected with the capability of these compounds to work as free radical scavengers. However, this kind of activity could not be prevented by metmyoglobin. These findings support the assumption that the inhibition of cell growth was not caused by an antioxidant effect on cells, but rather, was caused through an indirect generation of H_2O_2 in the growth medium. Our previous data demonstrated that metmyoglobin interacts rapidly with H_2O_2, forming oxoferryl myoglobin (*24*). Although oxoferryl myoglobin may cause oxidative damage under some circumstances (*25*), we clearly show that in our system, which contains phenolic substances, the effect is far less toxic than in the absence of metmyoglobin. This is most probably because oxoferryl myoglobin is very efficiently reduced by the phenolic antioxidants, and thus works as a pseudo-peroxidase, as summarized below:

$$\text{phenolics} + \text{culture media} \rightarrow H_2O_2 \quad (1)$$

$$MbFe^{3+} + H_2O_2 \rightarrow {}^{\bullet}MbFe^{4+}{=}O + H_2O \quad (2)$$

$${}^{\bullet}MbFe^{4+}{=}O + \text{phenolics} \rightarrow$$
$$MbFe^{3+} + \text{oxidized phenolics} \quad (3)$$

where ${}^{\bullet}MbFe^{4+}{=}O$ is oxoferryl myoglobin.

This activity of metmyoglobin decreases H_2O_2 levels in the medium containing a reducing compound and prevents probable generation of hydroxyl radicals through the Fenton reaction (*24*). Because myoglobin is a cationized protein, its interaction with membranes is more efficient than that of catalase and could better reduce H_2O_2 generated in the vicinity of membranes. Interestingly, in the presence of metmyoglobin, low concentrations of apple extract increased HepG2 cell proliferation above control levels. This effect seems to result from the presence in untreated culture media (without cells) of a low concentration (11−17 μM) of H_2O_2, which may cause some growth inhibition. The H_2O_2 concentration in the media in the presence of untreated

cells was below the detection level, probably because of the enzymatic activity of the cells.

Hydrogen peroxide has many effects on cells, and these effects are dependent on its concentration and on the cell type. Hydrogen peroxide could activate hemeproteins such as myoglobin and hemoglobin, as well as several types of enzymes such as peroxidases, lipoxygenases, and cyclooxygenases (*26*). In addition, H$_2$O$_2$ was found to raise intracellular Ca^{2+} levels (*27, 28*); activate transcription factors (*29, 30*); suppress signal transduction factors (*27, 30*); promote or decrease apoptosis (*28, 31*); and increase or repress proliferation (*32, 33*). In parallel, in various cell lines, flavonoids and phenolic compounds were found to suppress protein kinase C activation (*34, 35*); to down-regulate activation of AP-1 (*36, 37*) and expression of ICAM-1 (*38*); promote apoptosis (*39, 40*); suppress cell proliferation (*41, 42*); inhibit cell growth (*43, 44*); and to be cytotoxic (*45, 46*).

While agreeing with Eberhardt et al. (*3*) that apples contain a high concentration of antioxidants, we wish to emphasize that the anti-proliferative effect of apple extract is not due to a direct action of antioxidants on tumor cells; rather, it is caused *indirectly* through generation of H$_2$O$_2$ and consequent oxidative damage to cells.

Moreover, it is possible that many of the previously reported effects on cultured cells of flavonoids and phenolics result from artifactual generation of oxidative stress. Our results strongly suggest that in order to prevent such artifacts, the formation of H$_2$O$_2$ and other active oxygen species should be determined prior to cell seeding. In addition, the use of catalase and/or myoglobin/reducing agent should be considered as a method to decompose H$_2$O$_2$ and prevent generation of other reactive oxygen species. The use of tumor cells and "nontumor cells" in a bioassay to measure antioxidant activity, in this context, is potentially misleading and should be applied with caution.

ABBREVIATIONS USED

DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; PBS, phosphate buffer saline; FCS, fetal calf serum; ICP−AES, inductively coupled plasma−atomic emission spectrometry.

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