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Artifacts in Cell Culture: Rapid Generation of Hydrogen Peroxide on Addition of (−)-Epigallocatechin, (−)-Epigallocatechin Gallate, (+)-Catechin, and Quercetin to Commonly Used Cell Culture Media

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There is considerable current interest in the possible beneficial health effects of quercetin, catechins, epigallocatechins, epigallocatechin gallates, and related phenolic compounds found in teas, wines, and other plant products. As a result, many laboratories are studying the effects of these compounds on cells in culture. The present paper shows that addition of these compounds to commonly used cell culture media leads to generation of substantial amounts of hydrogen peroxide (H_2O_2). Dulbecco's modified Eagle medium gives the highest H_2O_2 level for all the compounds tested, with levels reaching >400 μM within 2 h for addition of 1 mM concentrations of gallic acid, epigallocatechin gallate, and epigallocatechin. Catechin and quercetin produced lower, but still significant, levels of H_2O_2. McCoy's 5A and RPMI 1640 media also promoted H_2O_2 production from the above phenolic compounds. This rapid generation of H_2O_2 could account for some or all of the reported effects of phenolic compounds on cells in culture.

There has been considerable recent interest in the beneficial health effects of diets containing fruits, vegetables, and certain beverages, including wines, green tea, and black tea (1–7). Many of these proposed health benefits, such as protective effects against the development of cancer and cardiovascular disease, have been attributed to the phenolic compounds present, with a particular focus on quercetin, catechin, epigallocatechin, and epigallocatechin gallate (1–8). As a result, many laboratories are examining the effects of these phenolic compounds on malignant, or "normal", cells in culture. For example, epigallocatechin gallate was reported to induce apoptosis in prostate cancer cell lines (9), WI38VA transformed fibroblasts (10) and U937 cells (11). Several polyphenolic compounds were reported as toxic to HL-60 cells, to an extent apparently related to their ease of oxidation (12). Quercetin promoted apoptosis in the HPB-ALL cell line (13) and in colorectal tumor cells (14). It also downregulated activation of the AP-1 transcription factor and expression of ICAM-1 in transformed human endothelial cells (15). Polyphenolic compounds such as the flavonoids can be oxidized and can exert pro-oxidant effects *in vitro* under some assay conditions (16–20), but most reported studies have emphasized their antioxidant effects (1–8).

In the present work we have identified an artifact that can affect studies of the action of quercetin, the catechins and probably most polyphenolic compounds on cells in culture: that these compounds interact with commonly used cell culture media to generate high levels of H_2O_2. Such H_2O_2 generation could explain many of the reported effects of polyphenolic compounds upon cells in culture.

MATERIALS AND METHODS

Reagents. Dulbecco's modified Eagle medium (DMEM) and RPMI 1640 cell culture media were obtained from Hyclone (Irvine, CA) and McCoy's 5A medium was from Gibco, BRL (Gaithersburg, MD). At least 5 different batches of each medium were examined, with no significant differences between batches. Phenolic compounds and other reagents (unless otherwise stated) were obtained from Sigma Chemical Pte Ltd., Singapore. They were dissolved in deionized water, except for quercetin which was first dissolved in ethanol to a final concentration of 10 mM. Catalase was Sigma Type C40 (specific activity 2500 units/mg protein).

Measurement of H_2O_2. This was carried out by the ferrous ion oxidation-xylenol orange (FOX) method (21). A sample of culture medium (90 μl) was mixed with 10 μl of methanol and incubated at room temperature for 30 min. The FOX reagent (21, 22) (0.9 ml) was

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TABLE 1

Generation of H_2O_2 in Cell-Culture Media

Compound added	$[H_2O_2]$ present in medium (μM)		
	DME	RPMI	McCoy
None	1.6 ± 0.7	1.1 ± 0.2	1.5 ± 1.1
Ethanol*	1.4 ± 0.6	1.3 ± 0.2	4.6 ± 1.3
Quercetin	25.4 ± 6.2	8.8 ± 2.9	11.8 ± 4.2
Catechin	6.1 ± 2.2	3.1 ± 1.1	4.11 ± 1.2
Epigallocatechin	115.0 ± 36.1	98.1 ± 21.5	104 ± 12.8
Epigallocatechin gallate	71.6 ± 8.2	69.9 ± 13.4	50.7 ± 10.2
Gallic acid	66.2 ± 12.7	25.0 ± 9.4	18.4 ± 6.3

Note. Phenolic compounds at a final concentration of 100 μM were added to culture media and incubated at room temperature for 1 h. H_2O_2 was then measured by the FOX assay. Data are means ± SD, $n \geq 3$.

* Ethanol at the same concentration used to dissolve quercetin. All other phenolic compounds were dissolved in deionised water before addition.

added, followed by vortexing and 30 min incubation. Solutions were then centrifuged at 15,000*g* for 10 min at room temperature and the absorbance at 560 nm was read against a methanol blank.

O_2 electrode assay (22). A Hansatech oxygen electrode (Hansatech, UK) was used. The electrode was stabilized for 30 min with 1.5 ml of air-saturated phosphate-buffered saline, pH 7.4 in the chamber. The buffer was then replaced by 1.5 ml of culture medium containing phenolic compounds as stated and the recorder pen adjusted to a position approximately 50% of full-scale deflection. 100 μl of catalase solution (containing 1000 units) in PBS was injected through the cap. The electrode was calibrated for O_2 evolution using freshly prepared solutions of H_2O_2 in each of the culture media.

Incubation of media. Phenolic compounds were added to cell culture media at the final concentrations stated in a total volume of 1.5 ml and incubated at room temperature for the times stated before assay of H_2O_2 by the FOX or O_2 electrode methods. Quercetin was dissolved in ethanol as indicated above; addition of equivalent amounts of ethanol to the culture media had no significant effects on the measurement of H_2O_2, nor did it lead to H_2O_2 generation in the media.

RESULTS

Three different media were examined in this study: Dulbecco's modified Eagles medium (DMEM), RPMI 1640 (RPMI) medium and McCoy's 5A medium (McCoy). Hydrogen peroxide was measured by the ferrous iron oxidation-xylenol orange (FOX) assay (21, 22)—preliminary experiments showed that none of the phenolic compounds or cell culture media examined had any effect on the measurement of H_2O_2 by this assay. When the phenols examined in the present study were dissolved in deionized water and allowed to stand at room temperature (or when quercetin was dissolved in ethanol to a final concentration of 10 mM and then diluted to 0.1 mM with distilled water), little, if any, H_2O_2 was detected (<5 μM after 2 h). The culture media contained little (≤ 9 μM) H_2O_2 even when exposed to air and ambient light intensity at room temperature for 2 h. Hydrogen peroxide added to any of the media was quantitatively recovered in the FOX assay, even after incubation at room temperature for 2 h (tested up to 1 mM H_2O_2). Hence the media show no significant H_2O_2-scavenging ability.

When quercetin, (+)-catechin, (−)-epigallocatechin and (−)-epigallocatechin gallate were added to cell culture media at a final concentration of 100 μM, and H_2O_2 assayed 60 min later, substantial amounts of H_2O_2 were detected by the FOX assay (Table 1). Addi-

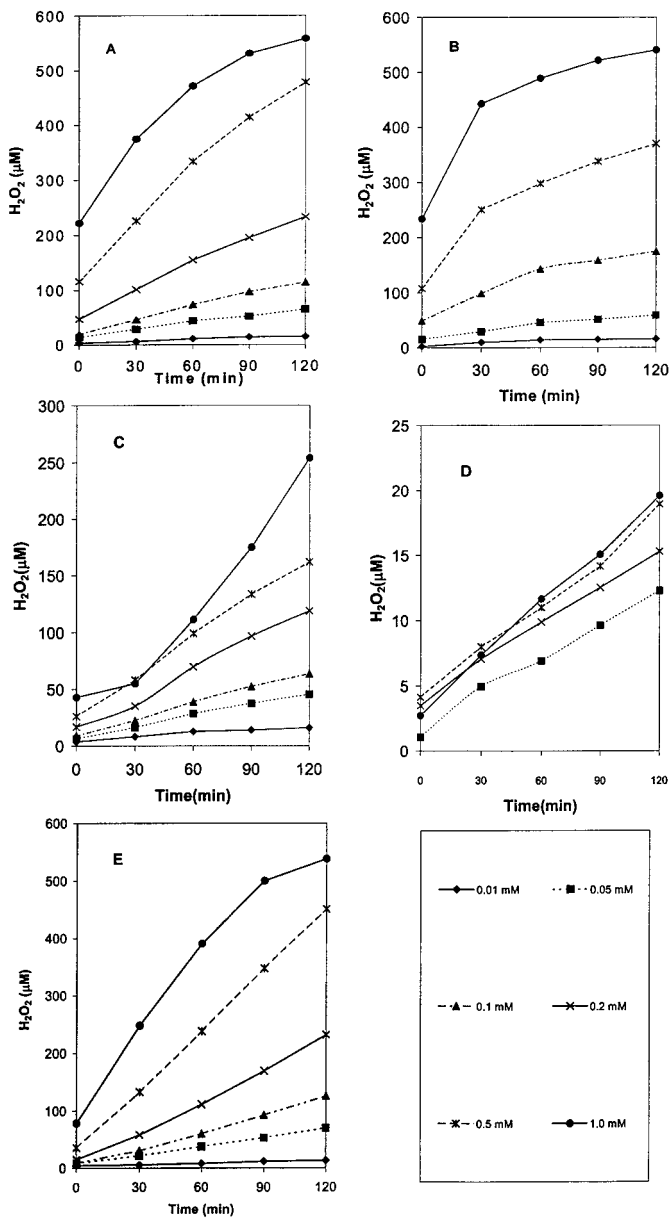

FIG. 1. Generation of H_2O_2 on addition of (A) epigallocatechin gallate, (B) epigallocatechin, (C) quercetin, (D) catechin, and (E) gallic acid to Dulbecco's modified Eagle medium. Concentrations stated are the final concentrations in the medium. SD values are not shown to avoid cluttering the figures, but some representative values are shown in Table 1.

tion of catalase abolished color development in the FOX assay, as expected. Levels of H_2O_2 were in general highest for DMEM, but also significant in the other two media. Quercetin was dissolved in ethanol, but addition of an equivalent amount of ethanol to any of the culture media did not lead to H_2O_2 generation.

The levels of H_2O_2 generated increased with time and with concentration of the phenolic compound added. For example, 1 mM epigallocatechin or epigallocatechin gallate in DMEM gave [H_2O_2] over 500 μM (Figs. 1A and 1B). Quercetin (Fig. 1C) and catechin (Fig. 1D) generated lower levels of H_2O_2, but they were still substantial; e.g., 1 mM quercetin in DMEM gave [H_2O_2] of about 250 μM (Fig. 1C). Gallic acid also generated substantial amounts of H_2O_2 (Table 1; Fig. 1E). Rates of H_2O_2 generation in the other media were also time-dependent and increased with concentration of the phenolic compound added (Table 1 shows representative data for 0.1 mM level at a 60 min time point). The generation of H_2O_2 from all the above compounds was confirmed using a different assay method based on use of an O_2 electrode. For example, after 60 min 0.1 mM quercetin generated 20 $\pm$ 9.19 μM H_2O_2 in DMEM, 9.15 $\pm$ 2.62 μM in RPMI and 11.67 $\pm$ 1.04 μM in McCoy media (mean $\pm$ SD, $n = 5$) as measured by the O_2 electrode method.

DISCUSSION

In the present paper we have shown that addition of phenolic compounds, especially epigallocatechin and epigallocatechin gallate, to cell culture media rapidly generates substantial amounts of H_2O_2. The gallate moiety seems especially important, since gallic acid also generated large amounts of H_2O_2 (Fig. 1E). Catechin and quercetin generated lower, but still substantial, levels of H_2O_2. Hydrogen peroxide has many cellular effects depending on the cell-type examined and the H_2O_2 concentration. For example, H_2O_2 can raise intracellular Ca^{2+} levels (23, 24), activate transcription factors (23, 25), repress expression of certain genes (26), promote (24, 27) or inhibit (27, 28) cell proliferation, be cytotoxic (29), activate (30) or suppress (26, 31) certain signal transduction pathways, promote or suppress apoptosis (28, 32–34, 36), affect expression of adhesion molecules (35), promote differentiation (36) or cell senescence (28) and affect ornithine decarboxylase activity (37, 38). The naturally-occurring phenolic compounds investigated in this paper, as well as related compounds, have been variously reported to induce apoptosis (9–11, 13, 14), be cytotoxic (12), downregulate activation of AP-1 (38) and expression of ICAM-1 (15), suppress cell proliferation (39) and protein kinase activation (40, 41) and inhibit cell growth (42). It is well-known that the responses of cells to H_2O_2 vary according to the cell studied, its levels of catalase and other H_2O_2-removing enzymes, the amount of H_2O_2 added, and whether the cell is transformed or not (29, 34, 43). Responses can include stimulation or inhibition of proliferation, signal transduction and gene expression. Thus effects of H_2O_2 resemble the reported effects of polyphenolic compounds reviewed above. It may be that most or all of the reported effects of polyphenolic compounds on cells are due to their oxidation in the culture media, leading to H_2O_2 generation. Indeed, DMEM and RPMI have been the media used for most studies of the cellular effects of polyphenolic compounds [e.g., Refs. 9–14, 44–46]. Several polyphenolic-rich beverages commonly drunk by humans, including green and black teas, have already been shown to contain substantial amounts of H_2O_2 (47). We recommend that in cell culture studies using DMEM, RPMI, McCoy and probably other media:

(i) that the fate of the added phenolic compound is examined, i.e., does it disappear?

(ii) that the formation of H_2O_2 and other reactive oxygen species in the culture medium is examined before adding any cells.

(iii) that the effect of catalase addition on apparent cellular effects of polyphenolic compounds be examined. More studies will be needed to differentiate direct cellular effects of phenolic compounds from effects caused by H_2O_2 generation in the culture media.

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