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Artefacts in cell culture: α-Ketoglutarate can scavenge hydrogen peroxide generated by ascorbate and epigallocatechin gallate in cell culture media

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ABSTRACT

Ascorbate and several phenolic compounds readily oxidise in cell culture media to generate hydrogen peroxide. However, addition of α-ketoglutarate, which is known to be released by several cell types, decreased the levels of H_2O_2, and the α-ketoglutarate was depleted and converted to succinate. These observations could account for previous reports of the protective effects of α-ketoglutarate in promoting the growth of cells in culture, and may contribute to explaining some of the variability in the literature in reported rates of H_2O_2 production from autoxidisable compounds in cell culture systems.

1. Introduction

Ascorbate and several polyphenolic compounds (e.g. epigallocatechin gallate, EGCG) undergo rapid oxidation in cell culture media to generate H_2O_2. This H_2O_2 and/or other oxidation products can contribute to, or account for, many of the observed effects of ascorbate and polyphenols on cells in culture [1–10]. Dulbecco's Modified Eagle's Medium (DMEM) frequently produces such artefacts [1,2,10], but they are also seen in several other media [2,10,11]. However, a wide range of variable results from different laboratories in terms of the extent and rate of H_2O_2 production in various cell culture systems has been reported (reviewed in [9], also several personal communications to Barry Halliwell). Even in our own laboratory, we find considerable batch-to-batch variations in the ability of media to promote oxidation of, and H_2O_2 production from, compounds added to cell cultures [2,5,6,11]. One factor contributing to this variability is the pyruvate content of the medium, since pyruvate can react rapidly with H_2O_2, is frequently added to cell culture media and is readily released by cells in culture [12–22].

Other keto-acids can also react with H_2O_2 [19,20,22]. In particular, α-ketoglutarate is decarboxylated by H_2O_2 to succinate, a reaction that has been used to measure H_2O_2 in biological systems [19,20,22–25]. Since α-ketoglutarate can also be released by certain cells in culture [26], its presence might affect the apparent toxicity of oxidisable compounds such as EGCG and ascorbate. The present paper examines this possibility.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose (25 mM) and with L-glutamine (Catalogue No. E15-810) was from PAA Laboratories GmbH Haidmannweg 9 A-4061 Pasching Austria. The DMEM also contained foetal bovine serum (FBS) (10%) and antibiotic antimycotic solution at a final concentration of onefold. FBS (Catalogue No. A11-151) was from PAA Laboratories and antibiotic antimycotic solution 100× (Catalogue No. A5955) was from Sigma–Aldrich Co., P.O. Box 14508, St. Louis, MO 63178, USA. The following reagents were also from Sigma–Aldrich Co.: catalase (Catalogue No. C40), (−)-epigallocatechin gallate (Catalogue No. E4143), (+)-sodium L-ascorbate (Catalogue No. A4034), α-ketoglutaric acid (Catalogue No. K-1875) and phosphate-buffered saline PBS (Catalogue No. P3813). Hydrogen peroxide was from Kanto Chemical Company Inc. (Japan) and dimethylsulphoxide (DMSO) was from Fisher Scientific Pte. Ltd. Singapore.

2.2. Measurement of H_2O_2

For EGCG, this was carried out by the ferrous ion oxidation–xylenol orange (FOX) method [2]. Briefly, a sample of culture medium (90 μl) was mixed with 10 μl of methanol and incubated at

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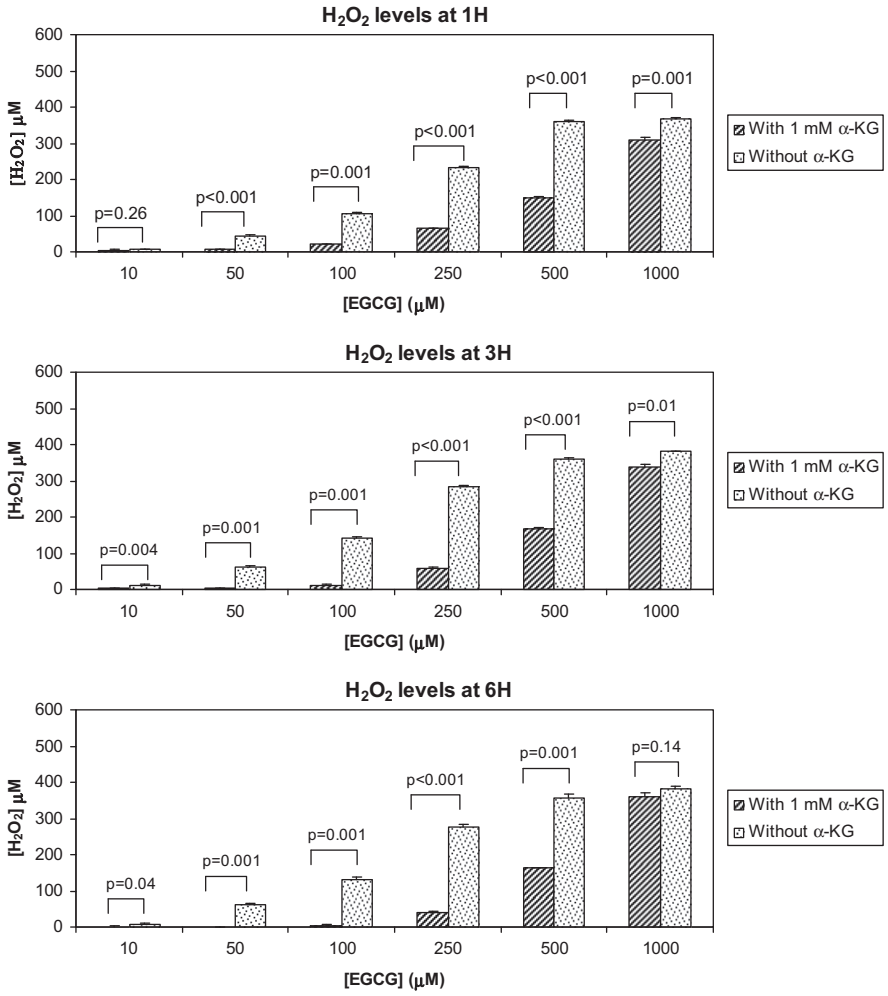

Fig. 1. Epigallocatechin gallate at various concentrations was added to DMEM with and without 1 mM α-ketoglutarate and incubated at 37 °C for 1, 3 and 6 h in a cell culture incubator under 95% air/5% CO_2. H_2O_2 was then measured by FOX assay. Data are means ± SD, $n \geqslant 3$. No significant level of H_2O_2 was detected in the absence of EGCG.

room temperature for 30 min. The FOX reagent (0.9 ml) was added, followed by vortexing and 10 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. Freshly prepared solutions of H_2O_2 were used to construct a calibration curve.

2.3. O_2 electrode assay

H_2O_2 production by ascorbate was measured as described in [27], since ascorbate interferes with the FOX assay [6]. Briefly, a Hansatech oxygen electrode (Hansatech Instruments, Narborough Road, Pentney, King's Lynn, Norfolk, England PE 32 1JL) was used. The electrode was stabilized for 30 min with 1.5 ml of air-saturated phosphate-buffered saline, pH 7.4 (0.01 M phosphate, 0.138 M NaCl and 0.0027 M KCl) in the chamber. The buffer was then replaced by 1.5 ml of culture medium. One hundred microliter of catalase solution (containing 1000 units of enzyme activity) in PBS was injected through the cap. The electrode was calibrated for O_2 evolution using freshly prepared solutions of H_2O_2.

2.4. Succinate assay

Succinate was measured using a commercial kit from Megazyme International Ireland Ltd. (Product No. K-Succ 11/05), Bray Business Park, Bray, Co., Wicklow, Ireland. The reactions can be summarised as follows:

$$\text{Succinate} + \text{ATP} + \text{CoA} \rightarrow \text{succinyl-CoA} + \text{ADP} + \text{Pi} \quad (1)$$

$$\text{ADP} + \text{PEP} \rightarrow \text{ATP} + \text{pyruvate} \quad (2)$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \textit{L}\text{-lactate} + \text{NAD}^+ \quad (3)$$

The amount of NAD^+ formed in the above coupled reaction pathway is stoichiometric with the amount of succinate. The NADH consumption was measured by the decrease in A_{340}.

2.5. α-Ketoglutarate assay

α-Ketoglutarate was measured using a commercially-available kit from Abcam plc. (Product No. ab83431) 330 Cambridge Science Park, Cambridge, UK. α-Ketoglutarate is transaminated with the generation of pyruvate which is oxidised by pyruvate oxidase to generate a chromogen which absorbs at 570 nm.

3. Results

As expected [2,10,21], epigallocatechin gallate (EGCG) added to DMEM underwent rapid oxidation to generate high levels of H_2O_2, close to 400 µM at 1 h from 1 mM EGCG. Fig. 1 shows the levels of H_2O_2 generated from EGCG at three time points, 1, 3 and 6 h. Little H_2O_2 (<3 µM) was detected in the absence of EGCG.

However, when α-ketoglutarate was present, the levels of H_2O_2 measured were lower. For concentrations of EGCG up to 100 µM, almost no H_2O_2 was detected. By contrast, for 1 mM EGCG no sig-

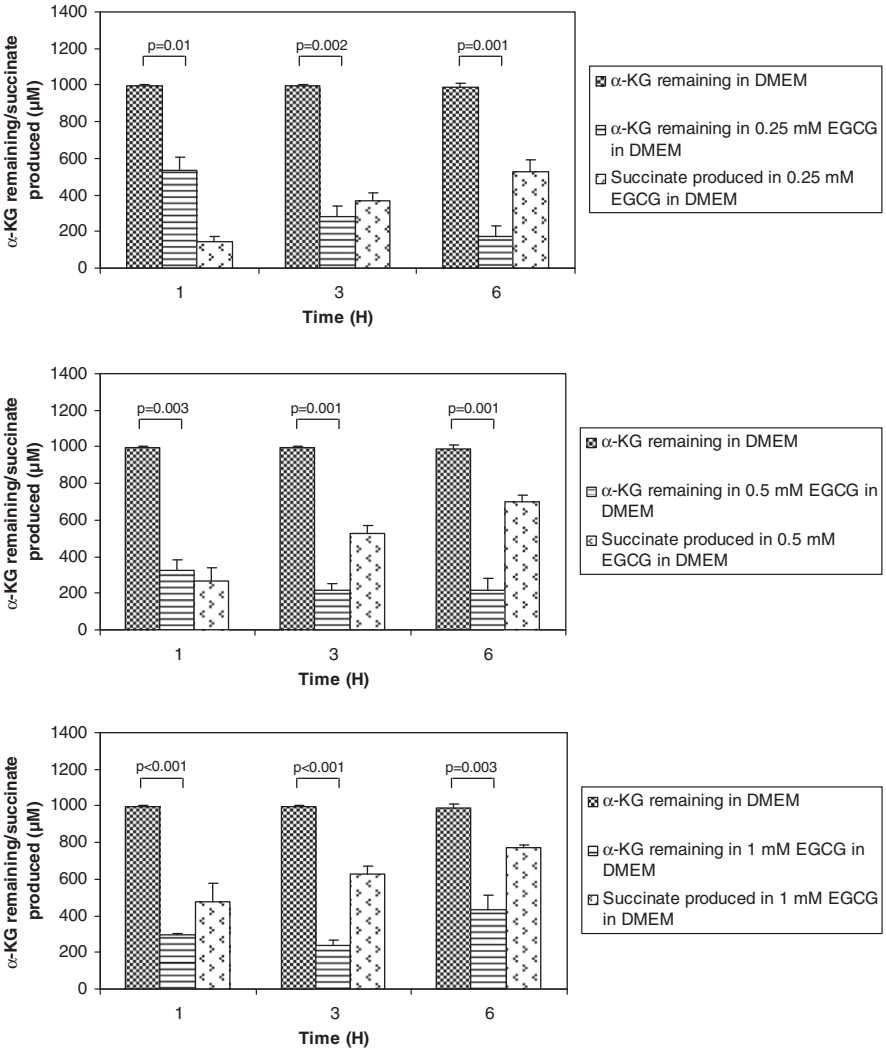

Fig. 2. Epigallocatechin gallate at the various final concentrations stated was added to DMEM with 1 mM α-ketoglutarate and incubated at 37 °C for 1, 3 and 6 h in a cell culture incubator under 95% air/5% CO_2. Loss of α-ketoglutarate was measured using a commercial kit from Abcam. Production of succinate was measured using a commercial kit from Megazyme. Data are means ± SD, $n \geqslant 3$.

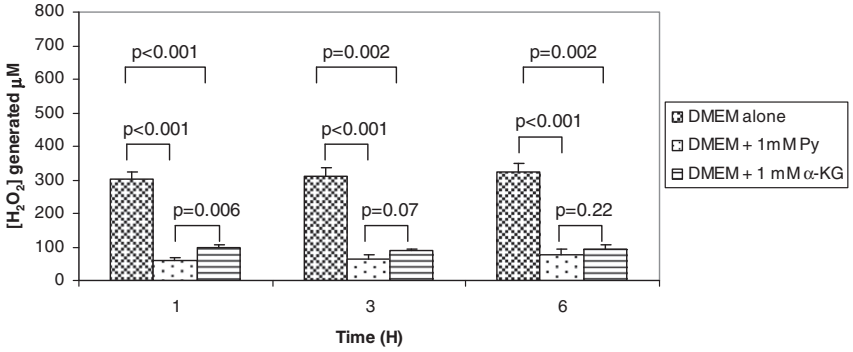

Fig. 3. Epigallocatechin gallate at a final concentration of 0.5 mM was added to DMEM with and without 1 mM pyruvate or 1 mM α-ketoglutarate and incubated at 37 °C for 1, 3 and 6 h in a cell culture incubator under 95% air/5% CO_2. H_2O_2 was measured by FOX assay. Data are means ± SD, $n \geqslant 3$.

nificant ($p > 0.05$) difference in the concentration of H_2O_2 was observed at 6 h, although it was significantly smaller at 1 and 3 h ($p < 0.05$). Addition of EGCG also caused a rapid loss of α-ketoglutarate from the medium (Fig. 2) accompanied by production of succinate (Fig. 2). Surprisingly however, approximately twofold more α-ketoglutarate was lost than succinate generated, suggesting that other reactions are taking place in addition (data not shown). α-Ketoglutarate was slightly less effective than pyruvate at decreasing the levels of H_2O_2 detected at the 1 h time point but not significantly different at later times (Fig. 3).

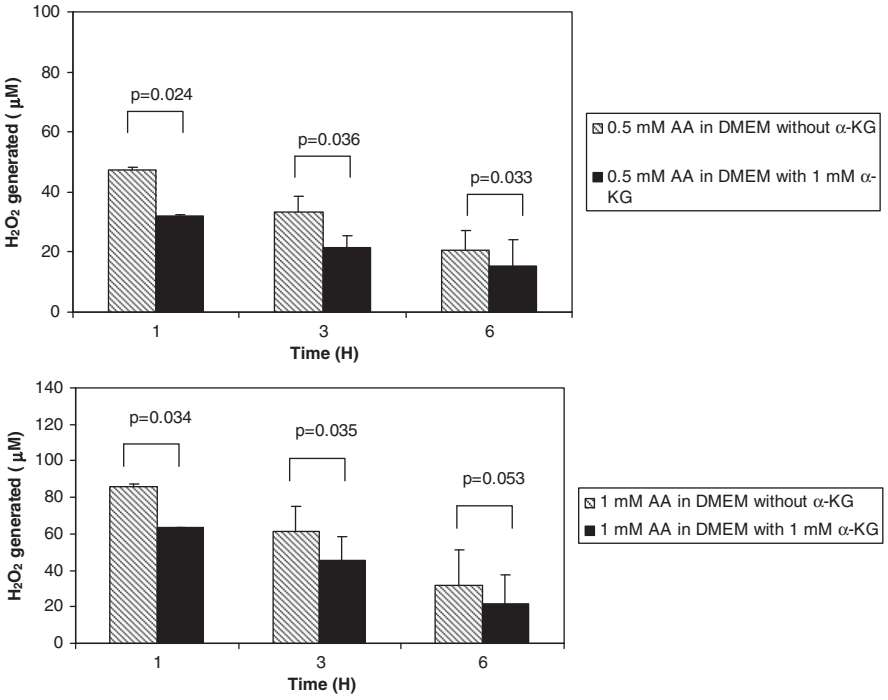

Fig. 4. Ascorbate at final concentrations of 0.5 and 1 mM was added to DMEM with and without 1 mM α-ketoglutarate and incubated at 37 °C for 1, 3 and 6 h in a cell culture incubator under 95% air/5% CO_2. H_2O_2 levels were highest at 1 h and then declined with time. Data are means ± SD, $n \geqslant 3$.No significant H_2O_2 was measured in the absence of ascorbate.

Ascorbate can also generate H_2O_2 in DMEM, although less extensively than for EGCG [2,6]. The presence of α-ketoglutarate in the medium also decreased the levels of H_2O_2 detected when ascorbate was added to DMEM (Fig. 4) and some of the α-ketoglutarate was lost with production of smaller quantities of succinate (Fig. S1).

4. Discussion

There is a growing (but still insufficient [28]) realisation that many compounds undergo rapid chemical reactions with cell culture media, and that H_2O_2 (or sometimes other oxidation products, such as quinones and semiquinones [9,29]) can exert potent cellular effects that can be mistaken for effects of the compounds itself [6,9–11]. The present paper highlights another important factor, that α-ketoglutarate in cell culture media can remove H_2O_2. This keto-acid can be released by certain cell types [20]. Indeed several earlier papers reported that addition of α-ketoglutarate to cell culture systems maintained cell stability and/or promoted cell growth [30–34], including cells used for monoclonal antibody production [31,34]. It was usually assumed that the α-ketoglutarate was acting by metabolic mechanisms. However, given the propensity of cell culture media contents to produce H_2O_2 [1–10,22,35], it may be that α-ketoglutarate was acting by scavenging this reactive oxygen species. Scientists must bear in mind that added compounds could be undergoing oxidation in cell culture media containing pyruvate or α-ketoglutarate, and this might not be detected by measuring H_2O_2. Reaction of α-ketoglutarate with H_2O_2 will generate succinate

$$COOHCH_2CH_2COCOOH + H_2O_2 \rightarrow COOHCH_2CH_2COOH + CO_2 + H_2O$$

and α-ketoglutarate will be depleted; both effects might cause metabolic disturbances in cultured cells that could confuse interpretation of cell behaviour in culture. Not all the α-ketoglutarate lost could be accounted for as succinate in our studies, however, suggestive of other reactions taking place in these complex cell culture media.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.091.

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