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The effects of oxaloacetate on hydrogen peroxide generation from ascorbate and epigallocatechin gallate in cell culture media: Potential for altering cell metabolism

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ABSTRACT

Several phenolic compounds as well as ascorbate can oxidise in certain cell culture media (especially Dulbecco's modified Eagle's medium (DMEM)) to generate hydrogen peroxide. Addition of oxaloacetate decreased the levels of H_2O_2 detected and the oxaloacetate was depleted. Oxaloacetate was approximately as effective as pyruvate in decreasing H_2O_2 levels and more effective than α-ketoglutarate. Our data raise important issues to consider when interpreting the behaviour and metabolism of cells in culture (which are both altered by the oxidative stress of cell culture) and their apparent response to addition of autooxidisable compounds such as ascorbate and epigallocatechin gallate.

1. Introduction

Hydrogen peroxide (H_2O_2) can readily be generated in cell culture media upon the addition of autooxidisable compounds such as ascorbate, or polyphenols such as epigallocatechin gallate, EGCG. This H_2O_2 and/or other oxidation products of such compounds can account for some or all of the observed effects of ascorbate and polyphenols on cells in culture [1–12]. Dulbecco's modified Eagle's medium (DMEM) frequently produces such artefacts [1,2,10], but they are also observed in several other media [2,10–12]. However, published results vary in terms of the extent and rate of H_2O_2 production in various cell culture media that are reported (reviewed in [9,11]). One factor contributing to this is the level of pyruvate in the medium, since pyruvate efficiently scavenges H_2O_2 [13–18]. Other keto acids, such as α-ketoglutarate can act similarly [15,16,18,19].

In mitochondria subjected to oxidative stress, oxidations of pyruvate, α-ketoglutarate and oxaloacetate have been described that can substantially perturb cell metabolism [20,21]. In particular, the oxidation of oxaloacetate generates malonate, which is an inhibitor of succinate dehydrogenase and thus of the mitochondrial electron transport chain [21]. *Escherichia coli* strains lacking malate dehydrogenase were more sensitive to H_2O_2 or ionizing radiating, indicating that oxaloacetate might act as an antioxidant *in vivo* [22]. Indeed, it has been reported that Chinese Hamster Ovary (CHO) cells in culture enhance oxaloacetate production [23], perhaps as an adaptation to the oxidative stress induced by cell culture [1].

In this paper, we therefore investigated the possibility that oxaloacetate in cell culture media (e.g. released as a result of spontaneous or damage-induced cell lysis) could scavenge H_2O_2.

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) at 10% and antibiotic antimycotic solution at a final concentration of onefold. FBS (Catalogue No. A11-151) and sodium pyruvate solution (Catalogue No. S11-003) were from PAA Laboratories. 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), oxaloacetic acid (Catalogue No. O7753), phosphate-buffered saline PBS (Catalogue No. P3813) and hydrogen peroxide 35 wt.% solution in water

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(Catalogue No. 349887). 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 as described in our previous papers [17,19,24]. H_2O_2 production by ascorbate was measured as described in [24], since ascorbate interferes with the FOX assay [6].

2.3. Oxaloacetate assay

Oxaloacetate was measured using a commercially-available kit from Abcam plc. (Product No. ab83428) 330 Cambridge Science Park, Cambridge, UK. Oxaloacetate is converted to pyruvate which is utilized to convert a nearly colourless compound to an intensely coloured product which absorbs at 570 nm.

3. Results

As expected [2,10,17,19], epigallocatechin gallate (EGCG) added to DMEM undergoes ready oxidation to generate high levels of H_2O_2, over 400 μM at 1 h from 1 mM EGCG. Fig. 1 shows the levels of H_2O_2 generated from EGCG at three selected time points, 1 h, 3 h and 6 h. Little H_2O_2 (<3 μM) was detected in the absence of EGCG. However, when oxaloacetate (final concentration of 1 mM) was added to DMEM, much less H_2O_2 was detected. In particular, for concentrations of EGCG up to 100 μM, almost no H_2O_2 was detected at all. By contrast, for 1 mM EGCG, no significant ($p > 0.05$) difference in the concentration of H_2O_2 was observed at 3 h or 6 h, although the level was less than half at 1 h. This is probably due to substantial loss of oxaloacetate by 3 h and 6 h (Table 1).

Fig. 2 shows one of a series of experiments showing that oxaloacetate is usually significantly ($p < 0.05$) more effective than α-ketoglutarate, also a powerful H_2O_2 scavenger, at decreasing

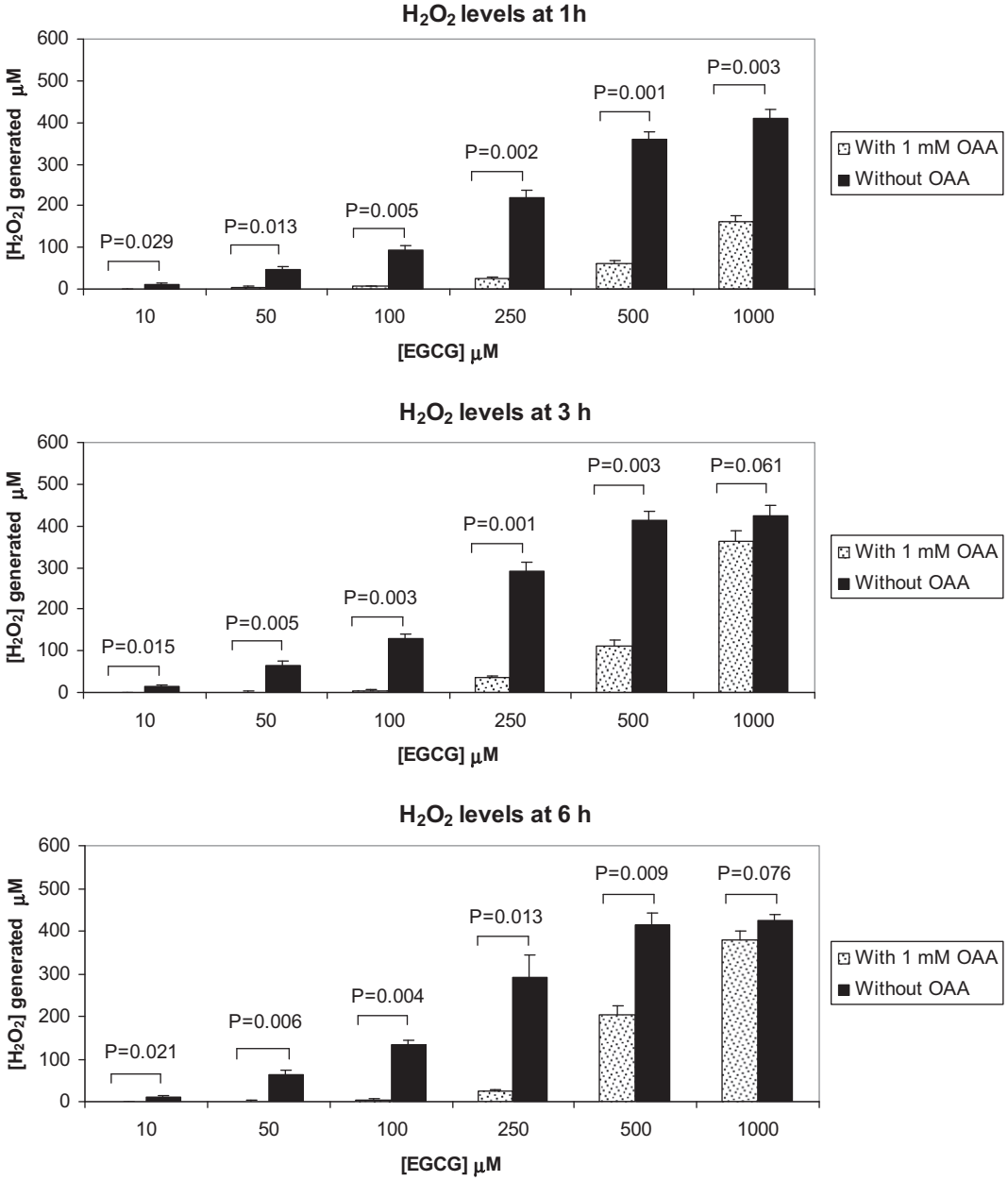

Fig. 1. Epigallocatechin gallate at the final concentrations stated was added to DMEM with and without 1 mM oxaloacetate and incubated at 37 °C for 1 h, 3 h and 6 h in a cell culture incubator under 95% air/5% CO_2. Data are mean ± SD, $n \geqslant 3$. Little (<3 μM) H_2O_2 was detected in the absence of EGCG.

Table 1
Loss of oxaloacetate and H_2O_2 production in DMEM containing EGCG at the final concentrations stated. For detailed reaction conditions, please see the legend to Fig. 1.

Concentration of EGCG (μM)	Loss of oxaloacetate (μM)		H_2O_2 (μM) Produced in DMEM − OAA		H_2O_2 (μM) Produced in DMEM + OAA		H_2O_2 apparently scavenged by oxaloacetate (μM)	
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
1 H								
10	82	43	11	3	0	0	11	3
50	255	58	46	7	4	1	42	8
100	327	68	94	10	7	2	87	11
250	515	125	221	17	26	4	194	16
500	622	150	358	19	60	7	298	13
1000	794	51	411	19	162	15	249	25
3 H								
10	95	41	15	3	1	1	14	3
50	229	141	66	8	2	1	65	8
100	400	121	128	12	5	1	123	11
250	723	115	292	21	34	5	258	16
500	883	60	415	21	111	14	304	27
1000	899	31	425	23	362	27	63	28
6 H								
10	107	25	11	2	1	0	11	3
50	241	84	65	8	1	1	64	8
100	438	69	132	14	5	1	127	14
250	747	97	290	52	25	4	265	54
500	879	30	413	28	204	22	209	35
1000	591	76	425	15	379	23	46	23

the levels of H_2O_2 detected. However, it is not significantly better than pyruvate in decreasing the levels of H_2O_2 ($p > 0.05$).

Addition of EGCG also caused a rapid loss of oxaloacetate from the medium (Table 1). The loss of oxaloacetate was in substantial molar excess (approximately threefold at 1 h) to the loss of H_2O_2, calculated as the difference between the H_2O_2 levels in medium without or with oxaloacetate (Fig 1).

Ascorbate can also generate H_2O_2 in DMEM, although less rapidly than for EGCG [6,17,19]. The presence of oxaloacetate in the medium also decreased the levels of H_2O_2 detected when ascorbate was added to DMEM (Fig. 3).

Rapid loss of oxaloacetate from the medium (again in considerable molar excess over the H_2O_2 apparently scavenged) was also observed for addition of ascorbate in DMEM (Fig. 4).

4. Discussion

Many (but not all, discussed in [12,25]) groups are now realising that many compounds undergo rapid chemical oxidation in cell culture media, and that the resulting H_2O_2 and/or other oxidation products (e.g. [26]) can exert cellular effects that have sometimes been mistakenly attributed to direct actions of the added compounds [1,6,9–12]. Keto acids such as pyruvate or α-ketoglutarate present in the medium (e.g. deliberately added by manufacturers and/or released from cells) can conceal this H_2O_2 production, although they do not prevent the oxidations and the keto acids are depleted [13–19]. Hence the metabolism of any cells present could be altered. Oxaloacetate can also scavenge H_2O_2 [15,16,18,20], and indeed we show here that oxaloacetate can remove H_2O_2 generated from ascorbate or EGCG (Fig. 1–3). Hence any oxaloacetate present in the cell culture media (e.g. released from cells) could not only mask H_2O_2 generation but could also be converted to malonate, a powerful inhibitor of mitochondrial function.

H_2O_2 can also diffuse into cells and react with intracellular oxaloacetate, which could seriously perturb cell metabolism [20–22]. Perhaps this is why some cells in culture appear to accelerate oxaloacetate production [23], yet another example of the many perturbations introduced by the cell culture process [1]. Oxaloacetate breakdown is increased in some tumor cells (e.g. [27]) and several of the metabolic perturbations described in such cells might perhaps relate to the increased oxidative stress that accompanies cancer [28,29]. The presence of oxaloacetate or other keto acids can also affect the survival of isolated neurons in culture [30],

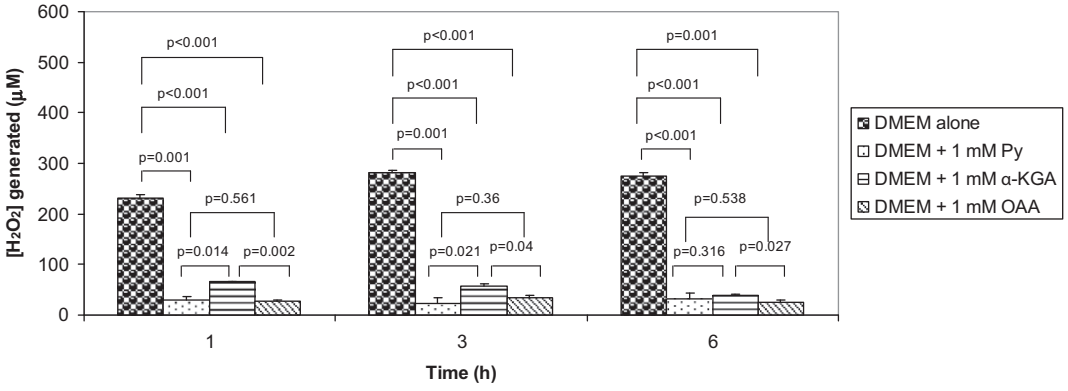

Fig. 2. Epigallocatechin gallate at final concentrations of 0.25 mM was added to DMEM with and without 1 mM pyruvate, 1 mM α-ketoglutarate or 1 mM oxaloacetate and incubated at 37 °C for 1 h, 3 h and 6 h in a cell culture incubator under 95% air/5% CO_2. H_2O_2 was then measured by FOX assay. Data are mean ± SD, $n \geqslant 3$.

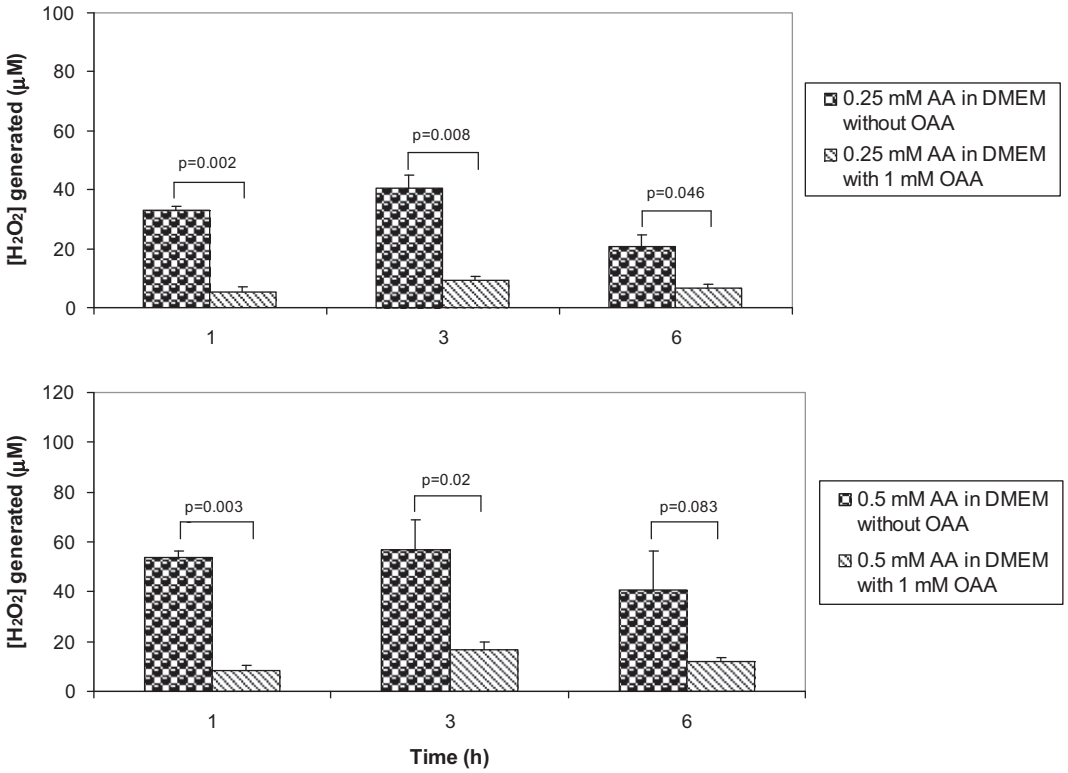

Fig. 3. Ascorbate (AA) at final concentrations of 0.25 mM and 0.5 mM was added to DMEM with and without 1 mM oxaloacetate and incubated at 37 °C for 1 h, 3 h and 6 h in a cell culture incubator under 95% air/5% CO_2. H_2O_2 levels were measured using an oxygen electrode [6]. Data are mean ± SD, $n \geqslant 3$. Little (<3 μM) H_2O_2 was measured in the absence of ascorbate.

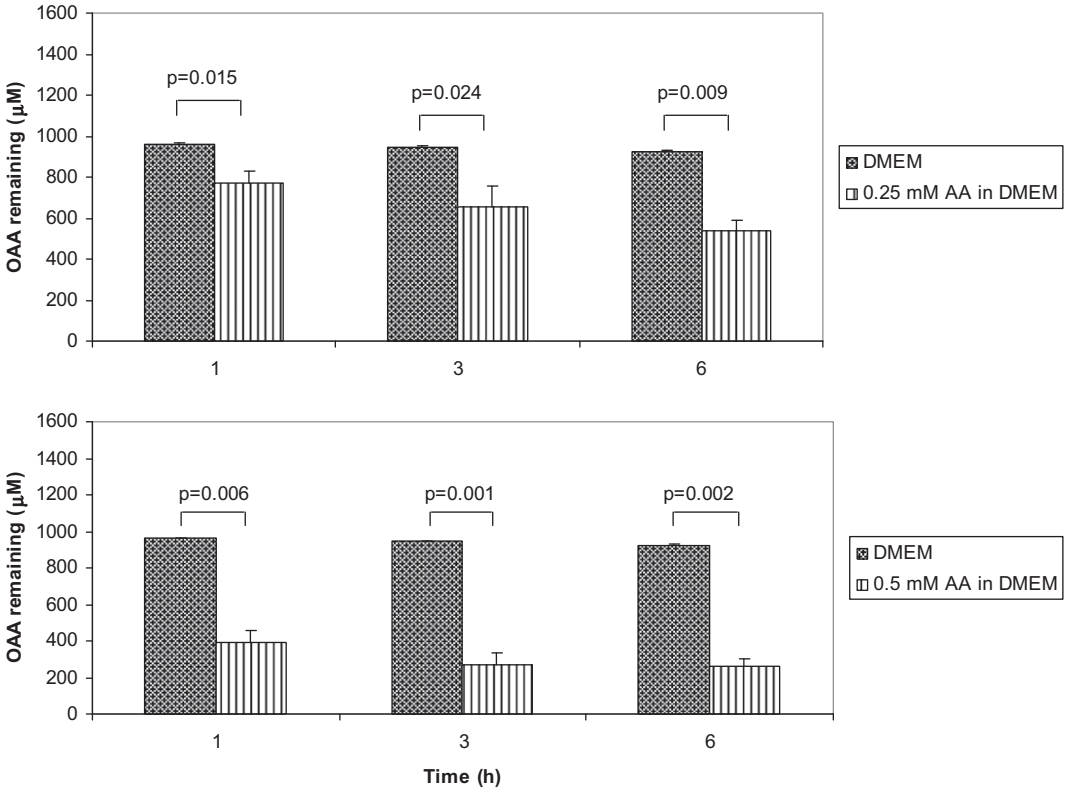

Fig. 4. Ascorbate at final concentrations of 0.25 mM and 0.5 mM was added to DMEM with 1 mM oxaloacetate and incubated at 37 °C for 1 h, 3 h and 6 h in a cell culture incubator under 95% air/5% CO_2. Loss of oxaloacetate was measured using a commercial kit from Abcam. Data are mean ± SD, $n \geqslant 3$.

and both pyruvate and oxaloacetate were highly effective in protecting neurons against the cytotoxicity of H_2O_2 [30]. α-Ketoglutarate was less effective, in agreement with the conclusions in this paper that it is less good as a H_2O_2 scavenger (Fig. 2).

In conclusion, the ability of keto acids to scavenge H_2O_2 can mask the generation of H_2O_2 in cell culture media and lead investigators to conclude that added compounds are not undergoing oxidation and resulting depletion. We have already stressed [3] that stability should be directly determined since H_2O_2 is not always generated. In addition, H_2O_2 diffusing into cells could seriously perturb their metabolism, depleting oxaloacetate, pyruvate [16] and other keto acids, and (in the case of oxaloacetate) generating the mitochondrial toxin malonate.

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