Biochemical and Biophysical Research Communications xxx (2017) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Pyruvate diminishes the cytotoxic activity of ascorbic acid in several tumor cell lines *in vitro*

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ARTICLE INFO

Article history:
Received 19 September 2017
Accepted 24 September 2017
Available online xxx

Keywords:
Ascorbic acid / pharmacology
Cancer
Cell death
Hydrogen peroxide
Prodrugs / therapeutic use
Pyruvate

ABSTRACT

The anticancer potential of ascorbic acid (AA) has been controversially discussed for decades. Although the cytotoxic effect of pharmacologic concentrations of ascorbic acid has already been successfully demonstrated in numerous studies *in vitro*, it could not be verified to the same extent *in vivo*. We propose that the ubiquitous metabolite pyruvate diminishes the effect of AA by reacting with its presumable cytotoxic mediator hydrogen peroxide (H_2O_2). MTT assays confirm that co-incubation with 1.4 mM pyruvate abolishes the cytotoxic effect of pharmacologic concentrations of AA in all cancer cell lines tested (human melanoma (WM451-Lu), breast (MCF-7) and hypopharyngeal cancer cells (FaDu)).

We further investigated whether pyruvate diminishes the anticancer effect of AA by interfering with the generation of H_2O_2. Therefore, we analyzed the concentration of AFR, a proposed intermediate in the AA-dependent formation of H_2O_2, by electron paramagnetic resonance spectroscopy, during incubation with AA and pyruvate in WM451-Lu cells as a model system. In addition, we measured H_2O_2 concentration by indirect detection with Clark-type oxygen electrode. AFR concentration was not significantly influenced by pyruvate, whereas H_2O_2 concentration was significantly reduced. In parallel, pyruvate concentrations of the stimulation medium declined with increasing AA and consequently H_2O_2 concentrations.

In summary, pyruvate diminishes the cytotoxic activity of ascorbic acid *in vitro*. The AFR concentration measured remains unaffected by pyruvate whereas the H_2O_2 concentration is reduced; confirming that pyruvate directly reacts with AA-induced H_2O_2, without influencing its formation. However, further experiments are needed to elucidate the complex mechanisms being responsible for the reduced efficacy of AA *in vivo*.

1. Introduction

Ascorbic acid (AA) functions as an antioxidant at physiological concentrations but exerts pro-oxidative anticancer effects in pharmacologic doses, as already demonstrated in numerous *in vitro* studies [1–3]. The underlying mechanism of selective cancer cell cytotoxicity is still unclear, but most likely appears to involve the oxidation of AA to ascorbyl free radical (AFR) and the subsequent formation and accumulation of hydrogen peroxide (H_2O_2) in extracellular fluid [1,4]. In blood, conversely, H_2O_2 is immediately eliminated by plasma catalase and glutathione peroxidase expressed in erythrocytes [1].

The anticancer potential of pharmacologic AA has been controversially discussed for decades. In the 1970s, clinical trials by Cameron have shown a positive effect of pharmacologic AA doses, improving survival and quality of life in terminal cancer patients [5,6]. However, two subsequent double-blind placebo-controlled studies conducted at the Mayo Clinic failed to verify the observed antitumor effect [7,8]. Therefore, AA was discarded as a potential anticancer agent. Decades later, pharmacokinetic studies of AA revealed that the administration route is a determinant factor for its anticancer potential [9], which was not taken into account in the studies at the Mayo clinic. Padayatty and coworkers demonstrated that intravenous administration of AA results in plasma concentrations substantially higher than after oral application [9]. While AA plasma concentrations do not exceed 220 μM after oral ingestion of pharmacologic AA doses, intravenous application can result in plasma AA concentrations higher than 15 mM. Hence, as AA exerts its cytotoxic effects only in a millimolar range, the

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https://doi.org/10.1016/j.bbrc.2017.09.138
0006-291X/

application route is crucial for the effectiveness.

In the meantime, the potential role of AA in cancer treatment was re-examined showing promising results *in vitro*: cancer cells, but not normal cells, underwent cell death after exposure to high doses of AA [1]. However, application of pharmacologic concentrations of AA decreased but did not inhibit growth of tumor xenografts in mice [2,10]. Therefore, the cytotoxic effect of high dose AA observed *in vitro* seems to be reduced *in vivo*.

In the context of another project, we performed some pretests to compare the effect of high dose AA in our cancer cell lines with the results published by others [1–3]. Surprisingly, we were not able to reproduce the cytotoxic effect of ascorbic acid up to concentrations of 25 mM in any cell line tested. Checking the exact test parameters, we found that the medium used for our pretests (DMEM) contained 1.4 mM pyruvate by default, whereas RPMI1640, a medium often used for cultivation of tumor cells, normally is pyruvate-free. As there are several publications showing that pyruvate reacts with H_2O_2, thereby reducing its cytotoxic effect [11–14], we hypothesized, that pyruvate also diminishes the cytotoxic effect of ascorbic acid by eliminating its supposed main mediator H_2O_2.

Therefore, we investigated the effect of pyruvate on the cell viability during a 2 h incubation period with both pharmacologic concentrations of AA or directly applied H_2O_2 in three different cell lines: human melanoma (WM451-Lu), breast (MCF-7) and hypopharyngeal cancer cells (FaDu). To elucidate whether pyruvate diminishes the anticancer effect of AA by interfering with the generation H_2O_2, we further analyzed the concentrations of AFR and H_2O_2 during simultaneous incubation with AA and pyruvate in WM451-Lu cells as a model system.

2. Materials and methods

2.1. Materials

L-(+)-ascorbic acid, hydrogen peroxide (30%), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS) and acetic acid were purchased from Carl Roth GmbH (Karlsruhe, Germany). RPMI 1640, sodium pyruvate, L-alanyl-L-glutamine and gentamicin were from Biochrom (Berlin, Germany). Fetal calf serum (FCS), penicillin and streptomycin were obtained from PAA Laboratories (Pasching, Austria). Catalase (bovine), β-nicotinamide adenine dinucleotide (NADH) and lactate dehydrogenase (LDH, rabbit muscle) were purchased from Sigma-Aldrich (Steinheim, Germany). Dipotassium hydrogen phosphate and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). 3-Carboxy-PROXYL was from abcr (Karlsruhe, Germany).

2.2. Cell culture and experimental conditions

Human melanoma (WM451-Lu), breast (MCF-7) and hypopharyngeal (FaDu) cancer cells were adapted to grow in pyruvate-free RPMI 1640 medium supplemented with 10% FCS, L-alanyl-L-glutamine (4 mM for WM451 and 2 mM for MCF-7 and FaDu) and 50 μg/mL gentamicin for WM451 or 100 U/mL penicillin and 100 μg/mL streptomycin for MCF-7 and FaDu, respectively. Cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C and split twice a week. For all experiments, cells were seeded in a defined number and grown for 3 days to reach 70–80% confluence. Experiments were performed in RPMI 1640 culture medium without FCS (stimulation medium). For co-incubation experiments, stimulation medium with additional sodium pyruvate (0.7 mM or 1.4 mM) was used. Ascorbic acid solutions were always prepared immediately before use under light protection and adjusted to pH 7.0.

2.3. Determination of cell viability (MTT assay)

For determination of cell viability, cells were grown in 24 well plates (WM451) or 35 mm culture dishes (MCF-7, FaDu). Cells were incubated in stimulation medium containing hydrogen peroxide (0.1–1 mM) or ascorbic acid (0.1–20 mM) in a humidified atmosphere with 5% CO_2 at 37 °C for 2 h. Subsequently, cells were washed twice with PBS^- and kept in culture medium for another 24 h. For MTT assay, cells were incubated in culture medium containing 0.17 mg/mL MTT for 1 h at 37 °C. After aspiration of the MTT solution, the produced formazan crystals were solubilized in DMSO with SDS (100 mg/mL) and acetic acid (0.6% v/v) and optical density was measured at 580 nm. Cell viability was calculated relating to an untreated control run with every experiment.

2.4. Measurement of AFR by electron paramagnetic resonance spectroscopy

To evaluate the amount of ascorbyl free radical generated, stimulation medium containing ascorbic acid (0.1–10 mM) was incubated in a humidified atmosphere with 5% CO_2 at 37 °C for up to 2 h in absence or presence of WM451 cells. Samples were taken every 15 min, instantly snap frozen in liquid nitrogen and stored at −80 °C. Immediately before analysis, samples were thawed in a water bath at 30 °C for 1 min, transferred into a quartz flat cell and measured in a Miniscope MS200 x-band spectrometer. Instrument settings were B(0)-field: 3340 ± 27.5 G, sweep time: 100 s, modulation: 1 G, microwave frequency: 9.43 GHz, microwave intensity: 10 mW. Calculation of AFR concentration was performed by using 3-Carboxy-PROXYL as a stable external standard.

2.5. Detection of H_2O_2 concentration

To detect the H_2O_2 concentration reached in the presence of AA, stimulation medium containing AA (0.1–10 mM) was incubated in a humidified atmosphere at 37 °C without additional CO_2 for up to 2 h in 35 mm culture dishes with or without WM451 cells. Every 15 min, a 1 mL sample was transferred into the chamber of a Clark-type oxygen electrode system (Oxygraph Plus, Hansatech Instruments Ltd, GB) and H_2O_2 concentration was determined indirectly via oxygen evolution after addition of 750 U catalase ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$). As the solubility of oxygen depends on the temperature of the samples, the chamber was maintained at 37 °C using a connected water bath. Calibration was performed using freshly prepared solutions of H_2O_2 (25–500 μM) in stimulation medium.

2.6. Detection of pyruvate concentration

To quantify the utilization of pyruvate in co-incubation experiments, samples were drawn every 15 min, stabilized with catalase (200 U/mL) to remove remaining H_2O_2 and kept on ice until analysis. Pyruvate concentration was detected indirectly based on the consumption of NADH after incubation with lactate dehydrogenase (pyruvate + NADH → lactate + NAD^+). Samples were mixed with NADH in potassium phosphate buffer (50 mM, pH 7.4) to a final concentration of 0.3 mM NADH. Optical density was measured at 340 nm and the enzymatic reaction was started by addition of LDH (final concentration: 3 U/mL). After 10 min of incubation, optical density measurement was repeated and NADH consumption was calculated. Freshly prepared solutions of sodium pyruvate (0.01–0.16 mM) were used for calibration.

Please cite this article in press as: S. Rodemeister, K. Hill, Pyruvate diminishes the cytotoxic activity of ascorbic acid in several tumor cell lines *in vitro*, Biochemical and Biophysical Research Communications (2017), https://doi.org/10.1016/j.bbrc.2017.09.138