Biochimica et Biophysica Acta 1830 (2013) 5267–5276

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Comparison between the effects of diallyl tetrasulfide on human retina pigment epithelial cells (ARPE-19) and HCT116 cells

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ARTICLE INFO

Article history:
Received 8 May 2013
Received in revised form 5 August 2013
Accepted 6 August 2013
Available online 12 August 2013

Keywords:
Reactive oxygen species
Thiols
ER stress signalling
Apoptosis
Antioxidants
Normal cells

ABSTRACT

Background: Diallyl mono- and polysulfanes from garlic are known to induce an adaptive cell response and the formation of antioxidants in cancer cells. In the case of a severe ER stress and a failure in the response, cancer cells eventually go into apoptosis. Only little is known about the response of normal cells upon treatment.
Methods: Normal ARPE-19 cells were treated with diallyl tetrasulfide to study their cellular response and the results were compared with those of HCT116 cancer cells. Cell viability was checked by an MTT assay and cytofluorimetry. The formation of superoxide radicals, H_2O_2 and thiols were determined and proteins involved in the ER stress response were also detected by Western blot analysis.
Results: We found that diallyl tetrasulfide induced reactive oxygen species (ROS) in normal cells similar to cancer cells in a time (0 to 60 min) and dose dependent manner (0 to 50 μM). The level of heme oxigenase-1 (HO-1) was up-regulated in both cell types. Initially, we found a decrease in the total thiol level in both cell types but in contrast to cancer cells, normal cells recovered from the decrease in the total thiol concentration within 60 min of treatment.
Conclusions: The recovery of the thiol concentration in normal cells treated with diallyl tetrasulfide seems to be responsible for the failure to induce the ER stress signalling pathway and finally apoptosis in normal cells.
General Significance: The difference in the recovery of the thiol status might be an explanation for the anti-carcinogenic effects of garlic compounds.

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1. Introduction

Garlic has long been used as a therapeutic agent over an extended period of time. High intake of garlic seems to be associated with a protective effect against different types of cancer. The anti-carcinogenic potential of garlic is, however, dependent on a great variety of different metabolites generated by crushing and eating garlic and it may be influenced also by a number of diverse dietary components. The anti-carcinogenic effect of garlic is often attributed to organosulfur compounds such as allicin, diallyl mono- and polysulfanes, ajoene and *S*-allyl cysteine [1]. From garlic gloves, garlic oil can be obtained by steam distillation. Diallyl monosulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) are the most abundant compounds in garlic oil. Nowadays, the highly active diallyl trisulfide is synthesized from allyl chloride, sodium thiosulfate and sodium sulfide according to the method of Milligan et al. [2]. A rather emerging polysulfide, namely diallyl tetrasulfide (DATTS), is synthesized from allyl mercaptan and disulfur dichloride according to Derbesy and Harpp [3]. There is an increasing number of publications showing that not only do diallyl polysulfanes suppress the growth of multiple cancer types in both *in vitro* and *in vivo* models (for review see: [4–6]), but also induce apoptosis. Growth arrest and apoptosis induction is achieved by multiple mechanisms including impairment of carcinogen activation, inhibition of post-translational modifications of proteins, histone modification, and inhibition of angiogenesis, and metastasis. The anti-cancer activity of the diallyl polysulfanes increases with the length of the sulfur chain *i.e.* diallyl tetrasulfide is more active than diallyl trisulfide which in turn is more active than diallyl disulfide and more active than diallyl sulfide [7–10]. Studies with normal, non-transformed cells are extremely rare. Some publications involving normal cells do, however, point to a rather selective cytotoxicity of such polysulfanes exclusively on cancer cells. Here, the underlying causes of such selectivity are still largely a mystery. We therefore decided to analyze the impact of diallyl tetrasulfide on

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http://dx.doi.org/10.1016/j.bbagen.2013.08.004

normal cells and compare the associated cellular events with the ones triggered in cancer cells. For these studies, we used ARPE-19 cells which are human retinal pigment epithelial cells. The retinal pigment epithelium is a monolayer of cells adjacent to the photoreceptors of the retina. The retinal epithelium plays a critical role in the development and maintenance of adjacent photoreceptors in the vertebrate retina [11]. It is implicated in the transport of nutrients from the vascular choroid, the formation of the blood–retina barrier and the absorption of scattered light. The cell line ARPE-19 is derived from a primary culture of retinal epithelium cells [11].

Some of the effects of diallyl polysulfanes on cancer cells have been associated with their capacity to generate reactive oxygen species (ROS). Other effects are due to reactions of the diallyl polysulfanes with thiol groups of structure forming or regulatory proteins [12]. Both effects induce cellular signalling pathways in order to cope with oxidative stress or with the in-activation of thiol containing proteins. Therefore, in the present study, we address the question whether ARPE-19 cells show an increase in the ROS level and whether the total thiol concentration is affected. Furthermore, we have also analyzed cellular signalling pathways *i.e.* ER stress or oxidative stress response pathways. For the first time, these studies with normal ARPE-19 cells were performed in parallel to experiments with colon cancer cells under identical conditions.

2. Materials and methods

2.1. Reagents and antibodies

Diallyl tetrasulfide (DATTS) was synthesized according to the method of Derbesy and Harpp [3] and purified by column chromatography [13]. Protease inhibitor cocktail Complete™ (Roche Diagnostics, Mannheim, Germany), ascorbic acid (ASC), *N*-acetyl cysteine (NAC), and anti-α-tubulin antibodies were obtained from Sigma-Aldrich (Munich, Germany), DMSO from Merck (Darmstadt, Germany). Antibodies against cytochrome *c*, GAPDH and Nrf2 were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The Amplex® Red Hydrogen Peroxide assay kit was purchased from Molecular Probes (Darmstadt, Germany), whilst Ellman's reagent was from Thermo Scientific (Schwerte, Germany). Anti poly(ADP-ribose) polymerase (PARP), anti-eIF2α, anti-phospho-eIF2α (Ser 51) and anti-HO-1 antibodies were purchased form Cell Signaling Technology (Frankfurt, Germany), whilst the caspase 3 antibody was purchased from Promega (Mannheim, Germany). Goat, mouse and rabbit secondary antibodies were all bought from Dianova (Hamburg, Germany).

2.2. Cell culture

HCT116 cells (ATCC Number: CCL-247) were maintained at 37 °C and 5% CO_2 in McCoy's 5A medium (PromoCell, Heidelberg, Germany) with 10% fetal calf serum (FCS). ARPE-19 cells (ATCC Number: CRL-2302) were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) supplemented with 2 mM L-glutamine, 1 mM gentamicin and 10% fetal calf serum (FCS). DATTS was dissolved in DMSO to an 80 mM stock solution, which was freshly prepared before use. NAC and ASC were each dissolved in distilled water to an 80 mM stock solution and applied to the cell culture medium 0.5 h before treatment (unless stated otherwise).

2.3. Evaluation of cell viability

In order to determine the effects of DATTS on ARPE-19 cells and on HCT116 colorectal cancer cells, cells were seeded at 1×10^4 cells per well to a final volume of 500 μl in a 24-well plate and incubated overnight. Cells were then incubated with 50 μM DATTS or the solvent DMSO (final concentration: 0.05%) alone, for 8, 24 and 48 h and the viability of the cells was determined by a colorimetric MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma) assay according to the manufacturer's instructions. One hour before the end of treatment, 50 μl MTT (5 mg/ml PBS) were added. The enzymatic reaction took place at 37 °C in a humidified atmosphere. Following 1 h MTT treatment, the media was disposed off and cells solubilised by adding 500 μl solubilising solution (10% (w/v) SDS in DMSO and 0.01% acetic acid) to each well and allowing the crystals to completely dissolve. The spectrophotometrical absorbance of the purple-blue formazan dye was determined in a TECAN Lumi multi-well plate reader at 595 nm. As a control, an MTT assay was performed without cells to exclude the possibility that DATTS alone would reduce the yellow MTT to the purple formazan.

2.4. Cell cycle analysis

Cell cycle analysis was carried out by flow cytometry using propidium iodide (PI) staining. The determination was based on the measurement of DNA content of nuclei labelled with PI [14]. ARPE-19 or HCT116 cells (5×10^4) were allowed to grow on a 10 cm Petri dish overnight. The medium was changed and cells were treated accordingly before being incubated for the various time periods. Cells were collected and washed two times with cold PBS before being resuspended in PBS and fixed with 70% ethanol. The cells were further incubated at 37 °C for 30 min with RNase and propidium iodide to label DNA. Cells were then analyzed in a cytofluorimeter (Guava easyCyte HT system, Millipore) according to the manufacturer's instructions.

2.5. Determination of $O_2^{\bullet -}$ in cell culture

Production of superoxide radicals was assessed by oxidation of dihydroethidium (DHE) to 2-hydroxyethidium. Seeded cells grown overnight were treated with 50 μM of DATTS, DMSO (0.05% final concentration) as solvent control or 25 μM amobarbital as a positive control in fresh culture media for 0, 10, 30, and 60 min. After removal of the cell culture medium, cells were washed with PBS, and 190 μl of 25 μM DHE dissolved in PBS was added to each well immediately before measurement. The transient increase in fluorescence (DHE ex/em: 520/602 nm) was measured using a TECAN fluorescence multi-well plate reader. In another experiment, cells were seeded as described above. After removal of the cell culture medium, cells were washed with PBS, and 190 μl of 25 μM DHE dissolved in PBS was added to each well. Immediately before measurement, increasing concentrations of DATTS, DMSO (0.05% final concentration) as solvent control or 25 μM amobarbital as a positive control were added. The dosage-dependent increase in fluorescence (DHE ex/em: 530/620 nm) was measured as described above.

In a third experiment, cells were seeded as above. Medium was changed and cells were pretreated with 5 mM NAC or 100 μM ASC for 0.5 h prior to exposure to 50 μM DATTS, DMSO or 25 μM amobarbital. After incubation for 30 min, cell culture medium was removed, cells were washed with PBS, and 190 μl of 25 μM DHE dissolved in PBS was added to each well immediately before the measurement as in the previous steps.

2.6. Determination of H_2O_2 in cell culture

The level of H_2O_2 produced in ARPE-19 cells or in HCT116 cells following treatment with DATTS was determined using an Amplex® Red hydrogen peroxide/peroxidase assay kit (Invitrogen, Darmstadt, Germany), according to the manufacturer's instructions. In brief, to detect hydrogen peroxide or peroxidase activity, Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and horseradish peroxidase (HRP) were added to the cells treated with varying concentrations of DATTS, DMSO as solvent control or 25 μM epigallocatechin-3-gallate (EGCG) (an inducer of H_2O_2) as positive control for 30 min.

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