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Glutathione: from cell death to detoxification

Ph.D. theses

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Background

Glutathione

Glutathione (GSH) is a nucleophilic tripeptide, composed of glutamate, cysteine and glycine, and it is found in most of the aerobic cells. This molecule plays an essential role in biochemistry: it is involved in the elimination of reactive oxygen species (ROS) formed during various physiological processes, in the defence against endogenous or xenobiotic electrophiles and redox signalling. Due to its diverse functions, glutathione is considered to be the most important non-protein thiol, and it is found in millimolar concentrations in both animal and plant cells[1].

A characteristic feature of glutathione is the linkage of the γ-carboxyl group of glutamate to the amino group of cysteine, forming an unusual peptide bond. This bond is thought to confer stability to the molecule since intracellular aminopeptidases cannot degrade it, only the γ-glutamyl transpeptidase[2].

The most important moiety of glutathione is the thiol group of cysteine. Deprotonation of this group results in a nucleophilic thiolate (GS^-) which can form a covalent bond with an electrophile or serve as an electron donor. The reactive glutathione intermediate generated after electron donation forms a disulphide bond with another intermediate of the same kind, resulting in a dimer called oxidised glutathione (GSSG) or glutathione-disulphide[3].

Oxidative stress may alter the level of glutathione, its redox state, and its distribution among cell organelles, making them good biomarkers of various biochemical processes. Thus, the precise measurement of glutathione may help us to understand the different stress responses better.

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[1] Noctor, Graham et al. 2012. "Glutathione in Plants: An Integrated Overview." *Plant, Cell and Environment* 35(2): 454–84.

[2] Lu, Shelly C. 2013. "Glutathione Synthesis." *Biochimica et Biophysica Acta (BBA) - General Subjects* 1830(5): 3143–53.

[3] Deponte, Marcel. 2013. "Glutathione Catalysis and the Reaction Mechanisms of Glutathione-Dependent Enzymes." *Biochimica et Biophysica Acta (BBA) - General Subjects* 1830(5): 3217–66.

Ferroptosis-like cell death

Ferroptosis is an iron-dependent, caspase-independent form of programmed cell death, which was identified in mammalian cells in 2012. It has unique morphological and molecular characteristics and has specific inducers (e.g. RSL3) and inhibitors (e.g. ferrostatin-1). Ferroptosis may be induced by the depletion of cellular glutathione by erastin or by the inhibition of glutathione peroxidase 4 (GPx4), that plays a crucial role in the elimination of lipid peroxides. Irrelevant of the mode of induction, the cell death process is characterised by increased ROS production, lipid peroxidation and the elevation of the labile iron pool. Due to these properties, ferroptosis can be suspended by lipophilic antioxidants (e.g. ferrostatin-1, liproxstatin-1, α-tocopherol) and by iron chelators (e.g. deferoxamine)[4].

Although the exact molecular mechanism of ferroptosis is still unknown, the damage of biological membranes due to lipid peroxidation may be responsible for cell death. Furthermore, the fragmentation of lipid alkoxyl radicals yields the production of reactive aldehydes such as 4-hydroxynonenal and acrolein. These molecules may also be involved in inducing cell death since they can carbonylate proteins and thereby alter their function[5].

In 2017, a ferroptosis-like cell death was described in plants[6]. Distéfano *et al.* subjected *Arabidopsis thaliana* root hairs to 55°C heat stress for 10 min. They reported, that pre-treating the root hairs with the ferroptosis inhibitor ferrostatin-1 or with ciclopirox olamine (iron chelator) resulted in a significant decrease in the rate of cell death caused by heat stress. In contrast, the same inhibitors did not have any effect against more severe (77°C) heat stress, hydrogen-peroxide treatment or salt stress. The inhibitors did not affect programmed cell death during vascular differentiation and development, from which the authors conclude that cell death induced by moderate heat stress is a unique process in plants.

Several biochemical similarities have also been observed between the cell death induced by heat stress and the ferroptosis of mammalian cells. Cell death was preceded by GSH depletion and ROS accumulation. Pre-treating the root hairs with cell death inhibitors could prevent ROS formation but not GSH depletion. Cell death could also be rescued by supplementation with deuterated polyunsaturated fatty acids which are more difficult to oxidise than normal

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[4] Galluzzi, Lorenzo et al. 2018. "Molecular Mechanisms of Cell Death: Recommendations of the Nomenclature Committee on Cell Death 2018." *Cell Death and Differentiation* 25(3): 486–541.

[5] Hirschhorn, Tal, and Brent R. Stockwell. 2019. "The Development of the Concept of Ferroptosis." *Free Radical Biology and Medicine* 133: 130–43.

[6] Distéfano, Ayelén Mariana et al. 2017. "Heat Stress Induces Ferroptosis-like Cell Death in Plants." *Journal of Cell Biology* 216(2): 463–76.

polyunsaturated fatty acids. These results suggest that lipid peroxidation plays a critical role in this form of cell death. Shrunken mitochondria have also been observed upon heat treatment, which is a unique morphological feature of ferroptotic cell death in mammalian cells.
Based on these results, heat treatment at 55°C induces programmed cell death in plants that is highly similar to ferroptosis, so the authors refer to it as ferroptosis-like cell death. However, it is important to note that on the contrary of the caspase-independent ferroptosis of mammalian cells, caspase-like activity seems to be involved in plant ferroptosis-like cell death.

Cyclophosphamide

In addition to cell death processes, glutathione plays an important role in the biotransformation of xenobiotics, including drugs. A good example of this is cyclophosphamide (CYC), which has long been widely used as an immunosuppressive agent and anti-cancer drug.
Cyclophosphamide is a prodrug, which is activated by cytochrome P450 (CYP) enzymes. 70-80% of the administered cyclophosphamide dose is hydroxylated by hepatic CYP enzymes, to form 4-hydroxycyclophophamide. Under physiological conditions, 4-hydroxycyclophosphamide exists in equilibrium with its aldehyde tautomer, aldophosphamide. This equilibrium mixture diffuses from the hepatocyte into the blood and is distributed throughout the body.
Aldophosphamide is a very unstable molecule and spontaneously breaks up into phosphoramide mustard and acrolein. Phosphoramide mustard, which is a bi-functional DNA alkylating agent, is the therapeutically active metabolite. It forms both intra- and interstrand DNA cross-links and DNA-protein cross-links. These cross-links result in the inhibition of DNA replication and cell death by apoptosis.
There are several possibilities to detoxify cyclophosphamide and its metabolites. However, glutathione-S-transferases (GST), which conjugate their substrates to glutathione, play a prominent role.
The level of the bioactive phosphoramide mustard in the body is determined by the rate of enzymatic activation and detoxification of CYC and its metabolites. The enzymes involved in CYC metabolism are known to be highly polymorphic and have alleles with increased, decreased or missing activity. For example, if a patient has a genotype in which the activity of CYPs is increased and/or the activity of GSTs is decreased, a larger amount of phosphoramide mustard is produced, and the cells are exposed to it for a longer time. The prolonged exposure to phosphoramide mustard may lead to an increased response to the therapy, but an increased

risk of adverse drug reactions must also be taken into account. The reverse of the same scenario is also conceivable, in which case the CYC therapy may be ineffective. Thus, the genetic differences of the patients may be responsible for the earlier observed large interindividual variations in both efficacy and toxicity of CYC treatment[7].

Objectives

In a part of our research work, we have dealt with a better understanding of ferroptosis-like cell death. Ferroptosis-like cell death is characterised by lipid peroxidation, and it is well known, that fragmentation of lipid peroxides yields the production of reactive carbonyl species, including acrolein. According to earlier observations[8], acrolein induced cell death is substantially similar to ferroptosis-like cell death. Thus, we have investigated the possible mediator role of acrolein in ferroptosis-like cell death.

In our research, we have also investigated the cause of significant interindividual variations in the response rate to cyclophosphamide therapy of various autoimmune diseases[9]. The enzymes involved in drug metabolism are known to be highly polymorphic and have alleles with decreased or missing activity. Thus, we hypothesised that genetic differences might be responsible for the earlier observed interindividual variations in the efficacy of cyclophosphamide treatment.

Glutathione has a prominent role in both topics, since ferroptosis-like cell death is characterised by glutathione depletion, and the metabolites of cyclophosphamide are detoxified partly by conjugation to glutathione. Studying these processes required the measurement of a large number of glutathione samples. However, glutathione is prone to autooxidation and decomposition during sample preparation[10]. Thus, we have investigated how these can be prevented.

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[7] de Jonge, Milly E, Alwin D R Huitema, Sjoerd Rodenhuis, and Jos H Beijnen. 2005. "Clinical Pharmacokinetics of Cyclophosphamide." *Clinical Pharmacokinetics* 44(11): 1135–64.

[8] Biswas, Md. Sanaullah, and Jun'ichi Mano. 2016. "Reactive Carbonyl Species Activate Caspase-3-like Protease to Initiate Programmed Cell Death in Plants." *Plant and Cell Physiology* 57(7): 1432–42.

[9] Pinto, Navin, Susan M Ludeman, and M Eileen Dolan. 2009. "Drug Focus: Pharmacogenetic Studies Related to Cyclophosphamide-Based Therapy." *Pharmacogenomics* 10(12): 1897–1903.

[10] Giustarini, Daniela et al. 2016. "Pitfalls in the Analysis of the Physiological Antioxidant Glutathione (GSH) and Its Disulfide (GSSG) in Biological Samples: An Elephant in the Room." *Journal of Chromatography B* 1019: 21–28.

Based on the above, our objectives were:

1. The comparison of two widely used bioanalytical methods for the determination of glutathione, and the investigation and optimisation of their applicability in plant suspension cell cultures. We also aimed to develop a method that can prevent the autooxidation and decomposition of glutathione during time-consuming organelle isolations, in order to acquire more accurate results.

2. The investigation of the possible relationship between the cell death induced by the lipid peroxide-derived reactive carbonyl species, acrolein and ferroptosis-like cell death in *Arabidopsis thaliana* suspension cell cultures.

3. To investigate the effect of polymorphisms of certain enzymes involved in the bioactivation and detoxification of cyclophosphamide and its metabolites on the cyclophosphamide therapy of autoimmune diseases. The activated metabolites of cyclophosphamide are distributed throughout the body by the blood and are detoxified partly by conjugation with glutathione. Thus, we have investigated if cyclophosphamide therapy affects blood glutathione levels.

Methods

Plant experiments

- *Arabidopsis thaliana* suspension cell cultures
- Organelle isolation from *Arabidopsis thaliana* suspension cell cultures
- Glutathione measurement by monochlorobimane (mBCl) derivatization and HPLC-fluorescent detection
- Glutathione measurement by 5,5'-dithio-bis(2-nitrobenzoic acid)-GSH recycling assay (DTNB-GSH method)
- Treatment of *Arabidopsis thaliana* cells with acrolein or RSL3, in the presence or in the absence of ferroptosis inhibitors
- Determination of the viability of *Arabidopsis thaliana* cells by triphenyl-tetrazolium chloride reduction assay
- Determination of ROS by 2',7'-dichlorodihydrofluorescein diacetate
- Determination of lipid peroxidation by TBARS assay

- Determination of caspase-3-like protease activity

Clinical survey

- DNA isolation from buccal cells of patients treated with cyclophosphamide for autoimmune diseases
- Genotyping with allele-specific PCR discrimination method
- Determination of blood glutathione content by mBCl-HPLC method
- Search for correlations between positive response to cyclophosphamide therapy and genotype and other biological characteristics

Results and discussion

Comparison and optimisation of glutathione determination methods

During our work, we have compared two bioanalytical methods to determine glutathione: the DTNB-GSH recycling assay and the mBCl-HPLC method.

The DTNB-GSH recycling assay is a high-throughput and relatively inexpensive method to determine glutathione. Based on our results, if immediate measurement is possible, it is well suited for the rapid determination of the glutathione content of whole cell homogenates. If the glutathione content of the samples is stabilised by acidification, then they must be adequately diluted with assay buffer before analysis to neutralise their pH. In the case of samples with low glutathione concentrations, this dilution may render their glutathione content unmeasurable.

The mBCl-HPLC method is a slower and more expensive technique to determine glutathione. On the other hand, mBCl-conjugated glutathione is stable in the dark for several days, so the samples can be stored if immediate measurement is not possible, which is a great advantage of this method. Based on our results, the addition of GST to the samples dramatically improves the sensitivity of the method and ensures the conjugation of the whole glutathione content of the samples. Our results also show, that the addition of mBCl to the organelle isolation buffer right after the homogenisation of the cells greatly protects the glutathione content of the samples from autooxidation and decomposition during time-consuming organelle isolations.

On the base of our experiments, the following method can be advised for the determination of glutathione for eco-toxicological and plant stress investigations: the immediate addition of 1 mM mBCl to the homogenisation buffer stabilises the glutathione for the whole time of cell organelle isolation. The following HPLC-fluorescence detection of the forming mBCl-GSH fluorescent adduct ensures the sensitive and automated determination of GSH.

Investigation of the possible mediator role of acrolein in ferroptosis-like cell death

Acrolein induced ROS generation, lipid peroxidation and cell death could be significantly mitigated by pre-treating the *Arabidopsis thaliana* cells with ferroptosis inhibitors, the acrolein scavenger carnosine and the cell-permeable caspase inhibitor Z-VAD-FMK. These observations strengthen our hypothesis that ferroptosis-like cell death is involved in acrolein induced cytotoxicity. The similar inhibitory profile of the known ferroptosis inducer RSL3 further confirmed that ferroptosis is, at least partly, responsible for the acrolein induced cell death in plant cells. Furthermore, the protective effect of carnosine in RSL3 treated cells also raised the possible involvement of reactive carbonyl species (acrolein) in the RSL3 induced (ferroptosis-like) cell death in plant cells. However, based on our and earlier observations, in contrast to the caspase-independent ferroptosis of mammalian cells, plant caspase-like proteases can be involved in ferroptosis-like cell death.

Investigation of the effect of genetic polymorphisms on the cyclophosphamide therapy of various autoimmune diseases

Patients diagnosed with various autoimmune diseases and treated with cyclophosphamide (CYC) were involved in our study. Several CYP isoforms are involved in the bioactivation of CYC and GSTs play a prominent role in the detoxification of its metabolites. During our work, we were searching for associations between the response to CYC therapy and certain polymorphisms of CYP and GST enzymes. Based on our results, a significantly higher response rate to CYC treatment could be observed in individuals carrying one or two copies of the gene of the less active variant GSTP1 I105V isoenzyme. Since GSTP1 is the main GST isotype in the erythrocytes, it is not surprising that the weakened first detoxification reaction may result in a higher amount of 4-hydroxycyclophospahmide transported into the proliferating cells, that can enhance its cytostatic effect. Our hypothesis is supported by the fact that no significant blood GSH depletion could be observed upon CYC treatment. This suggests that glutathione is not a limiting substrate for the biotransformation.

Theses

1. The addition of monochlorobimane at a final concentration of 1 mM to the cell (tissue) lysates right after the homogenisation of the cells during organelle isolation significantly inhibits the autooxidation and decomposition of glutathione. Thus, the level and redox state of glutathione in the subcellular organelles can be determined more precisely. Based on our results, the tendencies and proportions of changes in glutathione content can be measured well by both 5,5'-dithio-bis(2-nitrobenzoic acid)-glutathione recycling (DTNB-GSH) assay and monochlorobimane derivatization-HPLC method. However, the measurable glutathione contents of the samples are always lower in the case of DTNB-GSH recycling assay than in the case of the monochlorobimane derivatization-HPLC method [Publication III]. It was shown, that the level, redox state and distribution of glutathione among subcellular organelles change during biotic and abiotic stresses [Publication III and IV]. Thus, these parameters are good biomarkers of these stresses.

2. The cytotoxic effect of acrolein could be significantly reduced by the pre-treatment of *Arabidopsis thaliana* cells with ferroptosis inhibitors (ferrostatin-1, α-tocopherol, glutathione and deferoxamine). Our results suggest that ferroptosis-like cell death is, at least partly, responsible for acrolein induced cell death [Publication II].

3. It was shown that the ferroptosis inducer RSL3 also induced cell death in plant cells. The cell death process was characterised by lipid peroxidation and activation of caspase-3-like proteases, and it could be inhibited by ferrostatin-1, liproxstatin-1, α-tocopherol and glutathione. Cell viability was also significantly enhanced by the reactive carbonyl species scavenging dipeptide, carnosine, suggesting that these compounds, including acrolein, play a role in RSL3-induced cell death [Publication II].

4. It was found that individuals carrying the variant GSTP1 I105V allele in at least one copy have a significantly higher response rate to the cyclophosphamide therapy of various autoimmune diseases than their wild-type homozygous counterparts. Based on our results, the glutathione content of the blood is not a limiting substrate for the conjugation of 4-hydroxycyclophosphamide, so the lower activity of the variant enzyme may be responsible for the observed higher response rate to cyclophosphamide treatment [Publication I.].

Possible application of the results

The level and redox state of glutathione is a good biomarker of several stresses in plants, including heavy metal pollution. Our results may contribute to the more precise measurement of glutathione, which may help us to understand the biochemical processes during stress responses better.

As the average temperature of Earth rises, the heat stress induced ferroptosis-like cell death of plants is expected to play an increasing role. We have shown that acrolein presumably plays a mediator role in this cell death. Our finding may provide a good defence opportunity against ferroptosis-like cell death, which may be important for agriculture.

Large interindividual variations have been observed in both efficacy and toxicity of cyclophosphamide therapy. Our results may contribute to the development of tailor-made therapies to increase the efficacy and reduce the toxicity of the therapies.

Publications

Publications in relation with dissertation:

I. Péter Hajdinák; Melinda Szabó; Emese Kiss; Lili Veress; Lívius Wunderlich; András Szarka
Genetic Polymorphism of GSTP-1 Affects Cyclophosphamide Treatment of Autoimmune Diseases.
Molecules 2020, 25, 1542;
DOI:10.3390/molecules25071542.
IF (2019): 3.267; IC:0; author contribution: 55%

II. Péter Hajdinák; Ádám Czobor; András Szarka
The potential role of acrolein in plant ferroptosis-like cell death.
PLoS One 2019, 14, e0227278;
DOI:10.1371/journal.pone.0227278.
IF (2019): 2.740; IC:0; author contribution: 60%

III. Péter Hajdinák; Ádám Czobor; Tamás Lőrincz; András Szarka
The Problem of Glutathione Determination: a Comparative Study on the Measurement of Glutathione from Plant Cells.
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DOI:10.3311/PPch.11785.
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IV. Ádám Czobor; Péter Hajdinák; András Szarka
Rapid ascorbate response to bacterial elicitor treatment in Arabidopsis thaliana cells.
Acta Physiologiae Plantarum 2017, 39, 62;
DOI:10.1007/s11738-017-2365-1.
IF (2017): 1.708; IC: 0; author contribution: 40%

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Glutation meghatározási módszerek összehasonlítása növényi szuszpenziós sejtkultúrákon
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Presentations in relation with dissertation:

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Other publications:

Ádám Czobor; Péter Hajdinák; Bence Németh; Borbála Piros; Áron Németh; András Szarka
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Other posters:

Ádám Czobor, Péter Hajdinák, András Szarka

Rapid ascorbate response to bacterial elicitor treatment in Arabidopsis thaliana cells

- OCC World Congress and Annual SFRR-E Conference – Metabolic Stress and Redox Regulation 2017, Berlin
- 47. Membrán-transzport Konferencia 2017, Sümeg

Péter Hajdinák, Ádám Czobor, Veronika Deák, Tibor Balogh, András Szarka

The effect of HrpWpto and HrpZpto treatment on ascorbate metabolism

- XIII. Oláh György Doktori Iskola PhD Konferencia 2016, Budapest
- International Conference for Plant Mitochondrial Biology 2015, Wroclaw
- 12th International Conference on Reactive Oxygen and Nitrogen Species in Plants: from model systems to field, 2015, Verona