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Research Paper

Hormesis enables cells to handle accumulating toxic metabolites during increased energy flux

Johanna Zemva[a,b,*,1], Christoph Andreas Fink[a,b,1], Thomas Henry Fleming[a], Leonard Schmidt[a], Anne Loft[c,d], Stephan Herzig[c,d], Robert André Knieß[b], Matthias Mayer[b], Bernd Bukau[b], Peter Paul Nawroth[a,c,d], Jens Tyedmers[a,b,*]

[a] Department for Internal Medicine I and Clinical Chemistry, Heidelberg University Hospital, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany
[b] Center for Molecular Biology of Heidelberg University (ZMBH) and German Cancer Research Center (DKFZ), DKFZ-ZMBH Alliance, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany
[c] Institute for Diabetes and Cancer (IDC), Helmholtz Center Munich, Neuherberg and Joint Heidelberg-IDC Translational Diabetes Program, Department for Internal Medicine I and Clinical Chemistry, Heidelberg University Hospital, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany
[d] German Center for Diabetes Research, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

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ABSTRACT

Energy production is inevitably linked to the generation of toxic metabolites, such as reactive oxygen and carbonyl species, known as major contributors to ageing and degenerative diseases. It remains unclear how cells can adapt to elevated energy flux accompanied by accumulating harmful by-products without taking any damage. Therefore, effects of a sudden rise in glucose concentrations were studied in yeast cells. This revealed a feedback mechanism initiated by the reactive dicarbonyl methylglyoxal, which is formed non-enzymatically during glycolysis. Low levels of methylglyoxal activate a multi-layered defence response against toxic metabolites composed of prevention, detoxification and damage remission. The latter is mediated by the protein quality control system and requires inducible Hsp70 and Btn2, the aggregase that sequesters misfolded proteins. This glycohormetic mechanism enables cells to pre-adapt to rising energy flux and directly links metabolic to proteotoxic stress. Further data suggest the existence of a similar response in endothelial cells.

1. Introduction

Energy flux is essential for life, but at the same time, it leads unavoidably to the generation of highly reactive metabolites, such as reactive carbonyl (RCS) and reactive oxygen (ROS) species. RCS and ROS cause cellular damage through the production of advanced glycation endproducts (AGEs) and oxidative stress [1,2] and are thought to contribute to ageing. This view is supported by the observation that long-lived mutants from different species produce decreased levels of ROS [3,4], and lowering the burden of oxidative stress leads to lifespan extension [5–12]. However, an increasing body of evidence indicates that ROS can also act as a secondary messenger to induce pathways that prolong lifespan in yeast and *C. elegans* [13–17] and may also be beneficial in mice [18,19]. This effect has been described as mitohormesis and has been defined as a non-linear response to increased levels of ROS [20].

ROS are only one type of reactive metabolites that have been reported to result from increased energy flux, and they are formed at a rather late stage of energy production, namely during the concerted four-electron reduction of molecular oxygen catalysed by cytochrome C oxidase of complex IV of the respiratory chain. Therefore, it is unclear whether mitohormesis alone would be sufficient to protect cells from metabolic stress. In comparison to ROS, the role and effects of RCS have received less attention. RCS are formed endogenously mainly by carbohydrate metabolism but also during lipid peroxidation and autoxidation of reducing substrates [21]. Several of the most reactive RCS including methylglyoxal (MG) are produced non-enzymatically during glycolysis, mainly from intermediates such as glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (triosephosphates) [22,23]. The production of RCS, such as MG poses more of an immediate threat to the integrity of the cell as they are produced at three metabolic steps upstream of the step producing ROS. Given its early production in energy metabolism upon increasing glucose levels, RCS would be appropriately positioned to act as a potential gatekeeper in an immediate

* Corresponding authors at: Department for Internal Medicine I and Clinical Chemistry, Heidelberg University Hospital, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany.
E-mail addresses: johanna.zemva@med.uni-heidelberg.de (J. Zemva), j.tyedmers@zmbh.uni-heidelberg.de (J. Tyedmers).
[1] These authors contributed equally to this work.

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feedback loop, signalling that an increase in metabolic stress is to be expected even before oxidative stress levels have augmented. It is currently unknown whether RCS, such as MG, can exert a hormetic effect. However, hormesis dose-response relationships have been reported for various preconditioning agents and can be regarded as a measure of biological plasticity [24,25]. Therefore it is probable, that cells can also respond to RCS or MG in a hormetic manner.

The major cellular detoxification system for MG is the glyoxalase system, which requires catalytic amounts of reduced glutathione (GSH) to convert MG in a two-step reaction to D-lactate [23,26]. In the absence of glyoxalase 1 (Glo1), however, MG can also be degraded via aldoketo-reductases to acetol [27,28]. As MG is very toxic, the capacity of corresponding detoxifying enzymes has to be tightly adapted to the energy flux. Indeed, Glo1 is highly efficient, with reaction kinetics close to the rate of diffusion [29]. However, when MG levels reach a saturating amount which overwhelms the intracellular GSH pool, Glo1 will become inactive [30]. Under such circumstances, intracellular MG would accumulate and could potentially cause downstream damage. To handle such a dynamic situation, it seems plausible that cells have evolved additional defence mechanisms.

Yeast is a model organism well suited for the study of MG metabolism, as it possesses all enzymes needed to deal with glucose metabolites similar to mammalian cells. It has been reported that during a shift from low to high glucose concentrations, MG levels increase and cause protein damage, visible through increased amounts of AGE modified proteins, such as argpyrimidine [31,32]. When MG is added to the medium or during high osmotic stress, Glo1 is induced via the High Osmolarity Glycerol 1/Mitogen-activated protein kinase (Hog1) pathway [33–35]. Additionally, increased levels of MG can activate the yeast oxidative stress transcription factor Yap1 [36]. Furthermore, a genome wide unbiased screen for gene deletions that render yeast cells more sensitive towards MG identified gene deletions conferring increased sensitivity to MG [37]. These were enriched for protein, mRNA and DNA metabolic processes, but most hits were never followed up upon in a systematic way. An important question that remained open is whether any of these processes could be modulated in response to MG stress.

In this study, the hypothesis was investigated that cells are equipped with an elementary, evolutionary conserved pathway that is triggered by RCS and enables cells to handle increasing accumulation of toxic metabolites on different levels, to prevent further formation of toxic metabolites, to detoxify metabolites, and to repair metabolite-induced damage. If existing, this pathway should render cells more resistant to the physiological relevant reactive dicarbonyl MG as well as to ROS upon increasing glucose levels. Our results suggest that, when reaching a threshold level, MG can initiate a positive feedback mechanism in a hormetic manner, leading to activation of a multi-layered defence response.

2. Materials and methods

2.1. Chemicals and antibodies

Methylglyoxal solution (40% w/v; M0252), 2-Deoxy-D-glucose (D8375), Aminoguanidine hydrochloride (396494), Hydrogen peroxide solution ≥ 30% (95302), Doxycycline hyclate (D9891) as well as Paraformaldehyde (16005) were purchased from Sigma-Aldrich. D(+)-Glucose was from Merck. Aprotinin was from Applichem, Pepstatin was from Pepta Nova GmbH, Leupetin was from Peptide Institute, Inc., HygromycinB was from InvivoGen, Nourseothricin (cloNAT) was from Werner BioAgents. Zymolyase 20T was purchased from Amsbio. DAPI solution was from Thermo Fisher scientific. The anti-MG-H1 antibody was generated as described previously [28]. The anti-GFP antibody was purchased from Roche (Cat. No. 11 814 460 001). The anti-actin antibody was from Millipore (clone C4, Cat. No. MAB1501). As secondary antibodies horseradish-linked goat anti-rat (Cell Signalling Technology, # 7077S) goat anti-mouse (Cell Signalling Technology, # 7076S) were used.

2.2. Yeast strains, plasmids, growth conditions and standard methods

Yeast strains used in this study are derivatives of the BY4741 strain (Euroscarf). Single Knock-outs of Glo1, Btn2, Msn2, Msn4, Yap1, Cad1, Rim101 and all double and triple Knock-outs were created freshly using a gene replacement strategy [38] and confirmed by PCR. All additional knock-outs mentioned derived from the yeast deletion library (Invitrogen). The tetracycline regulatable Sln1 construct was from the "Yeast Tet promoters Hughes Collection (yTHC), Dharmacon". The sln1-gene was repressed in the presence of doxycycline with a final concentration of 50 µg/ml in the growth medium for 12 h [39]. The plasmid for galactose-based overexpression of Ssa4 derived from a yeast ORF overexpression library [40]. The Heat shock reporter construct consists of a heat shock element (HSE) cloned in front of the Cyc1 minimal promoter sequence followed by 4 tandem repeats of sfGFP. To gain a stronger fluorescent signal, the reporter plasmid contains 2 copies of these expression cassettes in inverted repeats. For a stable expression pattern, the construct was integrated into the genome. All yeast cultures were grown in standard rich media (YPD; BD Difco 1% yeast extract, 2% peptone and 2% glucose) or plated on standard YPD-plates.

2.3. MG tolerance test in yeast cells

For preconditioning with low concentrations of MG or H_2O_2, cells growing in logarithmic growth phase were divided into two samples. One sample was left untreated (control), while MG or H_2O_2 was added to the second aliquot and the cells were further incubated at 30 °C for 45 min. After that, the two samples were adjusted to the same OD_{600} of 0,4, divided into aliquots and treated with increasing concentrations of MG for 60 min at 30 °C prior to harvesting the cells and resuspending them in fresh YPD such that each aliquot contained the same OD_{600}. Subsequently, cells were serially diluted in 1:5 steps, spotted onto YPD plates and incubated for 48 h at 30 °C to determine cell survival.

2.4. Preparation of total/cytosolic yeast protein extracts

50 ml of a yeast culture in logarithmic growth phase at an OD_{600} ~ 0.4–0.6 were harvested and the pellet was transferred to 1.5 ml eppendorf tubes, resuspended in 100 µl of lysis buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1% NP40, 1 mM PMSF, protease inhibitors) and dripped in liquid nitrogen present in an 2 ml round bottom eppendorf tube that contained a 7 mm stainless steel ball. After boiling out of the liquid nitrogen, the tubes were closed and placed in an adaptor for 2 ml tubes into a Retsch mixer Mill MM 400 and agitated for 2 × 2 min at 30 Hz. The sample was cooled in liquid nitrogen in between the two rounds of agitation. The resulting powder of lysed cells was transferred into a 1.5 ml tube and resuspended into 500 µl of lysis buffer. Samples were centrifuged (2 × 5.000 rpm for 3 min at 4 °C) and supernatant was used for protein determination and further analysis. All protein concentrations were determined using the Bradford technique and BSA as calibration standard as described previously [41].

2.5. Western blotting

15 µg protein was incubated in 2x Laemmli buffer (Sigma) at 95 °C for 10 min and separated by a Mini-PROTEAN® TGX (Bio-Rad) precasted gel (4–20% acrylamide). Proteins were then transferred to a PVDF membrane and blocked with 2% dry milk (in PBS) or in the case of MG-modifications with a Pierce® protein-free blocking buffer (Thermo) at room temperature for 1 h. Membranes were then incubated overnight at 4 °C with antibodies against MG-H1 in protein-free blocking buffer (1:500 dilution), Actin in 2% dry milk containing PBS