Methionine Metabolism Alters Oxidative Stress Resistance via the Pentose Phosphate Pathway

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Abstract

Nutrient uptake and metabolism have a significant impact on the way cells respond to stress. The amino acid methionine is, in particular, a key player in the oxidative stress response, and acting as a reactive oxygen species scavenger, methionine is implicated in caloric restriction phenotypes and aging. We here provide evidence that some effects of methionine in stress situations are indirect and caused by altered activity of the nicotinamide adenine dinucleotide phosphate (NADPH) producing oxidative part of the pentose phosphate pathway (PPP). In Saccharomyces cerevisiae, both methionine prototrophic (MET15) and auxotrophic (met15Δ) cells supplemented with methionine showed an increase in PPP metabolite concentrations downstream of the NADPH producing enzyme, 6-phosphogluconate dehydrogenase. Proteomics revealed this enzyme to also increase in expression compared to methionine self-synthesizing cells. Oxidant tolerance was increased in cells preincubated with methionine; however, this effect was abolished when flux through the oxidative PPP was prevented by deletion of its rate limiting enzyme, ZWF1. Stress resistance phenotypes that follow methionine supplementation hence involve the oxidative PPP. Effects of methionine on oxidative metabolism, stress signaling, and aging have thus to be seen in the context of an altered activity of this NADP reducing pathway.

Introduction

The ability of cells to counteract oxidative stress is fundamental for survival in an ever changing environment and is implicated in growth and aging (3, 5). Metabolism is of importance for cellular tolerance to oxidants, providing the reducing power for the antioxidant machinery, including the glutathione and thioredoxin systems, and acting as a main source of intracellular oxidants itself (3, 4, 7). A central player in stress resistance is the amino acid methionine. Methionine is a direct target of reactive oxygen species (ROS), as its sulfur can be oxidized to sulfoxide; furthermore, via repair of methionine sulfoxide through methionine sulfoxide reductases, methionine can act as scavenger and protect cells from oxidative stress (2). Recent studies analyzing proteomic methionine usage show this amino acid to be enriched in protein at sites of major free radical production, such as the mitochondrial respiratory chain (6). Methionine has therefore been exploited by cells, acting as an ROS scavenger to protect proteins from oxidation, and has been implicated in caloric restriction phenotypes and aging (2, 3).

Genetic experiments have connected methionine biosynthesis to the pentose phosphate pathway (PPP), a pathway with essential roles in the antioxidative metabolism (5, 7). This connection appears to be predominantly caused by the redox cofactor nicotinamide adenine dinucleotide phosphate (NADP). De novo methionine biosynthesis, via the assimilation of inorganic sulfate, requires three molecules of NADPH per molecule of methionine, and the PPP is key to regenerating NADPH and maintaining redox balance (7). Two PPP enzymes responsible for NADPH production are glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH). When budding yeast is deleted for G6PDH (ZWF1), the rate limiting enzyme of this NADPH producing oxidative PPP, cells are no longer able to synthesize methionine and become methionine auxotrophs (8). Growth of zwf1Δ
Innovation

Methionine plays an essential role in oxidant resistances as it can be oxidized and has been implicated in caloric restriction and aging. We observed altered activity in the oxidative branch of the pentose phosphate pathway (PPP) after increasing the supplementation of methionine, detecting elevated levels of PPP metabolites, and increased abundance of the nicotinamide adenine dinucleotide phosphate (NADPH) producing enzyme 6-phosphogluconate dehydrogenase (6PGDH). Methionine preincubation also increased cellular tolerance to the thiol oxidizing agent diamide in dependency of the oxidative pentose phosphate. At least some oxidative stress resistance phenotypes caused by methionine hence involve the PPP.

cells therefore depends on supplementation with organic sources such as cysteine and methionine (8).

Results and Discussions

We hence questioned whether the effect of methionine on the PPP was related to the stress response. Direct NADPH quantification in yeast provides limited information about the PPP as (i) a major fraction of NADPH is localized to the vacuole or protein bound, (ii) different NADPH sources exist, and (iii) NADPH is readily oxidized upon cell lysis (4).

We therefore addressed this problem by combining mass spectrometry-based metabolite quantification, protein quantification, and genetics. First, PPP metabolite abundance was determined using a targeted LC-MS/MS method, which gives absolute quantities for the majority of PPP metabolites (Table 1).

Wild-type cells supplemented with methionine showed a distinct increase in PPP metabolites downstream of the NADPH producing enzyme 6PGDH (Fig. 1A, left panel). Auxotrophic yeast, unable to synthesize methionine by themselves, possessed similar concentrations confirming that the effect is mediated by methionine supplementation (Fig. 1A, right panel). Next, protein expression was analyzed by SWATH-MS, a target proteomics method based on LC/MS-MS (9). We detected a clear upregulation of 6PGDH in methionine-supplemented cells (Fig. 1B). Methionine treatment thus increases both metabolite concentration and enzyme expression in the oxidative PPP.

Finally, we tested for a causal relationship between methionine and the PPP by testing oxidant resistance upon methionine supplementation. The thiol oxidizing agent diamide was considered ideal for testing PPP-dependent oxidant resistance, as cells have increased resistance to this oxidant when PPP activity is increased; however, in contrast to hydrogen peroxide or other peroxides, diamide does not block glycolysis or activate the PPP directly (5). When methionine supplementation was increased from 0 to 200 mg/L, resistance to diamide was increased (Fig. 1C). The upregulated 6PGDH cannot be fully deleted in yeast as it is an essential gene; therefore, to prevent flux in the oxidative PPP, ZWF1 was deleted instead. In zwf1Δ cells, methionine no longer increased diamide tolerance (Fig. 1C).

Stress resistance phenotypes that follow methionine supplementation hence involve the oxidative PPP. Effects of methionine on oxidative metabolism, stress signaling, and aging have thus to be seen in the context of an altered activity of this NADPH providing pathway.

Notes

Yeast strains, plasmids, and growth media

All yeast strains and plasmids used are listed in Table 2. For metabolomic and proteomic studies, YSBN5, a prototrophic haploid variant of Saccharomyces cerevisiae S288c, was used alongside its met15Δ derivative. Deletion of MET15 in this strain was performed by homologous recombination using a kanMX marker (Table 3). To determine the effect of methionine supplementation on oxidative tolerance, BY4741 complemented with the centromeric vector (minichromosome) pHUM (Addgene ID #40276) was used alongside its zwf1Δ derivative. Yeast was cultivated if not otherwise indicated at 30°C, in minimal supplemented synthetic media (SM; YNB yeast nitrogen base [6.8g/L; Sigma]), with 2% glucose (Sigma) as the carbon source.

| Compound name | Compound abbreviation | YSBN5 (μM) | YSBN5 + methionine (μM) | (met15Δ) + methionine (μM) |
|---------------|-----------------------|------------|-------------------------|---------------------------|
| Pyruvate      | Pyr                   | 190.13 ± 19.31 | 241.95 ± 17.87 | 214.23 ± 9.15 |
| Glucose 6-phosphate/fructose 6-phosphate | G6P/F6P | 160.50 ± 3.66 | 188.38 ± 11.54 | 194.43 ± 4.50 |
| Sedoheptulose 7-phosphate | S7P | 126.10 ± 15.24 | 138.53 ± 8.73 | 153.80 ± 21.04 |
| Ribose 5-phosphate | R5P | 2.73 ± 0.15 | 5.55 ± 1.03 | 5.50 ± 1.71 |
| Fructose 6-phosphate | F6P | 94.90 ± 6.10 | 123.90 ± 11.92 | 123.23 ± 24.97 |
| Glyceraldehyde 3-phosphate | G3P | 8.27 ± 0.23 | 10.95 ± 0.64 | 10.67 ± 0.60 |
| Xylulose 5-phosphate/ribulose 5-phosphate | X5P/R15P | 9.20 ± 1.04 | 21.65 ± 4.31 | 18.10 ± 2.76 |
| Dihydroxyacetone phosphate | DHAP | 4.97 ± 1.66 | 17.48 ± 5.72 | 16.33 ± 9.15 |
| 6-Phosphogluconate | 6PG | 43.03 ± 1.71 | 51.90 ± 2.97 | 48.93 ± 0.92 |
| 2-Phosphoglycerate/3-phosphoglycerate | 2-PG/3-PG | 69.30 ± 10.37 | 89.50 ± 8.46 | 81.23 ± 8.02 |
| Phosphoenolpyruvate | PEP | 1.37 ± 0.32 | 1.63 ± 0.15 | 2.03 ± 0.38 |
| Fructose 1,6-bisphosphate | F1,6BP | 274.80 ± 20.22 | 343.08 ± 13.15 | 294.27 ± 19.55 |

Absolute quantification of PPP metabolites was conducted on the prototrophic yeast strain YSBN5 (MET15) ± methionine supplementation and its MET15 knockout derivative (met15Δ) grown with methionine supplementation (n = 3). Error = ±SD.

PPG, pentose phosphate pathway.
FIG. 1. Methionine supplementation alters intracellular PPP activity. (A) Methionine exposed wild-type (MET15) and met15Δ cells have increased concentrations of PPP metabolites (n = 3). Absolute metabolite concentrations are shown in Table 1. Glycolysis: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F1,6BP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate. Pentose phosphate pathway: 6PG, 6-phosphogluconate; Rl5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; X5P, xylulose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate. (B) Top: Reaction scheme of 6-phosphogluconate (6PG) to D-ribulose 5-phosphate (Rl5P) catalyzed by 6-phosphogluconate dehydrogenase (6PGDH), yielding NADPH. 6PG and Rl5P are highlighted in green and 6PGDH in brown, here and in (A). Bottom: The expression level of 6PGDH in wild-type (MET15) as well as in methionine supplemented wild-type and met15Δ cells, as determined by SWATH-MS. Bottom left: Reconstruction of SWATH-MS chromatographic spectra in Skyline; shown are two transitions of the representative 6PGDH peptide DYFGAHTFR. Bottom center: Expression level (fold change to wild type) of 6PGDH. c.p.s., counts per second. n = 3, error bars, ± SD. (C) Increased resistance of wild-type cells (ZWf1Δ) to diamide upon methionine supplementation. This phenotype is lost in cells deleted for the rate-limiting oxidative PPP enzyme G6PDH (zwf1Δ). PPP, pentose phosphate pathway. G6PDH, glucose 6-phosphate dehydrogenase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
The software package (Agilent) was used for data analysis. Energies were determined separately. MassHunter Workstation software (Agilent) was used for calculating peptide fold changes. To correct for matrix effects, interfering fragment ions were removed using Skyline software, and the three most abundant fragment ions were used for calculating peptide fold changes. To correct for matrix effects, interfering fragment ions were removed using Skyline software, and the three most abundant fragment ions were used for calculating peptide fold changes. To correct for matrix effects, interfering fragment ions were removed using Skyline software, and the three most abundant fragment ions were used for calculating peptide fold changes. To correct for matrix effects, interfering fragment ions were removed using Skyline software, and the three most abundant fragment ions were used for calculating peptide fold changes. To correct for matrix effects, interfering fragment ions were removed using Skyline software, and the three most abundant fragment ions were used for calculating peptide fold changes.

### Table 2. Strains and Plasmids Used in This Study

| Name         | Description                                      |
|--------------|--------------------------------------------------|
| Strains      |                                                  |
| YSBN5        | MATa, FY3 ho::Ble                                |
| YSBN5 met15Δ | YSBN5 met15::kanMX4                              |
| BY4741       | MATa, his3Δ1 leu2Δ0 met15Δ ura3Δ0 (ATCC® 201388™) |
| BY4741 zwf1Δ | BY4741 zwf1::kanMX4                              |
| Plasmids     |                                                  |
| pHLUM        | Yeast centromeric vector with HIS3, URA3, LEU2, and MET15 markers (minichromosome). (Addgene number: 40276) |

Quantification of glycolytic and PPP metabolites by LC-MS/MS

Intracellular levels of sugar phosphates were quantified using a previously published LC-MS/MS method (1). 7.5 OD<sub>595</sub> units of cells (YSBN5 MET15 ± methionine [20 mg/L] or YSBN5 met15Δ + methionine [20 mg/L]) were harvested in the exponential growth phase (OD<sub>595</sub> = 1.5 ± 0.05) following cold methanol quenching. Cell pellets were resuspended in 200 μL of extraction buffer (75:25 [v/v] acetonitrile:methanol, 0.2% formic acid) and lysed during three FastPrep-24 (MP Biomedicals) cycles for 20 s at 6.5 m/s. After centrifugation for 5 min at 16,000 g, the pellet was extracted again with 200 μL ultra high pressure liquid chromatography-grade water. Combined supernatants from both extractions were evaporated in a Concentrator plus SpeedVac (Eppendorf), resuspended in 100 μL 7% acetonitrile, centrifuged, and submitted to LC-MS/MS analysis. One microliter of the metabolite extract was injected on a C8 column (ZORBAX SB-C8 Rapid Resolution HD, 2.1 × 100 mm, 1.8 μm (Agilent); column temperature: 20°C) for liquid chromatography separation (Agilent 1290). Water/acetonitrile mixtures (A: 10% [v/v] acetonitrile, B: 50% [v/v] acetonitrile) that were used as mobile phases contained 750 mg/L octylammoniumacetate as the ion pairing reagent. After 3.5 min of isocratic flow at 12% acetonitrile, a 2.5-min gradient to 38% acetonitrile was used to elute the sugar phosphate analytes, followed by a washing step to 42% acetonitrile for 0.5 min and re-equilibration at starting conditions. Quantification was achieved with an online coupled triple quadrupole mass spectrometer (Agilent 6460) operating in the selective reaction monitoring (SRM) mode. Identification of metabolites was ensured by comparison of retention times and selective reaction monitoring (SRM) mode. Identification of metabolites was ensured by comparison of retention times and selective reaction monitoring (SRM) mode. Identification of metabolites was ensured by comparison of retention times and selective reaction monitoring (SRM) mode. Identification of metabolites was ensured by comparison of retention times and selective reaction monitoring (SRM) mode. Identification of metabolites was ensured by comparison of retention times and selective reaction monitoring (SRM) mode. Identification of metabolites was ensured by comparison of retention times and selective reaction monitoring (SRM) mode. Identification of metabolites was ensured by comparison of retention times and selective reaction monitoring (SRM) mode. Identification of metabolites was ensured by comparison of retention times and selective reaction monitoring (SRM) mode. Identification of metabolites was ensured by comparison of retention times and selective reaction monitoring (SRM) mode.

### Table 3. Oligonucleotides Used to Generate YSBN5 Met15Δ by Homologous Recombination

| Name       | Sequence                                                                 |
|------------|--------------------------------------------------------------------------|
| Met15-fw   | GTCGATACATAGATACAAAATTCATTACCCCTCCATCCATACAAGCTTGCCCGCTCCCGCCGCTCA     |
| Met15-rv   | GAGAAGTAGGTTTATACATATAATTGTTTACACACTCATTACACACTGCAACTGGAATGGCAGGCTTTAGATC |
with 20 and 200 mg/L of L-methionine [Sigma], respectively, as the zwf1A strain is auxotrophic for organic sulfur (8). Cell cultures were then normalized to 3.6e+06 cells in 200 L SM and spotted in 1:5 serial dilutions on SM solid media with l-methionine (200 mg/L) – diamide (Sigma). Growth was documented after 3 days incubation at 30°C.

Acknowledgments

The authors thank the Wellcome Trust (RG 093735/Z/10/Z), the ERC (Starting grant 260809), the Isaac Newton Trust (RG 68998), and the Austrian Science Funds (FWF, for a postdoctoral fellowship [J3341] to M.A.K.). M.R. is a Wellcome Trust Research Career Development and Wellcome-Beit Prize fellow.

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Date of first submission to ARS Central, September 29, 2015; date of final revised submission, November 16, 2015; date of acceptance, November 20, 2015.

Abbreviations Used

6PGDH = 6-phosphogluconate dehydrogenase
G6PDH = glucose 6-phosphate dehydrogenase
NADPH = nicotinamide adenine dinucleotide phosphate
PPP = pentose phosphate pathway
ROS = reactive oxygen species
SM = synthetic media
SRM = selective reaction monitoring