Excess PPI arrests cell cycle and induces autophagy

Inorganic pyrophosphatase defects lead to cell cycle arrest and autophagic cell death through NAD⁺-depletion in fermenting yeast

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Background: The cellular consequences of inorganic pyrophosphate excess in a eukaryotic cell are unknown.

Results: Saccharomyces cerevisiae cells depleted of inorganic pyrophosphatase Ipp1p on respiratory carbon sources arrest cell cycle but fermenting cells undergo NAD⁺-depletion-induced autophagy and die.

Conclusion: Inorganic pyrophosphatase depletion can cause cell death through autophagy.

Significance: This is the first work detailing the cellular consequences of intracellular pyrophosphate accumulation in eukaryotes.

SUMMARY

Inorganic pyrophosphatases are required for the anabolism to take place in all living organisms. Defects in genes encoding these hydrolytic enzymes are considered inviable, although its exact nature has not been studied at cellular and molecular physiology levels. Using a conditional mutant in IPP1, the Saccharomyces cerevisiae gene encoding the cytosolic soluble pyrophosphatase, we show that respiring cells arrest in S-phase upon Ipp1p deficiency but they remain viable and resume growth if accumulated pyrophosphate is removed. However, fermenting cells arrest in G₁/G₀ phase and suffer massive vacuolisation and eventual cell death by autophagy. Impaired NAD⁺ metabolism is a major determinant of cell death in this scenario since demise can be avoided under conditions favouring accumulation of the oxidised pyridine coenzyme. These results posit that the mechanisms related to excess pyrophosphate toxicity in eukaryotes are dependent on the cell's energy metabolism.

Many biosynthetic reactions produce inorganic pyrophosphate (PPI)⁴ as a by-product. The advantage of this seems to lie on a greater ∆G⁰ stored in the α,β phosphoanhydride bond of nucleoside triphosphates, compared to that in the β,γ one, and the fact that PPI is rapidly hydrolysed to orthophosphate, driving both the
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kinetics and energetics of anabolic reactions towards biosynthesis (1). For this reason, PPi homeostasis is exceedingly important for the cell. Removal of PPi from the cytosol is carried out by two main non-homologous enzyme types: soluble inorganic pyrophosphatases (sPPases) and proton-translocating membrane-bound pyrophosphatases (H+-PPases) (2,3). The first of these is found as the sole enzyme that keeps cytosolic PPi at very low levels in animals and fungi. Soluble PPases may share this function with their membrane-bound counterparts in plants, protists and some bacteria and archaea, albeit until now only the involvement and proper location of the membrane-bound H+-PPases has been proved in these organisms (2,3). Not surprisingly then, a genetic defect leading to absence of sPPase activity has been found to cause Escherichia coli to stop cell proliferation (4) and to be inviable in the budding yeast Saccharomyces cerevisiae (5-8). In the case of E. coli, this growth defect was shown to be non-lethal (4). However, no study to date has dealt with the physiological basis accounting for the lethality of sPPase deficiency in eukaryotes or how it affects the cell cycle in either prokaryotes or eukaryotes.

Up to now, the cellular importance of PPi homeostasis has only been patchily and scantily studied. In bacteria, it has been observed that the level of intracellular PPi does not correlate directly with that of sPPase polypeptide; on the contrary, it remains constant until the amount of this enzyme drops below a minimum threshold (9), suggesting post-translational regulation of its catalytic activity or a constitutive excess of this enzyme under physiological conditions. In mammals, alteration of sPPase levels is associated to several illnesses, many related to calcium phosphate homeostasis (10,11). Interestingly, human sPPase PPA1 is now being found associated with tumour cells. Thus, cytosolic levels of this protein have been shown to be increased in several proteomic studies dealing with cancer tissues from different origins, like lung and colon (12,13). Besides, plants have a very intricate PPi metabolism: they possess a number of structurally and catalytically diverse PPi-utilising proteins, like the membrane-bound H+-PPases, several PPi-dependent glycolytic enzymes, and multiple sPPase isoforms (2,3,14). It has been observed that photosynthetic carbon assimilation and, in general, plant metabolism is greatly affected by changes in the levels of soluble PPases (15-17). However, we cannot start to understand plant PPi metabolism, and its influence on economically important issues such as seed-oil or carbohydrate yield, if we are unsure how simpler unicellular organisms behave in this respect.

In this report, we show for the first time that a conditional genetic defect in the yeast IPP1 gene induces massive cell death in fermenting cultures, but only induces cell cycle arrest in respiring cells. Furthermore, we describe the physiological mechanism for cell death and the metabolic step that causes it. In addition we show that, under respiratory conditions, cell cycle arrest is reversible. These results may open the way to new approaches in metabolism-related cell bioengineering, chemotherapy, and underline the importance of so-called house-keeping cell processes like PPi homeostasis.

**EXPERIMENTAL PROCEDURES**

Yeast strains, plasmids and growth conditions – All S. cerevisiae strains are derivatives of W303-1a (MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-200 trpl-901 lys2-801 suc2-901 GAL). Relevant genotypes are as follows: YPC3: W303-1a ipp1UAS::GAL1 UAS IPP1-HIS3 (18); SAH6: YPC3 vph1Δ::KanMX4; SAH16: SEY6210 ipp1UAS::GAL1 UAS IPP1-HIS3; SAH17: SAH16 atg81Δ::LEU2. A multicopy plasmid bearing NQR1 under the control of the ADH1 promoter and its empty parental were kind gifts of Dr Carlos Santos-Ocaña and are described in (19). Introduction of plasmids into yeast cells was done by the lithium-acetate method (20). Cells were routinely grown on standard YP medium supplemented with appropriate carbon sources. Unless otherwise stated, all determinations were done on exponentially-growing cells (OD600 of cultures not greater than 0.5). To maintain cultures for several hours below OD600 = 0.5, they were routinely diluted with fresh medium every 2-3 h until the end of the experiment (semi-continuous culture). In experiments where a single time point was assayed, all determinations were done on cells incubated for 18 h under SG (standard glucose, 2% glucose) or CR (caloric restriction, 0.5% glucose) conditions but for 36 h under MR (mandatory respiration, 3% glycerol) condition, unless indicated otherwise. Nitrogen starvation conditions were induced by incubating cells for 6 h in 0.17 % yeast nitrogen base w/o amino acids or ammonium sulphate...
and supplemented only with 2% galactose. No loss in viability due to these starvation conditions was observed, as assessed by cfu counting.

**Cell death and viability assays** - Cell viability was assessed by colony forming ability. Briefly, ca 500 cells, as estimated by OD<sub>600</sub>, were plated onto appropriate solid media and survival scored as the percentage of colonies formed from the total of inoculated cells. Since different conditions could lead to alterations in the number of yeast cells estimated by absorbance due to size differences, estimations were corrected by counting cells on a hemocytometer. Death was estimated on propidium iodide-stained cells by flow cytometry as cells bearing DNA contents less than 1C (subG<sub>1</sub>) (21).

**Flow cytometry and microscopy** - Cells were stained with propidium iodide essentially as described by Sazer and Sherwood (22), and analysed on a Coulter Epics XL apparatus as described (23).

For microscopical analysis, cells were visualized using a Leica DM 6000B type fluorescence microscope. Cell size was evaluated from differential interference contrast microphotographs as the apparent area covered by the cell evaluated using NIH ImageJ program (24). Percentages of budded cells were estimated from microphotographs; a minimum of 150 cells per treatment were counted. TUNEL assays were done essentially as described in (25) using a commercial kit (Roche Applied Science, #11684817910). Cells were dihydrorhodamine-123 stained by incubating with 5 μg ml<sup>-1</sup> DHR123 for 2 h under appropriate growth conditions and then viewed under a rhodamine filter. FM4-64 staining was done as in (26); estimation of the percentage of cells showing solid vacuoles on FM4-64 staining was done as for budded cells above.

**Cell extracts and enzymatic assays** - Cytosol enriched fractions were obtained from exponentially-growing cells by breaking them using glass beads (0.5 mm Ø) in a buffer containing 0.33 M sorbitol, 25 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 mM benzamidine, 1 mM ε-aminocaproic acid. Cells were subjected to five 1-min bursts on a vortex mixer at full speed with 1-min intervals on ice between bursts. Total homogenates were cleared of unbroken cells and cell debris by low speed centrifugation (5 min, 500 g) followed by high speed centrifugation for 15 min (20,000 g). Pyrophosphatase activity assay was done according to (8) and released phosphate was determined as in (27). Activity in the presence of 2 mM potassium fluoride was subtracted from the data as corresponding to unrelated diphosphatase activities. These activities represented less than 5% of total PPI hydrolytic activity measured in control cells.

**Protein determination and Western blotting** - Protein determination was done using a dye-binding based assay from Bio-Rad (#500-0006) according to manufacturer instructions and using ovalbumin as a standard. Proteins were separated in SDS-PAGE gels using standard procedures. Proteins were then transferred to nitrocellulose filters and probed with antibodies raised against the sPPIase PPA I from the microalga *Chlamydomonas reinhardtii* (14) or a commercial anti-GFP antibody (Clontech #632460). Proteins were visualised on X-ray films using horseradish peroxidase-coupled secondary antibodies and a chemiluminescence kit (Millipore, #WBKLS0100).

**Quantitation of metabolites** - For PPI quantitations, YPC3 cells were grown on appropriate media for 24 h on semi-continuous culture conditions as stated above. Aproximately 10<sup>8</sup> cells were collected by centrifugation, washed with ice-cold water and extracted with 1 ml of 4% perchloric acid. To aid cell breakage, cells were subjected to three one-min vortex burst in the presence of glass beads (0.5 mm Ø). After decanting glass beads and debris by centrifugation, extracts were neutralised by adding sequentially 140 µl of 5 M KOH and 60 µl of 1 M Tris-HCl (pH 7.5) and centrifuged again at top speed in a table-top centrifuge for 30 min to decant potassium perchlorate salts. PPI was measured in the supernatants using a commercial kit (Molecular Probes, E-6645) according to manufacturer instructions. Pyridine-nucleotide coenzyme extractions were as follows: briefly, YPC3 and W303-1a cells were grown on semi-continuous conditions for 18 h (SG and CR conditions) or 36 h (MR and galactose controls); then ca 2.5x10<sup>7</sup> cells were washed with ice-cold water and resuspended in 200 µl of either 50 mM NaOH (NADH extraction) or 50 mM HCl (NAD<sup>+</sup> extraction) and heated at 60 °C for 30 min on a heat block. After this, extracts were

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neutralised and clarified by centrifugation. Coenzymes were determined using a coupled assay consisting of 10 mM bicine pH 8.0, 15 units/ml of yeast alcohol dehydrogenase (Sigma-Aldrich, A-7011), 0.4 mg/ml phenazine methosulfate, 0.25 mg/ml thiazolyl blue formazan and 3% ethanol. Pyridine nucleotide coenzyme levels were spectrophotometrically estimated at 570 nm as the kinetic production of reduced formazan.

RNA methods - Yeast RNA was extracted using the hot phenol method (28). One microgram of RNA was subjected to cDNA synthesis using Quantitect Reverse Transcription Kit (QIAGEN, #205311) according to manufacturer instructions. PCR was done using one µl of newly synthesized cDNA in a 20 µl tube. Sequence of the oligonucleotides used as primers is available upon request. Total number of cycles was set to 25 to avoid saturation. Amplified DNA was separated on agarose gels under standard conditions, photographed using a Gel Doc XR+ System (Bio-Rad, #170-9600) and quantified using Quantity One v4.6.2 software (Bio-Rad, #170-8195) and assayed, unless otherwise indicated, all determinations were done on YPC3 cells incubated for 18 h under SG and CR conditions after 48 h (Fig. 1D). In subsequent experiments, where only a single time point was assayed, unless otherwise indicated, all determinations were done on YPC3 cells incubated for 18 h under SG or CR conditions but for 36 h under MR condition.

**RESULTS**

Effect of Ipp1p deficiency on cell viability - We have previously shown that a *S. cerevisiae* engineered strain, where the gene *IPPI*, encoding its cytosolic sPPase is governed by a *GALI* inducible/repressible promoter (8), is viable as long as a plasmid-borne heterologous H’-PPase is expressed when the *GALI* promoter is repressed. An improved version of this mutant (YPC3) (18) has been used to study the kinetics of events related to Ipp1p deficiency in order to delineate the cellular importance of PPi homeostasis. Phosphate and carbon metabolisms are long known to interact in their regulation (see for example (29)). Also, viability is increasingly associated to carbon source quality and availability in many organisms, in particular to the dichotomy between fermentation and respiration and, more recently, to calorie availability (30-32). Having this in mind, we compared YPC3 control cells (grown on *GALI* inducing conditions: 2% galactose) to others placed under three different repressing carbon source conditions: standard glucose concentration (SG, 2% glucose), caloric restriction conditions (CR, 0.5% glucose) and standard mandatory respiratory conditions (MR, 3% glycerol). As expected, after 24 h, YPC3 cells accumulated 0.31±0.05, 0.31±0.05, 0.21±0.02 and 0.02±0.01 nmol PPi per 10⁵ cells in SG, CR, MR and galactose control conditions, respectively; i.e, SG and CR cells displayed ca 10-fold and MR cells ca 10-fold greater levels of PPi than those observed under galactose conditions. On the other hand, Ipp1p polypeptide levels decreased sharply upon switch of YPC3 cells from galactose (control) to glucose, irrespective of its concentration (SG and CR, Fig. 1A). Besides, switch to MR conditions induced a slower rate of Ipp1p disappearance. This is in agreement with *GALI* promoter being inactive due to lack of its transcription factor, Gal4p, when cells are grown on this carbon source, but not actively repressed by Gal80p and Mig1p, as it is under glucose conditions (33). In any case, Ipp1p polypeptide levels were undetectable on *Western blots* 6 h after switch to glucose and 24 h after switch to MR. These results were confirmed by PPi hydrolytic activity assays (Fig. 1B). After 6 h from the switch to SG or CR, or 24 h after switch to MR, no sPPase activity was observed. Concomitant with this, growth was slowed to a halt after 18 h under SG and CR conditions and 36 h under MR condition (Fig. 1C), while galactose controls showed a constant growth rate even after 48 h of semi-continuous culture.

In order to ascertain if viability was compromised and the stage of sPPase activity at which this reduction occurs, we also followed the ability of these cells to form colonies (Fig. 1D). Albeit enzymatic activity was undetectable much earlier, cell viability was not observed to drop until 18 h after switching to SG or CR conditions and after 21 h it was observed to plateau and to reach a minimum at ca 27% of plated cells. Similarly, the viability of MR cells dropped severely at 36 h and reached the same lowest value as SG and CR cells after 48 h (Fig 1D). In subsequent experiments, where only a single time point was assayed, unless otherwise indicated, all determinations were done on YPC3 cells incubated for 18 h under SG or CR conditions but for 36 h under MR condition.
Effect of Ipp1p deficiency on cell cycle - We were interested in determining the consequences that a defect in PPI homeostasis had on the cell cycle. To this end, we compared the cell cycle profiles of cells incubated under control, SG, CR and MR conditions (Fig. 2A, left panel). In this case, a differential behaviour was evident between fermenting and respiring cells. Under both fermenting conditions (SG and CR) cells arrested their cycle abruptly; additionally, a great proportion of cells showed a DNA content smaller than that corresponding to an haploid intact genome (SubG1), indicative of cell death (21). Conversely, MR cells showed no accumulation of SubG1 cells despite displaying a nearly equally severe cell cycle arrest. In the case of SG and CR, a discernible subG1 population increase over control cells could be observed as early as 18 h after carbon source switch (Fig. 2A, right panel) and reach up to 25% of the cells in a culture. However, under MR conditions, no meaningful increase in the proportion of subG1 cells, with respect to control cells, was observed even 56 h after the glycerol switch (6.5%±1.3 and 2.2%±0.2 for MR and control cells, respectively; n=10,000 cells). Since propidium iodide staining does not provide enough accuracy to discern between a G1 and an S-phase cell cycle arrest by flow cytometry in budding yeast, we measured the amounts of CLN3, CLB5 and CLB3 cyclin mRNAs by semiquantitative RT-PCR as markers of G1, S and G2 phases of the yeast cell cycle, respectively (Fig. 2B). Both SG and CR conditions showed a marked depletion of all cyclins studied, a situation usually associated to G0 (39). On its turn, MR cells displayed an accumulation of CLB5 cyclin mRNA and an overall similar profile to that observed in hydroxyurea treated cells, indicative of S-phase arrest, and in sharp contrast with the profile observed in cells where G1 arrest was induced by addition of a-factor (Fig. 2B, right panel).

Morphology and characteristics of Ipp1p-deficient SG and CR cells - In agreement with cyclin mRNA results, under the microscope (Fig. 3A), most MR cells showed large buds, compared with control cells (percentages of budded cells ±SD from a representative experiment were 46.1±5.9% and 72.2±5.8% for galactose control and MR, respectively; n=2), while SG and CR cells were largely un budded (5.0±3.1% and 5.0±5.2% of total cells were budded for SG and CR, respectively). Strikingly, they also showed a characteristic morphology: they were much bigger than control or MR cells and appeared to contain an enlarged vacuole that filled much of the cell interior. When the apparent cellular area observable under the microscope was quantified, MR and control cells showed no statistically significant differences among themselves, while SG and CR cells were ca 2-fold larger than controls (Fig. 3A, bottom panel). Nuclear DNA degradation and, hence, large subG1 populations are characteristic features of apoptosis. In this case, nuclear DNA degradation frequently gives rise to the appearance of a characteristic laddering on genomic DNA agarose gels due to internucleosomal fragmentation. However, when genomic DNA from SG ad CR cells were electrophoresed, a continuous smear was observed instead (Fig. 3B). Tunel assays were also negative on SG, CR and MR cells (data not shown). Reactive oxygen species (ROS) are also typical inducers and markers of programmed cell death (34). However, both SG and CR cells did not show any discernible increases in ROS, as measured by dihydrorhodamine 123 fluorescence (Fig. 3C). In marked contrast, MR cells showed a clear fluorescent staining indicative of ROS accumulation; this feature was not observed on the parental W303-1a strain grown under the same conditions.

The prevalence of vacuolisation in SG and CR cells led us to test if autophagy was induced. To test this hypothesis, a GFP-ATG8 fusion construct was transformed into YPC3 and the proteolytic processing of ATG8 was followed by Western blot as a marker of autophagy. Control cells kept in galactose and MR cells did not show any processing of the GFP-Atg8p fusion while SG and CR cells showed a clear band corresponding to liberated GFP in addition to the full fusion. Also, bands of intermediate molecular weight were visible, probably reflecting partial degradation of GFP-Atg8p (Fig. 4A). A similar band pattern was displayed by a nitrogen-starvation positive control of autophagy. The fluorescent vital dye FM4-64 has been shown to stain differentially the acidic vacuoles of autophagic yeast cells due to accumulation of membrane material (26). When FM4-64 was added to cultures of SG and CR cells, a pattern of solid vacuoles was observed, similar in appearance to what is observed in nitrogen-starved yeast cells (Fig. 4B). The proportion of cells showing solid vacuoles under these conditions was similar to that of cells bearing subG1 amounts of DNA.
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On the contrary, galactose-grown control and MR cells did not show any apparent accumulation of FM4-64 inside vacuoles and retained a normal morphology.

Reversibility of cell cycle arrest in MR cells – In contrast to SG and CR cells, yeast under mandatory respiration (MR) did not show any signs of cell death. We therefore tested if these cells were in fact arrested in a reversible way and if removal of excess PPI could promote recovery. We first tested if cells grown under MR conditions for 48 h and then placed in a fresh YPGalactose liquid medium could resume growth. Indeed, while cells maintained under MR conditions (fresh YPGlycerol medium) could not proliferate, OD$_{600}$ clearly increased in YPGalactose cultures (Fig. 5A). Nevertheless, these cultures showed a ca 6h-lag phase, probably while MR cells diffused their cytosolic PPI and had their GAL1 promoters released from repression (Fig 5A). We also tested if the capacity for producing colonies was restored. Cells kept on MR conditions for 48h that were placed in a releasing liquid medium (YPGalactose) prior to plating them on galactose-containing agar plates increased its capacity to form colonies in a time dependent manner, as opposed to those kept in YPGlycerol (Fig. 5B). This recovery was exclusive of cells that had been subjected to MR conditions. When cells under SG or CR conditions were subjected to a 8h-release in fresh YPGalactose medium, no recovery was observed in their colony forming capacity (Fig. 5C).

Molecular mechanisms causing cell death under SG and CR conditions - Yeast fermentative metabolism is stoichiometrically taut regarding NAD$^+$/NADH homeostasis. Since NAD$^+$ biosynthetic enzymes have long been known to be sensitive to excess PPI (35), we hypothesized that the cell levels of pyridine nucleotide coenzymes could be affected in YPC3 cells under SG, CR and MR conditions. Indeed, levels of NAD$^+$ dropped significantly in YPC3 cells grown for 18 h under SG and CR, and 24 h MR conditions, compared to a parental strain grown in the same conditions (Table 1). Concomitantly, YPC3 cells showed decreased NAD$^+$/NADH ratios with respect to wild-type cells when grown under SG and CR conditions. Strikingly, YPC3 MR cells displayed a NAD$^+$/NADH ratio close to that observed in wild-type cells under the same conditions; this is due to a reduction in the levels of NADH that is not observed under SG or CR conditions. Depletion of cellular NAD$^+$ levels have been described to induce massive autophagy and cell death in mammalian cells (36). In order to test if such a mechanism was responsible for the induction of cell death in YPC3 yeast cells under SG and CR conditions, we supplemented the growth culture medium with 10 mM acetaldehyde so that yeast cells could oxidise additional amounts of NADH independently from fermentation. Under these conditions, CR cells showed a three-fold increase in colony forming capacity, reaching levels close to those found for control cells (Fig. 6A, top panel). Cells kept under SG conditions showed a similar behaviour (16.3%±0.3 and 63.0%±3.5 for untreated and acetaldehyde-treated cells 18h after medium switch, respectively). This effect was accompanied by a drastic reduction on the percentage of cells displaying subG$_1$ characteristics as well as a cell size that was indistinguishable from cells kept in galactose (Fig. 6A, middle and bottom panels, respectively). Moreover, this pattern was repeated in cells transformed with an expression plasmid carrying the ORF of the gene NQR1, encoding a plasma membrane-bound NADH oxidoreductase (19). Cells overexpressing this enzyme oxidise greater amounts of NADH under fermenting conditions in a coenzyme Q-dependent manner, but independently of alcohol dehydrogenase. Yeast YPC3 cells transformed with this construct and subjected to caloric restriction conditions showed again clear differences in survival rate, proportion of cells in SubG$_1$ and cell size (Fig. 6B, top, middle and bottom panels, respectively). However, under standard glucose concentrations (SG), cells displayed smaller differences despite the overexpression of NQR1 (survival figures were 54.4%±2.3 and 68.4%±3.0 for empty plasmid-bearing and NQR1-overexpressing cells 18h after medium switch, respectively).

Cell death process under SG and CR conditions - Autophagy has been shown to be a cellular process that, under certain circumstances, can lead to cell death (37). We investigated the involvement of autophagy in the cell death induction and execution taking place under SG and CR conditions. In order to ascertain if autophagy was a cause of death and not simply a concomitant process, we made use of two YPC3-derived yeast strains affected in their capability to complete autophagy. Strains defective for ATG8 are unable to promote the formation of the autophagosome.
(38). We also deleted VPH1, an essential subunit of the vacuolar V-ATPase; this H⁺-pump is required for vacuolar acidification and was early shown to be paramount for completion of the autophagic process (39). Cells that had their IPP1 gene under the control of GAL1 promoter and devoid of Atg8p or vacuolar V-ATPase activity showed no loss of viability when placed under SG conditions, as measured by their capacity to form colonies (Fig. 7A and B, top panels). Furthermore, they showed dramatic decreases in subG₁ cells under flow cytometry (Fig. 7A and B, middle panels) and the cell size was reduced to almost control levels when compared with galactose-grown control cells (Fig. 7A and B, bottom panels). Similar results were obtained under CR conditions (survival figures were 42.5%±3.2 and 88.9%±7.0 for wild-type and atg8Δ cells 18h after medium switch, respectively, on the one hand and 17.2%±3.9 and 111.8%±6.3 for wild-type and vph1Δ cells 18h after medium switch, respectively, on the other).

**DISCUSSION**

Despite intensive research, no thorough information exists yet as to the cellular implications of some basic metabolic processes. One such field is PPI metabolism. Despite it being involved in pathophysiological processes to excess PPI seems to be the homeostasis of PPi producing reactions (1). Moreover, even unrelated reactions are affected by the side effects of attaining high concentrations of PPI, such as sequestration of Mg²⁺ ions (1), making elimination of excess PPI a critical issue in cell metabolism. In this regard, nicotinic acid mononucleotide adenyltransferase step in the biosynthesis of NAD⁺ was the first major biochemical reaction described to both produce this metabolite and to be inhibited by it (35). Other PPI producing biological reactions are also affected by an excess of PPI, for example, the polymerisation of macromolecules such as proteins, RNA or DNA. However, despite an important and still open discussion on the influence of PPI levels on fidelity of nucleotide or amino acid incorporation into macromolecules (40,41), it is unknown if any of them is specially sensitive to excess PPI and, therefore, candidates for the growth defects observed in bacteria and yeast. As we show in the present work, under some conditions, one of the most sensitive cellular processes to excess PPI seems to be the homeostasis of nicotinamide.

In yeast, fermentation places cells in a delicate redox equilibrium: on the one hand, nicotinamide coenzyme concentrations are low ([NAD⁺]+[NADH] is ca 1 mM) and, as a result, most of the cytosolic coenzyme is probably committed to cycle between its oxidised and reduced forms in glycolysis; on the other, this process is stoichiometrically rigid, i.e., the capacity to oxidise NADH by acetaldehyde reduction using alcohol dehydrogenase is equivalent to the amount that was previously reduced by the glyceraldehyde-3-dehydrogenase and, thus, the cell lacks any flexibility to alter its NAD⁺/NADH ratio through metabolism. This means that any further needs for NAD⁺ must be covered by biosynthesis of new coenzyme molecules. Noteworthy, in the cell there are several reactions that consume NAD⁺ and eventually destroy it, e.g. ADP-ribose transferases, cADP-ribose synthases, sirtuins and, in the case of mammals, poly(ADP-ribose) polymerases. At least in mammals, under some conditions, such as DNA damage by genotoxic drugs, these processes can even deplete cellular NAD⁺ contents (42). Both known pathways for NAD⁺ biosynthesis, salvage and de novo biosynthesis, include steps where PPI is formed and, hence, where an excess of this compound can act as an effective inhibitor. Noticeably, the rate-limiting step in NAD⁺ biosynthesis, shared by both de novo and salvage pathways, is the adenylation of nicotinic acid mononucleotide, a reversible reaction among the first...
ones described as sensitive to excess PPi (1,35). In addition to this, the transfer of a phosphoribosyl moiety to yield nicotinamide/nicotinic acid mononucleotide and PPi in the salvage pathway is reversible too (43). The salvage pathway is considered the primary pathway for NAD$^+$ synthesis in yeast (44). Which of these steps is primarily responsible for the observed cell death induction in fermenting cells is still unknown. Research on this subject may offer potential as a way to control cell proliferation.

Caloric restriction has been shown to lengthen life-span and affect many cellular processes in nearly all living model systems, with the exception of plants (44). The best accepted mechanism relies on the increase of the concentration of NAD$^+$ at the expense of NADH. Sirtuins, NAD$^+$-dependent histone deacetylases, are derepressed by the excess NAD$^+$, gene expression is altered through transcriptional silencing and this brings about changes in life-span. In yeast, the presence of glucose at a low concentration (typically 0.5 %) is enough to induce a mixed metabolism comprising fermentation and respiration that helps maintaining a high NAD$^+$/NADH ratio and increase life-span (45,46). In the case of cytosolic sPPase depletion, we have shown that increasing NAD$^+$ availability in the cell inhibited the induction of autophagy and cell death. Despite this, caloric restriction could not increase survival, extent of cell death induction or its time course. Noticeably, overexpression of an alternative NADH-oxidase such as Nqr1p, was only able to alleviate cell death under caloric restriction conditions. This suggests that nicotinamide coenzyme homeostasis must be sternly pushed towards NAD$^+$ production or NADH reoxidation (e.g. addition of high concentrations of acetaldehyde) in order to alleviate cell death effectively and that milder strategies, such as caloric restriction and Nqr1p overexpression, are only effective when in combination.

Although cell death is usually accompanied by prior cell cycle arrest, a general inhibitory effect of anabolism by excess PPi could be perfectly understood with an asynchronous arrest of cell functions. Our data show that, under both fermentative (SG and CR) and respiratory (MR) conditions, cell cycle arrest is part of the cellular responses to cytosolic sPPase depletion. In the case of fermentative conditions, G0/G1 arrest is observed. This could be a response to a deficiency in nicotinamide coenzymes. Subsequent induction of autophagy seems to reinforce this idea. In the case of cells maintained in glycerol as a carbon source, an S-phase arrest may suggest that DNA duplication is, together with nicotinamide coenzyme biosynthesis, one of the most sensitive biochemical processes to an excess of PPi in the cell. Further work is being carried out in our laboratory to shed light into this topic.

All in all, the present report constitutes the first cell physiology study on the alteration of PPi homeostasis in eukaryotes, in terms of its effects on cell cycle and cell death induction. These data have clear importance to understand which processes are most likely affected by an excess of cellular PPi and may also be useful to design new alternative therapeutic approaches against diseases like cancer or bone disorders.

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**FOOTNOTES**

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*Abbreviations used: SG: standard glucose concentration (2% glucose); CR: caloric restriction (0.5% glucose); MR: mandatory respiration (3% glycerol); sPPi: soluble inorganic pyrophosphate; PPI: inorganic pyrophosphate.*
Excess PPI arrests cell cycle and induces autophagy

### TABLE 1. Pyridine nucleotide coenzyme contents in yeast cells grown on different carbon sources

|        | W303-1a | YPC3   |
|--------|---------|--------|
|        | NAD⁺    | NAD⁺   |
| 2% Galactose | 1.51±0.28 | 1.85±0.25 |
| 2% Glucose  | 1.28±0.21 | *0.72±0.12 |
| 0.5% Glucose| 1.38±0.18 | *0.71±0.11 |
| 3% Glycerol | 1.51±0.29 | *0.54±0.11 |
| NADH    | 1.17±0.26 | 1.39±0.18 |
| NAD⁺/NADH| 1.30     | 1.32    |
|         | 2.58     | 1.67    |
|         | 3.94     | 2.26    |
|         | 2.09     | 1.92    |

Data in μmol/10⁶ cells±standard error of the mean (n=3). Asterisks denote statistically significant differences compared with W303-1a cells, as evaluated by t-tests.
FIGURE LEGENDS

FIGURE 1. Depletion of Ipp1p sPPase in yeast YPC3 strain as a function of carbon source and time. A. Time course of Ipp1p depletion. Levels of Ipp1p polypeptide were evaluated by Western blot. Each lane was loaded with 50 µg of protein from a total cell lysate and probed with an antibody raised against a sPPase from Chlamydomonas reinhardtii (see Materials and Methods). B. Time course depletion of PPase enzyme activity. Whole cell lysates were assayed for phosphate-release activity from PPI as indicated in Materials and Methods. Error bars denote standard error of the mean (n=3). C. Changes in growth rates for YPC3 cells maintained in liquid culture. Velocities were estimated as the increment in OD_{600} per unit of time. A representative experiment is shown (n=2). D. Survival ability of YPC3 strain measured as the capacity to form individual colonies (colony forming units, cfu) as a function of carbon source and time (n=3). Maximal values (100%) correspond to 563, 482, 478 and 523 colonies for Control, SG, CR and MR, respectively. Legend for B, C and D panels: solid circles, 2% galactose control (Control); open circles, mandatory respiratory condition (MR, 3% glycerol); solid squares, standard glucose condition (SG, 2% glucose); open squares, caloric restriction (CR, 0.5% glucose).

FIGURE 2. Effects of Ipp1p depletion on cell cycle. A. Cell cycle profiles. Left panel, propidium iodide-flow cytometry histograms of YPC3 cells. YPC3 yeast cultures were kept in galactose (Control), standard glucose (SG, 2% glucose for 18 h), caloric restriction (CR, 0.5% glucose for 18 h) or mandatory respiratory conditions (MR, 3% glycerol for 36 h) at OD ≤ 0.5 prior to analysis. Right panel, quantification of subG1 populations from histograms on the right. Solid bar, control; oblique hash, SG; open bar, CR; horizontal hash, MR. Ten thousand cells were analysed in each case. Error bars denote standard error of the mean (n=3). B. Cyclin levels in YPC3 cells as a function of energy metabolism. Left panel, semiquantitative RT-PCR products obtained using 1 µg of total RNA. Right panel, quantification of cyclin abundance on the left normalised using ACT1 as a standard. Maximal values (100%) correspond to 69.5, 35.4, 33.3 and 61.4 A.U. For CLN3, CLB5, CLB3 and ACT1, respectively. Identity of samples as in panel A. HU, hydroxyurea (0.1 M, 2 h); α-factor (10 µg/ml, 2 h). Legend: solid bars, CLN3; open bars, CLB5; hashed bars, CLB3. Both panels: error bars denote standard error of the mean (n=4).

FIGURE 3. Major cell biological effects of Ipp1p sPPase depletion in YPC3. A. Left panel, morphology of YPC3 cells as a function of energy metabolism; right panel, quantitation of apparent cell size as the area observed under the microscope. Solid bar, control; oblique hash, SG; open bar, CR; horizontal hash, MR. Error bars denote standard error of the mean (n=3). Asterisks denote significant statistical differences (p ≤ 0.05). B. Electrophoresis of genomic DNA obtained from YPC3 cells under different metabolical conditions. Each lane corresponds to 25 µg of DNA; leftmost lane: Lambda DNA digested with EcoRI and HindIII as a size standard. C. ROS accumulation in YPC3 cells. Presence of reactive oxygen species was evaluated using dihydrorhodamine-123. Cells were stained with 5 µg ml⁻¹ for 2 h under appropriate growth conditions and then viewed under a rhodamine filter. Oxygen peroxide controls were done by subjecting YPC3 cultures to 1 mM H₂O₂ for 3h. All cells are YPC3 except where indicated otherwise. DHR, dihydrorhodamine 123 fluorescence; BF, bright field microscopy.

FIGURE 4. Autophagy in Ipp1p-depleted YPC3 cells. A. Processing of a GFP-Atg8p translational
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Western blot of YPC3 whole cell extracts probed with a commercial antibody recognising GFP. Acidic compartments were stained using the red fluorescent vital dye FM4-64 (Calbiochem, #574799). Cells were incubated under standard glucose (SG, 2% glucose), caloric restriction (CR, 0.5% glucose) or mandatory respiratory conditions (MR, 3% glycerol) for 18 (SG and CR) or 36 h (MR) and later stained with the vital dye FM4-64 to display acidic compartments. DIC, differential interference contrast; FM4-64, fluorescence of FM4-64. Numbers in brackets denote percentage of cells showing solid vacuoles ± standard deviation in a representative experiment (n=3).

FIGURE 5. Reversibility of cell cycle arrest in Ipp1p-depleted MR yeast cells. A. Growth of MR YPC3 cells in fresh media. YPC3 cells were kept under mandatory respiratory (MR) conditions for 48 h and later inoculated into fresh complete medium supplemented with a carbon source as stated below at the indicated OD_600. Culture turbidity was monitored for 8 h. Solid circles, 3% glycerol; open circles, 2% galactose. B. Ability to form individual colonies of cells released in liquid medium. YPC3 cells kept under MR conditions for 48 h were later incubated in fresh complete medium supplemented with 2% galactose (open bars) or 3% glycerol (solid bars) for the indicated times. Survival of cells grown on 2% galactose and later refreshed again in galactose-medium for the duration of the experiment (mock) was considered 100% (357 colonies). Asterisks denote significant statistical differences (p ≤ 0.05). C. Irreversibility of cell cycle arrest under glucose conditions. YPC3 cells were kept under control (2% galactose), SG (standard glucose, 2% glucose), CR (caloric restriction, 0.5% glucose) and MR (mandatory respiration, 3% glycerol) conditions for 24 h (SG and CR) or 48 h (MR) prior to being inoculated into fresh complete medium identical to the initial (- release) or supplemented instead with 2% galactose (+ release); after the indicated times, the ability to form individual colonies was tested. Survival of cells grown on 2% galactose and later refreshed again in galactose-medium for the duration of the experiment (mock) was considered 100% (380 colonies). Closed bars, 0 h in release medium; open bars, 8 h in release medium. All panels, error bars denote standard error of the mean (n=3).

FIGURE 6. NAD⁺ homeostasis and cell death. A. Alteration of NAD⁺ metabolism using acetaldehyde. YPC3 cells were incubated in the presence or absence of 10 mM acetaldehyde while kept under control (2% galactose, solid bars) or caloric restriction conditions (0.5% glucose, open bars). Top panel, ability to form individual colonies (colony forming units, cfu); legend: open symbols: Galactose; solid symbols: CR; squares: wild type control (YPC3); circles: acetaldehyde. Colonies representing 100% survival were 709 and 245 for untreated and acetaldehyde-treated control grown cells, respectively. Middle panel, proportion of cells showing DNA contents smaller than Gap-1-cells (subG_{1}) observed in propidium iodide FACS histograms, with respect to the whole population (10,000 cells analysed). Bottom panel, apparent cell size: area, in µm^2, observed under the microscope. Legends for middle and bottom panel: Solid bar, Galactose; open bar, CR. B. Alteration of NAD⁺ metabolism by overexpression of an alternative NADH-oxidase. YPC3 cells carrying an empty expression vector (vector) or the plasma membrane-bound NADH Coenzyme Q reductase 1 gene (NQR1) were placed under control (2% galactose, solid bars) or caloric restriction conditions (0.5% glucose, open bars). Legends and experiments as in A. Colonies representing 100% survival on middle panel were 503 and 525 for YPC3 and NQR1 cells, respectively. Then thousand cells per case were analysed by flow cytometry. In all experiments, cells were incubated in glucose containing media for 18 h and kept at OD ≤ 0.5 prior to analysis. Asterisks denote significant statistical differences (p ≤ 0.05). All panels, error bars correspond to standard error of the mean (n=3).
FIGURE 7. Effect of autophagy deficiency on cell death. A. Effect of a mutation in the early steps of autophagy. Wild-type and mutant *atg8Δ* strains correspond to SAH16 and SAH17, respectively (Materials and Methods). Top panel, ability to form individual colonies (colony forming units, cfu); legend: open symbols: Galactose; solid symbols: SG; squares: wild type strain; circles: *atg8Δ*. Colonies representing 100% survival were 198 and 187 for wild-type and *atg8Δ* strains grown under control conditions, respectively. Middle panel, proportion of cells showing subG\(_1\) DNA contents with respect to the whole population (10,000 cells analysed). Bottom panel, apparent cell size: area, in \(\mu\)m\(^2\). Legends for middle and bottom panel: Solid bars, Galactose; open bars, SG. B. Effect of a blockage in the late steps of autophagy. Mutant *vph1Δ* was constructed on a YPC3 background and the latter is used as a wild type strain (Materials and Methods). Legends and experiments as in A. Colonies representing 100% survival on top panel were 709 and 789 for YPC3 and *vph1Δ* cells, respectively. In all experiments, cells were incubated in glucose containing media for 18 h and kept at OD ≤ 0.5 prior to analysis. Asterisks denote significant statistical differences (p ≤ 0.05). All panels, error bars correspond to standard error of the mean (n=3).
Figure 1

A. Western blot analysis of Control, SG, CR, and MR samples at different time points (0, 3, 6, 9, 12, 18, 24 h).

B. PPase specific activity (µmol P_i min^{-1} mg^{-1} protein) over time (5 to 25 h).

C. Growth rate (A.U.h^{-1}) over time (10 to 50 h).

D. Survival (cfu, %) over time (10 to 50 h).
Figure 2

A

Control

SG

MR

CR

B

CLN3

CLB5

CLB3

ACT1

Relative abundance (fold)
Figure 4

A

| Condition   | GFP-Atg8p | GFP |
|-------------|-----------|-----|
| N-starvation|           |     |
| Control     |           |     |
| SG          |           |     |
| CR          |           |     |
| MR          |           |     |

B

| Condition   | DIC          | FM4-64          |
|-------------|--------------|-----------------|
| N-starvation| ![DIC image](image1) | ![FM4-64 image](image2) |
| Control     | ![DIC image](image3) | ![FM4-64 image](image4) |
| SG          | ![DIC image](image5) | ![FM4-64 image](image6) |
| CR          | ![DIC image](image7) | ![FM4-64 image](image8) |
| MR          | ![DIC image](image9) | ![FM4-64 image](image10) |

7.5 µm
Figure 7

A

Survival (cfu, %)

SubG₁ (%)

Cell size (µm²)

WT

atg8Δ

WT

atg8Δ

B

Survival (cfu, %)

SubG₁ (%)

Cell size (µm²)

WT

vph1Δ

WT

vph1Δ

*
Inorganic pyrophosphatase defects lead to cell cycle arrest and autophagic cell death through NAD⁺-depletion in fermenting yeast
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