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Effect of the periodic properties of toxic stress on the oscillatory behaviour of glycolysis in yeast-evidence of a toxic effect frequency

C. André, F. Gagné *

Aquatic Contaminants Research Division, Environment and Climate Change Canada, 105 McGill St., Montréal, QC H2Y 2E7, Canada

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ABSTRACT

Starving and nondividing yeast cells induce changes in the electron donor nicotinamide adenine dinucleotide (NADH) levels in a cyclic and wave-like manner for over 90 min. Yeast suspensions were used to examine the toxic effects of contaminants on the cyclic behaviour of metabolite changes during anaerobic glycolysis. The cyclic behaviour NADH levels in yeast cell suspensions starved for 2 to 5 h was studied after the addition of 10 mM glucose for 5 min followed by 10 mM KCN to block aerobic glycolysis. The effects of three toxic elements ($CuSO_4$, silver nanoparticles-nAg, and $GdCl_3$), known for their potential to alter glycolsysis, on NADH levels over time were examined during the 3-h starvation step. The data were analyzed using spectral analysis (Fourier transformation) to characterize the cyclic behaviour of NADH levels during anaerobic glycolysis. Increasing the starvation time by 3 h increased the amplitude of changes in NADH levels with characteristic periods of 3 to 8 min. Longer starvation times decreased the amplitude of oscillations during these periods, with the appearance of NADH changes at higher frequencies. Moreover, the amplitude changes in NADH were proportional to the starvation time. Exposure to the above chemicals during the 3-h starvation time led to the formation of higher frequencies with concentration-dependent amplitude changes. In comparison with nAg and Gd^{3+}, Cu^{2+} was the most toxic (decreased viability the most) and produce changes at higher frequencies as well. It is noteworthy that each element produced a characteristic change in the frequency profiles, which suggests different mechanisms of action in which the severity of toxicity shifted NADH changes at higher frequencies. In conclusion, the appearance of synchronized oscillations in dense yeast populations following synchronization stress could be induced by starvation and exposure to chemicals. However, synchronicity could be abolished when cells desynchronize as a result of loss of cell viability, which contributes to heterogeneity in yeast populations, translating into NADH changes at higher frequencies. This is the first report on the influence of environmental contaminants on the cyclic or wave-like behaviour of biochemical changes in cells.

1. Introduction

Toxicity bioassays are routinely used to detect and predict the negative impacts of chemical pollution on individuals and populations. In ecotoxicology, toxicity in various species is determined usually following a stepwise food chain gradient. Bioassays based on eukaryotes are usually conducted in invertebrates (microcrustaceans, annelids, and mollusks) and vertebrates (fish and amphibians), and such bioassays are time-consuming and become rapidly cost-prohibitive. Bioassays on microorganisms are attracting increased interest and are rapid and cost-effective alternatives (Blaise and Gagné, 2009). Among the simplest unicellular eukaryotes, yeasts have been widely used over the ages in baking and the production of alcoholic beverages from fermentation. The yeast *Saccharomyces cerevisiae*, commonly known as baker's yeast, has been proposed as a bioassay organism to determine the toxicity of various environmental contaminants (Estève et al., 2009; Rumlova and Dolezalova, 2012). For example, yeast microbioassays were previously compared with standard toxicity tests using *Daphnia magna* and the bioluminescent bacteria *Aliivibrio fisheri* (formerly *Vibrio fisheri*) on five toxic compounds: atropine, fenitrothion, potassium cyanide, mercuric chloride, and lead nitrate. The yeast test system proved cheaper and was considerably faster than the *D. magna* test and had speeds comparable to those of the bioluminescent bacteria test (5-to-30-min exposure times). The sensitivity of the yeast microbioassay was similar to or higher than that of the bacterial test depending on the test substance. Yeast reproduction occurs by budding, which can be used as an additional chronic endpoint. The yeast test was also previously used to examine the toxicity of pesticides (Azoxystrobin, Cymoxanil, and Diuron) (Estève et al., 2009). The toxic endpoint was the production of ATP levels in cells during respiration and glycolysis. The assay proved less sensitive than the *D. magna* test but was more sensitive than the test with the bioluminescent bacteria *A. fisheri*. Single-cell eukaryotes such as yeasts are potentially sensitive

* Corresponding author.
E-mail address: francois.gagne@canada.ca (F. Gagné).

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and cost-effective models for assessing chronic toxicity and sublethal effects (Ribeiro et al., 2000). Yeast cells are very easy to grow and maintain in the laboratory under controlled conditions. Yeasts are considered to be fundamental eukaryote models in human health studies (Menacho-Márquez and Murguía, 2007). Indeed, yeasts are sometimes used in anticancer drugs and pharmaceuticals research as indicators of cell proliferation inhibition. Yeasts are also amenable to toxicogenomic studies for both human health and ecotoxicological investigations. For example, yeast mutants in the *Yap1* gene (yeast-associated proteins are transcription factors involved in the control of oxidative stress response) were found to be more sensitive to genotoxic compounds using transcriptomic profiles (Zhang et al., 2016). *Saccharomyces cerevisiae* was recently used for ecotoxicogenomic investigations (dos Santos and Sá-Correia, 2015) to determine the toxicological outcomes of exposure to environmental pollutants in risk assessment, bioremediation, plant biotechnology, and biofuels with applications in drug toxicity screening and the food industry. Yeast is sometimes used as an initial screening tool, to limit the use and cost of animal models (dos Santos et al., 2012). Given its position at the forefront of systems biology research, the yeast model contributes to the overall understanding of toxicological responses and resistance mechanisms with respect to pharmaceutical drugs, such as anticancer agents, and environmental pollutants, such as pesticides.

An interesting feature of yeast (but not specific to it) is that glycolysis exhibits wave-like oscillatory behaviour. Under specific conditions, the concentrations of various metabolites involved in anaerobic and aerobic glycolysis exhibit sustained oscillations (Pye and Chance, 1966; MacDonald et al., 2003). In *S. cerevisiae*, for example, growth, harvest, and starvation conditions have been found to reproducibly induce sustained oscillations in nondividing cells. Oscillations in anaerobic glycolysis were found to involve the allosteric regulation of phosphofructokinase in yeast cell lysates (Goldbeter and Lefever, 1972). This enzyme is negatively regulated by ATP levels and positively regulated by NADH levels, which forms the basis of oscillations in cells. Oscillations can be observed in many other cells, especially those under constant energy demands such as cardiomyocytes, myocytes, and pancreatic islet cells (MacDonald et al., 2003; Cortassa et al., 2004). Mitochondrial activity can oscillate in citrate (and some other intermediates of the citric acid cycle), in NADH, and even at the membrane potential level. Oscillations in the production of reactive oxygen species and levels of glutathione have also been observed. The activity of peroxidase, an enzyme involved in the oxidation of miscellaneous substrates by H_2O_2, can also show oscillations in its activity in NADH levels in the presence of O_2 (Bronnikova et al., 2001). One interesting feature of these wave-like behaviours is that individual yeast cells can synchronize their oscillations in the presence of starvation stress (Bier et al., 2000). Acetaldehyde, a metabolite of glycolysis, was found to be the major synchronization signal in yeast cell populations. At cell densities that permit optimal communication between cells, oscillations in NADH levels are reproducibly observed in the presence of added glucose and an added respiratory inhibitor (KCN).

Given that the production and metabolism of reactive oxygen species could behave as a wave-like function, the role of these oscillations from an ecotoxicological perspective is an unanswered question at this time. Oscillations are ubiquitous at the biochemical and cellular levels and form the basis of circadian rhythms and biological clocks. More attention needs to be paid to the effects of chemical pollutants on the cyclic properties of metabolite changes in organisms, in order to better understand the subtle, long-term impacts and cumulative effects of environmental pollution. A recent study reported that biochemical changes (biomarkers) at the gene expression level often display wave-like behaviours and can be analyzed as waves (Gagné, 2016). Changes in stress-related gene expression display specific wave-like properties, such as oscillations in amplitude, frequency, and phase (coherence), and some of these changes are highly predictive of toxicity (decreased survival) and effects at a higher level of biological organization. This suggests that the cyclic nature of biochemical changes is more completely described as waves (instead on intensity) and could be altered by exposure to chemicals which can results in toxicity.

The purpose of this study was therefore to examine the effects of certain contaminants on the oscillatory behaviour of NADH in yeast cell suspensions during anaerobic glycolysis. The cyclic changes in NADH levels were analyzed using Fourier transformation analysis to identify periodic changes in anaerobic glycolysis induced by the following contaminants: copper (Cu^{2+}), silver nanoparticles (nAg, 20 nm), and gadolinium chloride (Gd^{3+}). The first contaminant, Cu^{2+}, is extensively used as antifungal agent in vine and other crops, which results in its release in the environment, where it is a common toxicant and is a well-recognized inhibitor of glycolysis in both prokaryotes and eukaryotes (Gebhard et al., 2001; Lauer et al., 2012). Nanoparticles such as nAg are used as bacteriostatic agents in personal care products and clothes and are released during washing. Silver nanoparticles were recently reported to decrease the release of lactate, the end product of anaerobic glycolysis, in mammalian cells (Lee et al., 2016). A paramagnetic rare earth element, Gd is used in diverse fields (e.g. green phosphor in TV screens), and an organic complex of Gd is found in contrast agents for magnetic resonance imaging (MRI) analysis in medicine. Recent evidence suggests that Gd^{3+} could also decrease carbohydrate metabolism in HeLa cells (Long et al., 2011). These three elements were used as environmentally relevant contaminants to examine the impacts of xenobiotics on the cyclic properties of NADH levels during anaerobic glycolysis.

2. Materials and methods

Saccharomyces cerevisiae yeast powder (Sigma Chemical Company, Mississauga, Ont, Canada) was suspended at 0.67% (w/v) in YPD culture medium (containing peptone, yeast extract, and glucose) and then incubated at room temperature under constant agitation (120 rpm) at 30 °C. The cells were maintained under those conditions for 18 h. After this period, the cells were centrifuged at 200 × *g* for 5 min and resuspended in 40 mL of 100 mM sodium phosphate buffer, pH 6.8. Cell density was adjusted to 30 million cells/mL based on cell counting and the pre-established relationship $A_{600} = 1$ for approximately 3×10^7 cells/mL. They were held in these "starvation" conditions for 2, 3, and 5 h at 30 °C under constant agitation. Cell density and viability were concomitantly determined using the methylene blue dye method. Briefly, 10 μL aliquots of 1/10, 1/20, and 1/50 dilutions of the cell suspension were prepared in 100 mM sodium phosphate buffer, pH 6.8, containing 0.004% methylene blue, pipetted on a hemocytometer, and counted under a binocular microscope at 200× enlargement. Live cells appeared clear and transparent, and dead cells were stained blue (unable to exclude the dye).

Glycolysis activity was determined in harvested yeast suspensions at a density of 30 million cells in dark microplates. Variations in reduced NADH were determined first in yeast suspensions that were "starved" in sodium phosphate buffer for 2, 3, and 5 h at room temperature. Anaerobic glycolysis was initiated by adding 10 mM glucose for 5 min, followed by the addition of 10 mM KCN to block aerobic glycolysis. The levels of NADH were then determined every 20 s for 120 min by fluorescence measurement at 360-nm excitation and 460-nm emission (Synergy 4 microplate reader; BioScan, USA). Variations in reduced NADH (anaerobic glycolysis) were also determined for $CuSO_4$, nAg (20-nm diameter; Ted Pella, USA), and $GdCl_3$ during the 3-h starvation step. During 3 h of incubation, the following were added: $CuSO_4$ to final concentrations of 1, 5, and 25 μM, nAg to final concentrations of 20, 100, and 500 μg/L (corresponding to 2, 10, 50 μM Ag; 50 μM Ag contain 1.125×10^{11} nanoparticles) and $GdCl_3$ to final concentrations of 2, 10, and 50 μM. After the incubation time, anaerobic glycolysis was monitored as described above (the addition of 10 mM glucose for 5 min followed by the addition of 10 mM KCN). The controls were subjected to the 3-h starvation time in sodium phosphate only. Time-point glycolysis activity with and without the addition of KCN was determined after 30 min of incubation to determine the total activity.