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Direct measurement of catalase activity in living cells and tissue biopsies

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ABSTRACT

Spatiotemporal regulation of enzyme-substrate interactions governs the decision-making steps in biological systems. Enzymes, being functional units of every living cell, contribute to the macromolecular stability of cell survival, proliferation and hence are vital windows to unraveling the biological complexity. Experimental measurements capturing this dynamics of enzyme-substrate interactions in real time add value to this understanding. Furthermore these measurements, upon validation in realistic biological specimens such as clinical biopsies – can further improve our capability in disease diagnostics and treatment monitoring. Towards this direction, we describe here a novel, high-sensitive measurement system for measuring diffusion-limited enzyme-substrate kinetics in real time. Using catalase (enzyme) and hydrogen peroxide (substrate) as the example pair, we demonstrate that this system is capable of direct measurement of catalase activity in vitro and the measured kinetics follows the classical Michaelis-Menten reaction kinetics. We further demonstrate the system performance by measuring catalase activity in living cells and in very small amounts of liver biopsies (down to 1 μg total protein). Catalase-specific enzyme activity is demonstrated by genetic and pharmacological tools. Finally we show the clinically-relevant diagnostic capability of our system by comparing the catalase activities in liver biopsies from young and old mouse (liver and serum) samples. We discuss the potential applicability of this system in clinical diagnostics as well as in intraoperative surgical settings.

1. Introduction

Aerobic metabolism defines the fundamental characteristic of life for the eukaryotic organisms [1]. Harnessing the available oxygen for utilization in bioenergetic and biosynthetic processes involves complex layers of enzymatic reactions in every living eukaryotic cell [2]. Cellular respiration (oxygen intake) is tightly regulated by subcellular compartments and in particular, mitochondria – which make the cellular energy currency, the adenosine triphosphate (ATP), by complete oxidation of oxygen via a series of redox steps. Electron leaks during these steps lead to the generation of oxygen free radicals. Evolutionarily, living cells have developed sophisticated enzymes and other non-enzymatic reaction partners that continuously scavenge these free radicals thereby detoxifying the cells for their survival and proper function [3,4]. These antioxidant enzymes vary in their subcellular location and modes of action. For instance, superoxide dismutase is a mitochondrially localized enzyme that converts toxic superoxide (negatively charged oxygen free radical) to less toxic hydrogen peroxide. Whereas catalase is the enzyme that converts hydrogen peroxide to water and predominantly is localized to peroxisomes and also in cytoplasm. By fine-tuning the rates and the magnitudes of these antioxidant enzymes, cell survival is regulated amidst dynamic changes in pro-oxidant balance. Deregulation of this balance has been implicated in a variety of diseases including diabetes, cancer and aging [5]. Experimental methods to monitor the pro-anti-oxidant balance in various tissues add significant value to not only disease diagnostics but also in treatment monitoring [6–9]. In this paper, we describe a novel method to directly measure the activity of a representative antioxidant enzyme, catalase in real time.

2. Materials and methods

2.1. Cells & reagents

The normal mammary epithelial MCF10A cells and the breast

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cancer cells, MDA-MB-231, MCF7 and BT-549 - were originally from ATCC and were cultured in DMEM with high glucose (4.5 g/l) and 10% FBS and antibiotics. MDA-MB-231 cells were transduced with lentiviral particles containing human Catalase expression vector and the MDA-MB-231 cells overexpressing catalase were selected using neomycin (3 μg/ml) antibiotics at least for 10 consecutive passages and the catalase overexpression was confirmed by western blotting. Immunofluorescence slides were prepared by standard procedure from monolayer cultures of parental (231-P) and catalase-overexpressing (231-CAT) breast cancer cells.

2.2. Animal tissues

Male FVB/N-J mice were originally purchased from Jackson Laboratory and further breeding was carried out in-house as approved by the institutional animal care and use committee. Just before the tissue enzyme activity measurements, animals of two age groups (4 weeks & 40 weeks) were sacrificed and the liver tissues were surgically excised. Immediately after the surgery, the liver tissues were homogenized in 2 ml microcentrifuge tube containing hypotonic buffer using a tissue lyser (Qiagen, 5 min, 30 Hz or 1800 oscillations/minute). Protein lysates were then used immediately for measuring catalase activity as described. In another set of experiments, 50 mg fresh liver biopsy was used to measure catalase activity in the presence and in the absence of catalase-specific pharmacological inhibitor 3AT (6 mM 3-Amino, 1,2,4, triazole, Sigma Aldrich, USA).

2.3. Catalase activity measurements

Viability and mitochondrial functional status were measured in all the cell lines and the tissue samples before measuring catalase activity. Mitochondrial oxygen consumption [pO_2] data were obtained with a clark-type oxygen microelectrode (Strathkelvin Instruments, Scotland) in a closed-cell respirometry design. The oxygen probes consist of electrolytes separated by a polypropylene membrane. Hermetically sealed probe holder allows dissolved oxygen measurement in a constant volume (typically 2 ml). The probe electrodes were calibrated with 5% sodiumthiosulfite solution (0% oxygen) and mammalian ringer solution (100% oxygen ~ 207 μmol/l) at 38 °C. All measurements were performed in phosphate buffered saline as described earlier [10]. After collecting basal respiration (in stirred medium) for about 10 min in the substrate-limiting conditions, 200 μM H_2O_2 was added and catalase-specific oxygen release rates were then calculated from the initial slopes of raw pO_2 data.

2.4. Statistics

Data presented are mean ± S.E from at least three independent experiments. Statistical significance was estimated based on Student's t-test ($p < 0.05$).

3. Results

3.1. In vitro catalase activity profiles follow Michaelis-Menten steady state kinetics

For in vitro validation study, we chose the Catalase (enzyme) −Hydrogen peroxide (substrate) system. Catalase converts the toxic hydrogen peroxide into water (H_2O) and oxygen (O_2). Fig. 1a shows representative oxygen concentration profiles for three different situations. Detailed characterization of concentration dependence of both the enzyme and substrate were done earlier in the laboratory (data not shown) and these data suggested the optimum concentrations of hydrogen peroxide (200 μM) and Catalase (50 U/ml) where one unit (U) is defined as the amount of catalase required to oxidize 1 μmol of hydrogen peroxide in one second at 24 deg C. As can be seen, the substrate-alone or the enzyme-alone did not give any appreciable oxygen release. The small change in oxygen release with hydrogen peroxide within the experimental duration is because of the known instability of the hydrogen peroxide upon dilution in micromolar range. However, the presence of catalase and hydrogen peroxide gave the largest change in oxygen release demonstrating the sensitivity of the system in measuring subtle changes in oxygen concentration in real time.

Any typical biochemical reaction involving an enzyme and substrate involves an initial binding step between them to form an intermediate complex which in turn, is converted to the product and the enzyme. Under certain assumptions (e.g., enzyme concentration << substrate concentration), most of the enzyme-substrate interactions follow the classical Michaelis-Menten equation which relates the reaction velocity (v) of the equation to the concentration of the substrate [S] as:

$$v = (d[P])/dt = (V_{max}[S])/(K_M + [S]) \quad (1)$$

where Vmax is the maximum reaction velocity achievable at saturation concentration of the substrate and K_M is the substrate concentration at which the reaction rate is half of V_{max}. In order to test if the oxygen release assay as described in our system follows similar model, we repeated the experiments by varying the substrate (H_2O_2) concentration systematically and measured oxygen release rate at each of these concentrations by keeping the catalase concentration fixed. Fig. 1b summarizes the results.

3.2. Direct measurement of label-free catalase activity distinguishes normal and cancer cells

Having established a robust assay for monitoring Catalase enzyme activity in in-vitro conditions, we next tested the efficacy of the system in detecting the catalase activity in living cells. In order to determine the catalase-specific effects in the measured oxygen release, we compared the rates of oxygen release upon H_2O_2 stimulus − in parental and catalase-overexpressing human breast cancer (MDA-MB-231) cells. As can be seen in Fig. 2a–c, catalase-overexpressing cells indeed showed a clear increase in oxygen release rate as compared to the parental cells-thereby validating the detection specificity in our method. Next, we wanted to test if the proposed assay can give valuable information on the basal antioxidant status in living cells. Among the cancer-associated changes in epithelial cells, deregulation in redox status and the disruption of pro-oxidant/anti-oxidant balance are the common metabolic phenotypes. Fig. 2d shows representative catalase activity in non-transformed human mammary epithelial cells, MCF10A and two canonical human breast cancer cells, MCF7 and MDA-MB-231 cells. Both the cancer cell lines showed a higher catalase activity as compared to the normal, MCF10A cells.

3.3. Catalase activity profiles in tissue biopsies reveal age-dependent compensatory metabolism

We next tested the utility of our method in measuring catalase activity in fresh liver biopsy specimens obtained from experimental mouse models. Catalase is an ubiquitous antioxidant enzyme that is present in almost all the body tissues although varying in its content and functional capacity in different tissues [3,11]. Liver has been reported to have one of the highest catalase and other antioxidant activity owing to its role in extensive detoxification processes in the body [12]. As approved by the institutional animal care