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Original Contribution

Detection of oxidative stress-induced carbonylation in live mammalian cells

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ABSTRACT

Oxidative stress is often associated with etiology and/or progression of disease conditions, such as cancer, neurodegenerative diseases, and diabetes. At the cellular level, oxidative stress induces carbonylation of biomolecules such as lipids, proteins, and DNA. The presence of carbonyl-containing biomolecules as a hallmark of these diseases provides a suitable target for diagnostic detection. Here, a simple, robust method for detecting cellular aldehydes and ketones in live cells using a fluorophore is presented. A hydrazine-functionalized synthetic fluorophore serves as an efficient nucleophile that rapidly reacts with reactive carbonyls in the cellular milieu. The product thus formed exhibits a wavelength shift in the emission maximum accompanied by an increase in emission intensity. The photochemical characteristics of the fluorophore enable the identification of the fluorophore-conjugated cellular biomolecules in the presence of unreacted dye, eliminating the need for removal of excess fluorophore. Moreover, this fluorophore is found to be nontoxic and is thus appropriate for live cell analysis. Utility of the probe is demonstrated in two cell lines, PC3 and A549. Carbonylation resulting from serum starvation and hydrogen peroxide-induced stress is detected in both cell lines using fluorescence microscopy and a fluorescence plate reader. The fluorescent signal originates from carbonylated proteins and lipids but not from oxidized DNA, and the majority of the fluorescence signal ($>60\%$) is attributed to fluorophore-conjugated lipid oxidation products. This method should be useful for detecting cellular carbonylation in a high-content assay or high-throughput assay format.

Introduction

Oxidative stress is a physiological condition that results from imbalance in the pro-oxidant/antioxidant homeostasis. An abnormal increase in oxidants, such as reactive oxygen species (ROS), or insufficient antioxidant defense culminates in oxidative stress in the cellular system. This failure of the cells' buffering system to maintain the redox equilibrium leads to diverse modifications in biomolecules that are a hallmark of disease conditions. Oxidative stress is often associated with the onset and/or progression of cancer [1], neurodegenerative diseases [2], diabetes [3], chronic obstructive pulmonary disease [4], and cardiac complications [5]. These diverse pathophysiological implications of oxidative stress thus make it a valuable target for diagnosis and prognosis of disease states.

Stable irreversibly carbonylated biomolecules such as proteins [6–9] and lipids [10,11] and, to some extent, DNA [12], serve as biomarkers for oxidative stress. For example, protein carbonylation can directly result from carbonylation of the side chains of amino acids, such as proline, arginine, lysine, and threonine [13,14], which may in turn cross-link intracellular proteins. Indirect means of protein modification include addition of oxidized products of carbohydrates or lipids [15,16]. Lipid peroxidation can result in aldehyde-containing products such as 4-hydroxynonenal [17], malondialdehyde, acrolein, and heptanal [18]. Aldehydes and ketones from lipid oxidation can subsequently react with proteins and nucleic acids to form covalent adducts that may retain the carbonyl functionality [19]. Hence, a well-accepted approach for evaluating the extent of oxidative stress is to detect the level of modified biomolecules.

Traditionally, cellular carbonyl detection is achieved by using chemical probes such as tritiated sodium borohydride [20,21], which reduces the carbonyls to alcohols, or a hydrazine or hydrazide-containing reagent, which forms hydrazones with the target carbonyl. Popular hydrazine/hydrazide methods use reagents, such as 2,4-dinitrophenylhydrazine (DNPH) [22–26], biotin-linked hydrazides [27], or fluorophore-hydrazides [16,28]. The resulting hydrazone can then be detected by fluorescence [25,28] or by analytical techniques

Abbreviations: CH, coumarin hydrazine (7-hydrazinyl-4-methyl-2H-chromen-2-one).

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such as enzyme-linked immunosorbent assay [23,29], Western blot [24], or mass spectrometry [27].

The extent and distribution of carbonylated species in fixed cells can be observed by fluorescence microscopy. Cellular carbonyl-containing molecules are first reacted with DNPH and are subsequently visualized by immunochemical analysis with anti-DNP antibodies [25]. Detection of carbonylation products in live cells, however, has not yet been reported. In principle, cell-permeable hydrazine or hydrazide-containing fluorophores could be used to covalently attach a fluorophore to carbonylated cellular components. A possible difficulty with this technique is background due to unreacted fluorophore in the cytosol [30] and the slow reaction between hydrazides and aliphatic aldehydes at neutral pH [31].

We have shown previously that reaction of an aromatic hydrazine-containing fluorophore with an aromatic aldehyde occurs at a reasonable rate at neutral pH ($\sim 30\ M^{-1}\ min^{-1}$) and that the reaction also occurs within live cells [32]. Moreover, the hydrazone thus formed has absorption and emission spectra that are red-shifted relative to the hydrazine and, importantly, a higher quantum yield. The molecule, 4-methyl-7-hydrazinyl coumarin (**1**, CH), was able to fluorescently label an aromatic aldehyde-containing protein in live cells. We reasoned if CH retained similar behavior with aliphatic aldehydes, then this molecule could be used to detect cellular carbonylation. In this work, we show that CH is suitable for visualizing carbonylation of lipids and proteins in live cells resulting from hydrogen peroxide treatment. Cellular carbonylation due to serum starvation can also be detected using CH both by confocal microscopy and by a fluorescent plate reader. This method of detecting carbonylation due to oxidative stress should be applicable for high-content screening [33] and forms the basis of a high-throughput assay for inducers of intracellular carbonylation.

Materials and methods

Reagents

Hydrogen peroxide (30%) was purchased from Thermo Scientific. Bovine serum albumin–fraction V (BSA) was purchased from Rockland. Sephadex G-25 fine resin was purchased from GE Healthcare. Fetal bovine serum (FBS) and penicillin–streptomycin were purchased from Atlanta Biologicals. Chloroform was purchased from Spectrum. The remaining chemicals were purchased from Sigma Aldrich unless otherwise stated. Coumarin hydrazine (7-hydrazinyl-4-methyl-2*H*-chromen-2-one, CH) was synthesized starting with 3-aminophenol as described in Banerjee et al. [32].

Cell culture

Prostate cancer (PC3) cells were purchased from the American Type Culture Collection (ATCC). Lung carcinoma (A549) cells (originally purchased from ATCC) were a gift from Professor Ming An. Cells were grown in F12K medium (ATCC) supplemented with 10% (v/v) FBS and 1% penicillin–streptomycin (standard medium) in a humidified incubator containing 5% CO_2 at 37 °C. Cells were seeded on 96-well plates (Greiner Bio-One or Corning), 6-well plates (Corning), or Lab-Tek II chambered coverglass (Thermo Scientific), and grown for the stated period of time.

Spectroscopy of the fluorophore

Absorption spectra were obtained at room temperature using a Hewlett Packard 8452A diode array spectrophotometer. Emission spectra were recorded at room temperature using a Jobin Yvon Horiba FluoroMax-3 spectrofluorometer. The samples were excited at 405 nm.

Stock solutions of the fluorophore were made fresh by dissolving the solid molecule in 10 mM phosphate buffer, pH 7.0 (PB). An extinction coefficient of $\varepsilon_{346} = 1.9 \times 10^4\ M^{-1}\ cm^{-1}$ was used to obtain the concentration of the solution. A propanal hydrazone of CH was prepared by mixing a large excess of propanal with CH in PB.

Detection of carbonyls in oxidized BSA

Making oxidized BSA

BSA (10 mg/ ml) was dissolved in oxidizing buffer (25 mM Hepes, 25 mM sodium ascorbate, 100 μM $FeCl_3$, pH 7.2) and incubated at 37 °C for 5 h. The sample was then subjected to rapid gel filtration chromatography using G-25 resin in HE buffer (50 mM Hepes, 1 mM EDTA, pH 7.2). The oxidized BSA thus obtained was stored as aliquots at -50 °C until use.

Detecting oxidized BSA by CH—SDS PAGE analysis

Oxidized BSA or unmodified BSA in HE buffer (final concentration 2 μg/μL) was allowed to react with CH (final concentration 300 μM) for 4.5 h at room temperature. The samples were then subjected to SDS PAGE analysis. The gel was first imaged under long wavelength UV to observe the fluorophore-conjugated protein and then Coomassie-stained. Densitometric analysis was performed using ImageJ (NIH). The graph represents the fluorescence signal of the BSA band normalized to the Coomassie signal, i.e., fluorescent band intensity due to CH-ligated BSA divided by the intensity of the Coomassie-stained band.

Standard curve for carbonyl detection in oxidized BSA

Making reduced BSA

Reduced BSA was prepared as described elsewhere [34], with minor modifications. Briefly, BSA in PBS (10 mM PB pH 7.4, 0.9% sodium chloride) was incubated with sodium borohydride (BSA: sodium borohydride = 1:2) for 40 min at room temperature. The protein solution was then subjected to gel filtration with G-25 resin in HE buffer. The reduced BSA thus obtained was stored as aliquots at −50 °C until use.

Standard curve

Oxidized BSA or reduced BSA (final concentration: 2.5 μg/μL) was incubated with 400 μM CH for 4.5 h at room temperature. The protein solutions were subjected to gel filtration chromatography using G-25 in HE buffer and their concentration was determined using a BCA assay kit (Thermo Scientific). Various proportions (0–100%) of protein samples (oxidized BSA+CH and reduced BSA+CH) were mixed together to generate a standard curve. The total protein concentration (0.2 μg/μL) was kept constant in all samples. The samples were excited at 364 nm and the emission was measured at 475 nm.

Detection of biomolecule carbonyls in cell lysate

Detection of protein carbonyls in cell lysate

A549 cells (500,000 cells per well) were grown overnight in a 6-well plate. The cells were then treated with or without 4 mM hydrogen peroxide for 4 h. CH to a final concentration of 20 μM was added to the cells and incubated at 37 °C for 2 h. The medium was discarded and the cells were washed twice with PBS. The cells were then lysed in PS buffer (10 mM PB, pH 7, 1% SDS) and subjected to SDS PAGE analysis. The gel was first imaged under long wavelength UV to observe fluorophore-conjugated proteins and then Coomassie-stained.

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