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Dihydrorhodamine 123 is superior to 2,7-dichlorodihydrofluorescein diacetate and dihydrorhodamine 6G in detecting intracellular hydrogen peroxide in tumor cells

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Abstract

Dihydrorhodamine 123 (DHR 123), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), and dihydrorhodamine 6G (DHR 6G) were evaluated as probes for detecting cellular hydrogen peroxide levels in SPC-A-1 lung adenocarcinoma cells. Imaging techniques and fluorescence-activated cell scan were used in the study of the probe responses. Obvious green fluorescence was established after a 25-min exposure. After staining with MitoTracker Orange CM-H2TMRos (a probe for mitochondria) and the abovementioned probes simultaneously, only the DHR 123 and DHR 6G groups exhibited legible green fluorescence in the mitochondrial regions. Furthermore, the DHR 6G group exhibited weaker fluorescence intensity. When 100 μM H_2O_2 was added to SPC-A-1 cells loaded with these probes, the intracellular fluorescence increased rapidly and significantly. Our results suggest that DHR 123 is superior for the instantaneous detection of cellular hydrogen peroxide in SPC-A-1 cells.
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Keywords: Dihydrorhodamine 123; 2,7-Dichlorodihydrofluorescein diacetate; Dihydrorhodamine 6G; Hydrogen peroxide

1. Introduction

Cell injury and carcinogenesis from oxidative stress have been implicated in diverse trauma and tumor disease. However, intracellular reactive oxygen species (ROS) may also function as secondary messengers in pathways, such as the Akt and Bcl-2 pathways (Hu et al., 2005). Our previous research also indicated that the elimination of intracellular ROS inhibits tumor cell proliferation (Lu et al., 2006). Thus, it is necessary to evaluate the cellular ROS levels exactly and also maintain them at a normal level. As one of the primary ROS in tumor cells, the detection of cellular $H_2O_2$by dihydrorhodamine 123 (DHR 123), 2,7-dichlorodihydrofluorescein (DCFH-DA) or dihydrorhodamine 6G (DHR 6G) has gained broad popularity (Royall and Ischiropoulos, 1993; Carter et al., 1994; Keston and Brandt, 1965; Ou and Huang, 2006; Edman and Rigler, 2000). These probes can easily penetrate the cellular membrane and be oxidized to green fluorescent substances that are non-permeant (Bass et al., 1983). Generally, DHR 123, DCFH-DA and DHR 6G can be oxidized to rhodamine 123 (Rh 123), 2,7-dichlorofluorescein (DCF-DA), and rhodamine 6G (Rh 6G), respectively (Fig. 1, intracellular esterases hydrolyze the acetate groups of DCFH-DA to DCFH before oxidation).

We earlier confirmed that cellular superoxide anions originate from mitochondria in SPC-A-1 cells, by tracing cells with a superoxide anions probe and a mitochondria probe simultaneously (Lu et al., 2006; Hennet et al., 1993). We also speculated that the mitochondria would be the likely source of H_2O_2 in SPC-A-1 cells. To confirm this, cells were stained with H_2O_2 probe and mitochondria probe simultaneously, and showed considerable differences among the characterizations of the three H_2O_2 probes. Cellular H_2O_2 was traced using these probes, and the laser confocal scanning microscope (LCSM) technique was used to analyze the kinetics of H_2O_2

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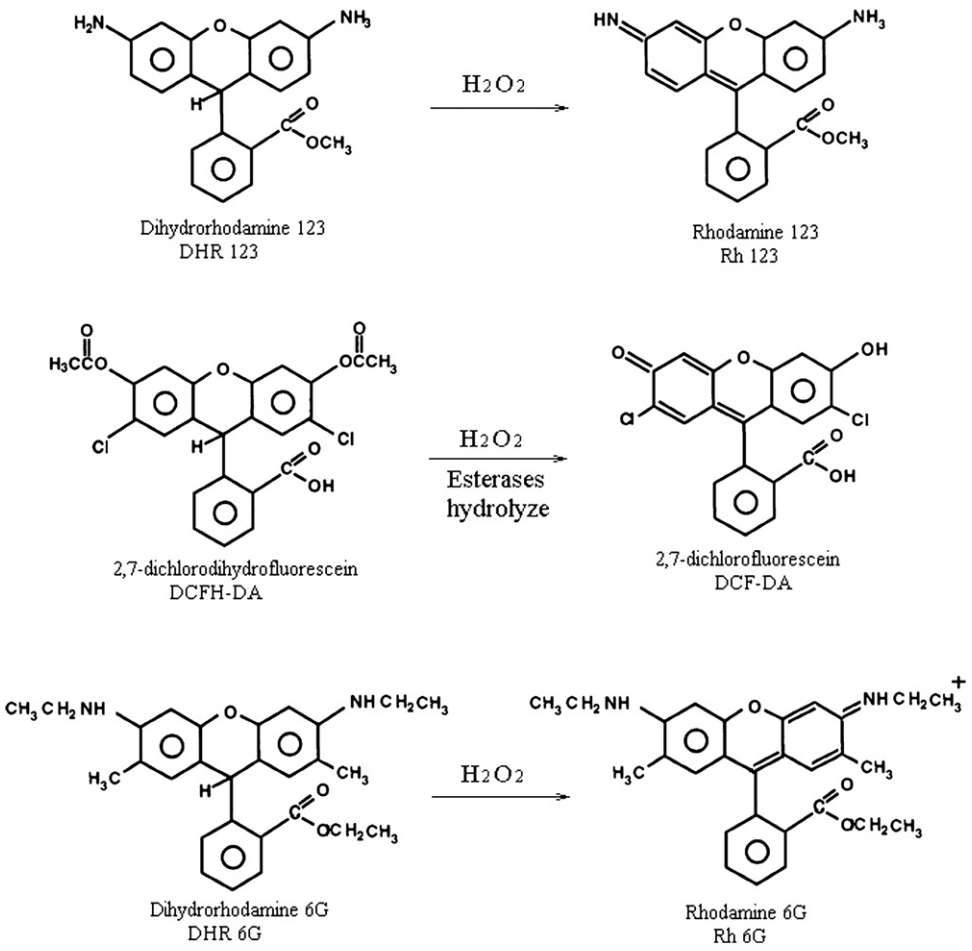

Fig. 1. Structures of three oxidant-sensitive fluorescent probes for H_2O_2. Reduced (non-fluorescent) and oxidized (fluorescent) structures of three fluorescent probes: dihydrorhodamine 123, 2,7-dichlorodihydrofluorescein diacetate and dihydrorhodamine 6G.

fluctuation and its subcellular distribution, aiming to find a superior probe for H_2O_2 in tumor cells.

2. Materials and methods

2.1. Reagents

Dihydrorhodamine 123 (#D632), 2,7-dichlorodihydrofluorescein diacetate (#D399), dihydrorhodamine 6G (#D633), and MitoTracker Orange CM-H2TMRos (#M7511) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). The SPC-A-1 lung adenocarcinoma cell line is available at our institute, cultured in RPMI 1640 medium, containing 10% fetal calf serum. Cells were maintained in a CO_2 cell culture incubator (Forma Scientific, 3154 CO_2 Water-Jacketed Incubator, USA) at 37 °C under 5% CO_2. Before every experiment, the cells were plated on glass coverslips in a 6-well plate until 70–80% confluent. The experiments were repeated at least three times.

2.2. Detection of probe sensitivity by fluorescence spectrophotometry

To detect the sensitivity of DHR 123, DCFH-DA, and DHR 6G, the fluorescence intensity of probe-loaded tumor cells was determinated by using a fluorescence spectrophotometer. Briefly, the prepared SPC-A-1 cells were washed three times with serum-free RPMI 1640 medium and incubated with 20 μM DHR 123, or 5 μM DCF-DA, or 10 μM DHR 6G (these concentrations are relatively in excess for intracellular H_2O_2, and will not harm the cell activity) at 37 °C for 25 min. The cells were then washed three times with PBS at 37 °C. Cells (5×10^6) were collected, and lysed with 0.1% SDS. The fluorescence intensity was analyzed by a fluorescence spectrophotometer (Hitachi, 850, Japan). Excitation was set at 488 nm and emission was at 543 nm.

2.3. Detection of probe distribution by laser confocal scanning microscope

To determine the intracellular distribution of the probes in tumor cells, their subcellular localization was processed by LCSM. Based on the findings of Lu et al. (2006), we speculated that the mitochondria would be the likely source of H_2O_2 in SPC-A-1 cells, indicating that these three probes should localize on mitochondria. In the study, the mitochondria district was visualized by CM-H2TMRos, which is a new red fluorescent probe developed recently that is more photostable and stable in mitochondria (Poot et al., 1996). Briefly, the prepared SPC-A-1 cells were washed three times with serum-free RPMI 1640 medium and incubated with PBS containing DHR 123 (or DCF-DA, or DHR 6G) and 0.5 μM CM-H2TMRos, at 37 °C for 25 min. The cells were then washed three times with PBS. The coverslips with the dye-loaded cells were removed and attached to the coverslip clamp chamber for the probe imaging analysis. Fluorescence measurements were made on an LCSM (Zeiss LSM510, Germany). The excitation and emission wavelengths of the three H_2O_2 probes were 488 and 543 nm, respectively; the excitation and emission wavelengths of CM-H2TMRos were 543 and 610 nm, respectively.

2.4. Kinetic measurement of intracellular H_2O_2 concentration

To monitor the changes of H_2O_2 in single cells undergoing extracellular H_2O_2 stimuli, the prepared SPC-A-1 cells on glass coverslips were loaded

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