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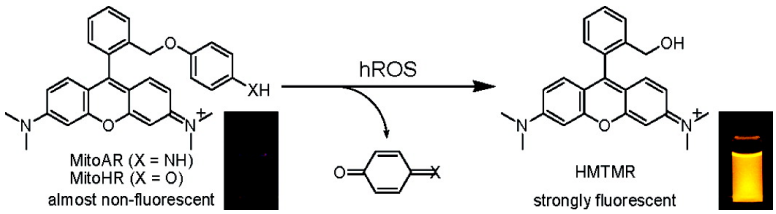

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Design and Synthesis of Fluorescent Probes for Selective Detection of Highly Reactive Oxygen Species in Mitochondria of Living Cells

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Reactive oxygen species (ROS) are important mediators in a variety of biological and pathological events;[1−6] for example, ROS generated in mitochondria play important roles in the modulation of signal transduction cascades and transcription factors. However, oxidative stress due to excessive generation of ROS is implicated in many human diseases, including acute and chronic inflammatory processes, ischemic stroke, diabetes, sepsis, ischemia-reperfusion injury, atherosclerosis, and neurodegenerative disorders.

Mitochondria are believed to be the major source of intracellular reactive oxygen species.[7] Superoxide anion ($O_2^{-\bullet}$) is first generated in the respiratory chain and can be converted to H_2O_2 in the intermembrane space. Even though $O_2^{-\bullet}$ and H_2O_2 are not strong oxidants, they are precursors of highly reactive oxygen species (hROS; for example, hydroxyl radical (•OH), peroxynitrite ($ONOO^-$), hypochlorite (OCl^-)). In the presence of transitional metal complexes, for example, iron or copper complexes, which are rich in the mitochondrial inner membrane, H_2O_2 can be converted to the highly reactive hydroxyl radical (•OH) via Fenton reaction.[8] $O_2^{-\bullet}$ can react with another free radical, nitric oxide (•NO), formed by mitochondrial nitric oxide synthase, to generate highly reactive peroxynitrite ($ONOO^-$).[9] hROS are strong oxidants that can directly oxidize DNA-duplex, proteins, and lipids. Despite their importance, however, the precise roles of mitochondrial hROS are still elusive.

One reason for this is that no method is yet available for direct detection of hROS selectively in mitochondria. Dihydrorhodamine has been widely used in conjunction with fluorescence microscopy to detect ROS. In general, a positive charge facilitates movement across phospholipid bilayers and accumulation into the mitochondrial matrix in response to the negative membrane potential, but dihydrorhodamine is not cationic before oxidation, and so would not be localized preferentially in mitochondria. It is therefore unsuitable for selective detection of ROS generation in mitochondria. Furthermore, dihydrorhodamine is extensively autoxidized, resulting in a marked increase of the fluorescence intensity. Therefore, there is a need to develop novel mitochondria-selective probes for hROS.

For this purpose, we chose rhodamine dye as a fluorophore, since it is already used as a probe for mitochondria.[10] Rhodamine dye has a positive charge, as well as many other favorable characteristics for biological applications, including high water solubility, high fluorescence intensity, long excitation and emission wavelengths (>500 nm), pH independent fluorescence, and tolerance to photobleaching. In short, it has excellent photochemical properties and is suitable for fluorescence microscopy studies.

Our group previously reported that 4-aminophenyl aryl ether and 4-hydroxylphenyl aryl ether react with hROS selectively over other ROS.[11] 4-Amino- or 4-hydroxylphenyl ether moieties are more electron-rich than the xanthene moiety and we considered that they could be used to regulate the fluorescence of rhodamine through photoinduced electron transfer (PeT).[12,13] To facilitate the PeT process, we designed molecules in which the electron donor (4-amino- or 4-hydroxyphenyl ether) was placed at the 2-position of the phenyl moiety of rhodamine, thereby being fixed in close proximity to the xanthene structure. Consequently the fluorescence of the dye should be efficiently quenched. Reaction with hROS should cause cleavage of the ether moiety (Scheme 1), affording HMTMR, which is highly fluorescent in an aqueous environment. Thus, we synthesized the two novel fluorescent probes MitoAR and MitoHR, which were expected to be selectively localized in mitochondria, and to react selectively with hROS.

Scheme 1. The Design of MitoAR and MitoHR

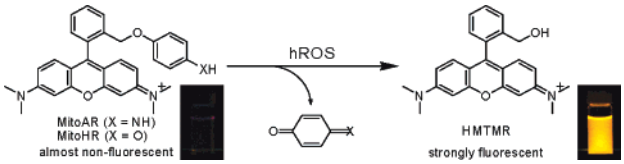

Figure 1 shows the reactivity of MitoAR and MitoHR with various ROS. When MitoAR and MitoHR reacted with hROS (•OH, $ONOO^-$, OCl^-), a large and immediate increase of fluorescence intensity was observed. In the case of MitoAR, the fluorescence intensities reached the maximum within a few seconds, whereas the reactions with other reactive oxygen species ($O_2^{-\bullet}$, •NO, H_2O_2) led to almost no fluorescence increase even after 30 min. Thus, MitoAR and MitoHR appear to be a highly selective for the detection of hROS. In addition, we examined the fluorescence response of MitoAR with enzymatic systems. In an HRP/H_2O_2

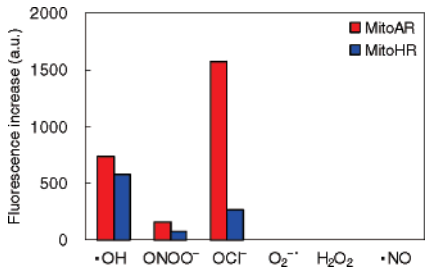

Figure 1. Reactivity of MitoAR and MitoHR with various ROS. MitoAR and MitoHR (final 10 μM, 0.1% DMF as a cosolvent) were dissolved in sodium phosphate buffer (0.1 M, pH 7.4). The figure shows the increase of fluorescence intensity, measured at 574 nm with excitation at 553 nm, in the presence of various ROS generating systems. •OH: Ferrous perchlorate (300 μM) and H_2O_2 (1 mM) were added at room temperature. $ONOO^-$: $ONOO^-$ (final 5 μM) was added and the mixture was stirred at 37 °C. OCl^-: NaOCl (final 5 μM) was added and the mixture was stirred at 37 °C. $O_2^{-\bullet}$: KO_2 (100 μM) was added and the mixture was stirred at 37 °C for 30 min. H_2O_2: H_2O_2 (100 μM) was added and the mixture was stirred at 37 °C for 30 min. •NO: NOC13 (5 μM) was added and the mixture was stirred at 37 °C for 30 min.

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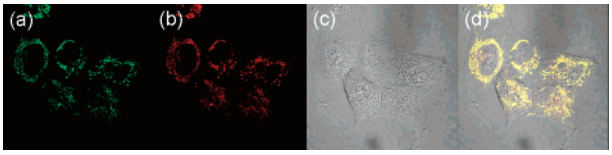

Figure 2. Localization of MitoAR in mitochondria in HeLa cells. MitoAR (1 μM) and MitoTracker Green FM (250 nM) were loaded into HeLa cells: (a) fluorescence image of MitoTracker Green FM; (b) fluorescence image of MitoAR; (c) brightfield image; (d) merged image.

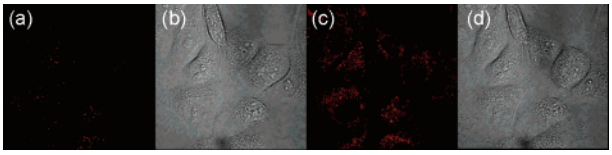

Figure 3. hROS detection by MitoAR in living cells: (a, b) fluorescence and brightfield images of HeLa cells loaded with MitoAR (1 μM) for 15 min; (c, d) after addition of NaOCl (10 μM).

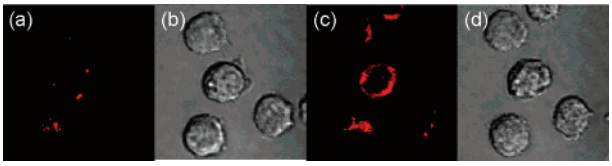

Figure 4. Detection of hROS generation in mitochondria: (a, b) fluorescence and brightfield images of HL-60 cells loaded with MitoAR (1 μM) for 15 min. (c, d) 10 min after addition of H_2O_2 (100 μM).

system, addition of H_2O_2 (5 μM) to MitoAR (10 μM) and horseradish peroxidase (0.2 μM) at 37 °C produced a 7.6-fold increase of fluorescence, while in an MPO/Cl^-/H_2O_2 system, which generates OCl^-, addition of H_2O_2 (5 μM) into MitoAR (10 μM), myeloperoxidase (11.2 nM) and NaCl (150 mM) at 37 °C caused a 10.4-fold increase of the fluorescence intensity. In an $O_2^{-\bullet}$ generating X/XO system, addition of xanthine (10 μM) to MitoAR (10 μM) and xanthine oxidase (1.73 m/mL) at 37 °C for 30 min produced a much smaller fluorescence increase (about 1.1-fold). Under these conditions, the amount of generated $O_2^{-\bullet}$ was 6.0 μM, as determined from the redox reaction of cytochrome *c*.

We next used confocal microscopy to assess the localization of MitoAR and its ability to sense hROS in HeLa cells. We selected MitoAR for this purpose because it has higher reactivity for hROS and better photochemical properties than MitoHR. First, we co-stained HeLa cells with MitoAR and MitoTracker Green FM, a mitochondrial stain (Figure 2). MitoAR is almost, but not completely, nonfluorescent and could be detected with the microscope. The fluorescence images of the two dyes were well merged, which indicates that MitoAR is localized selectively in mitochondria. Next, NaOCl was added to MitoAR-loaded HeLa cells to examine whether MitoAR could detect hROS in living cells. The addition of 10 μM NaOCl resulted in a large increase of fluorescence (Figure 3). Compared with Figure 2b, there appears to be some diffusion of dye out of the mitochondria after reaction with NaOCl. This could be due to a loss of mitochondrial membrane potential after NaOCl treatment, allowing a small part of the resulting fluorescent product to leak out. These results showed that MitoAR has the ability to detect hROS in mitochondria.

With these results in hand, we examined hROS generation inside the cells. When we stimulated MitoAR-loaded HeLa cells with H_2O_2, which does not react with MitoAR at all, no fluorescent increase was observed even at the concentration of 1 mM (see Supporting Information). However, in the case of MitoAR-loaded HL-60 cells stimulated with 100 μM H_2O_2, the fluorescence intensity increased gradually and strong fluorescence was seen after 10 min (Figure 4). HL-60 cells, but not HeLa cells, contain myeloperoxidase in azurophilic granules, and H_2O_2 activates myeloperoxidase to produce HOCl, which is generated outside the mitochondria. This is presumably the reason why only HL-60 cells showed a large fluorescence increase. We also examined the sensitivity of MitoAR by using various concentrations of H_2O_2, and found a 1.2-fold enhancement of fluorescence intensity in HL-60 cells even after adding only 10 μM H_2O_2 (see Supporting Information). Thus, different cell types appear to generate different levels of hROS in response to H_2O_2-induced stress, and MitoAR can detect this with high sensitivity.

In summary, we have designed and synthesized a fluorescent probe, MitoAR, for the selective detection of hROS generation in mitochondria in real time. MitoAR has excellent properties for biological applications, including tolerance to autoxidation and photobleaching during laser irradiation for fluorescence microscopy. We confirmed that MitoAR can sensitively and selectively detect mitochondrial hROS generation in living cells. This probe is expected to be a useful tool for a range of biological and pathological investigations.

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Supporting Information Available: Synthesis, HPLC charts, experimental details and characterization of MitoAR and MitoHR, and experiments using living cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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