Modulation of Subcellular Redox Homeostasis with Trialkylphosphines

Jade Nguyen*

SCS-DSM Award for best oral presentation in Chemical Biology

Abstract: Redox homeostasis is essential for cell function and its disruption is associated with multiple pathologies. Redox balance is largely regulated by the relative concentrations of reduced (GSH) and oxidized (GSSG) glutathione. In eukaryotic cells, this ratio is different in each cell compartment. There is a lack of chemical probes able to modulate GSH/GSSG to study the impact of redox stress in an organelle specific manner. Here, we highlight the importance of trialkylphosphines to induce reductive stress and how it can be targeted to a specific organelle. Our probe is selectively activated by endogenous nitroreductases, and releases tributylphosphine to trigger redox stress in mitochondria. Mechanistic studies revealed that the induced stress activates a cellular response orchestrated by transcription factor ATF4, which upregulates genes involved in glutathione catabolism.

Keywords: Glutathione · Mitochondria · Redox homeostasis · Reductive stress · Trialkylphosphine

1. Introduction

1.1 Subcellular Redox Homeostasis

Redox homeostasis is the state of cellular health that is maintained by the adjustment of redox species and is controlled at the subcellular level. Each cellular compartment has a specific redox environment, matching the need for the specific organelle’s functions. Disruption of this redox homeostasis impairs the organelles and can lead to diseases. It is thus essential to study the underlying principles of redox stress responses in order to develop new therapeutic targets.

1.2 Role of Glutathione

Intracellular redox balance is primarily regulated by the relative concentrations of glutathione (GSH) and its oxidized, disulfide-bonded dimer (GSSG). Multiple physiological processes, ranging from cell signaling to protein folding, depend on redox homeostasis. Being able to modulate the GSH/GSSG ratio in an organelle-specific manner would allow us to investigate the relationship between cellular redox stress responses and the spatial origin of the imbalance, enriching our understanding of subcellular compartmentalization of redox signaling. Consequently, several pathological conditions such as cancer, diabetes and neurodegenerative diseases have been associated with redox imbalance. In eukaryotes, this homeostasis is controlled at the level of subcellular compartments and each organelle possesses its own redox environment.

1.3 Phosphines as Reducing Agents

We envisioned that the GSH/GSSG ratio could be manipulated by direct reduction of the disulfide bond in GSSG. This disulfide can be efficiently and rapidly reduced to GSH by trialkylphosphine derivatives, which have no appreciable reactivity against other amino acids.

1.4 Mitochondria Targeting

Mitochondria perform multiple essential tasks in the cell that depend on redox modulation. Disruption of this homeostasis leads to pathologies such as insulin resistance, obesity, and type II diabetes. We hypothesized that we could achieve mitochondria-specific reductive stress by taking advantage of the activity of enzymes that are present only in these organelles to trigger the release of tributylphosphine from a masked precursor (Scheme 1). Here, we report the development of such a probe, its validation in live human cells, and its application to characterize the cellular response to mitochondrial reductive stress.

*Correspondence: J. Nguyen, E-mail: jade.nguyen@epfl.ch, Laboratory of Chemical and Biological Probes, EPFL, Station 6, CH-1015 Lausanne
2. Results and Discussion

2.1 Activation in Live Cells

Upon enzymatic reaction by mitochondrial nitroreductases (NTRs), the strong electron-withdrawing nitro group of the non-fluorescent probe 1 is converted to the corresponding amine intermediate (not observed). This newly formed electron-rich substituent can donate electron density to the π-conjugated system, cleaving the weak C–P bond to release the reducing agent PBu3, and the fluorescent reporter 2 (Scheme 1). Probe 3, essentially lacking the phosphine moiety, is likewise converted to 2 and used as a control probe to verify enzymatic activation by NTRs and fluorescence distribution without releasing the reducing agent PBu3.

Due to the presence of NTRs in mitochondria, we expected probes 1 and 3 to be exclusively activated in this organelle to yield the fluorescent product 2 and reducing agent PBu3. Confocal microscopy experiments employing HEK293 cells revealed that both probes 1 and 3 are activated in live cells under normoxic conditions (Fig. 1A). A timelapse experiment shows activation of probe 1 over 30 min with significant fluorescence from reporter 2 already in the first 5 min of incubation, confirming cellular entry of 1 and activation by NTR.

Owing to the different cellular uptake of probes 1 and 3, we performed titration experiments (not shown) to estimate which incubation conditions produced similar intracellular concentrations of dye 2, the enzymatic product of both probes 1 and 3. We determined that extracellular concentrations of 15 μM and 5 μM, for probe 1 and 3 respectively, resulted in similar intracellular concentrations of dye 2. Therefore, these values were used in all further cell experiments.

To confirm that the fluorescence signal from reporter 2 is indeed located in mitochondria, we conducted colocalization experiments of the activation of probes 1 and 3 with the small-molecule mitochondrial stain MitoTracker™ deep red FM (MDR, Fig. 2).

2.2 Redox States – GSH levels

To assess the redox states in mitochondria, we developed a mitochondria-targeted small-molecule ratiometric GSH sensor based on a reported cytosolic probe (Fig. 3A). The accumulation of the sensor in mitochondria is achieved by modification of the reported cytosolic probe3 with a cationic triphenyl phosphine moiety.

GSH reacts with the small-molecule sensor via Michael addition, which blueshifts its green fluorescence. The probe is ratiometric by excitation and emission and an increase in the blue/green ratio indicates a shift toward a more reduced state. The addition of GSH is reversible and therefore directly correlates to the amounts of GSH present.

We observed that cells treated with probe 1 for 1 h displayed a significantly increased mitochondrial blue/green ratio compared to control probe 3, indicative of a change toward a more reduced state (Fig. 3B). When cells were treated with 1 for a longer time (1.5 h), this effect faded, suggesting that redox homeostasis had been restored. After an even longer incubation (not shown), it led to oxidative stress. We next aimed to understand how a reducing agent can cause oxidative stress. Previous studies have reported...
that an increase in GSH may lead to oxidative stress through the accumulation of intracellular superoxide ($O_2^-$),[10] which would explain our observations.

### 2.3 Redox States – Superoxide Levels

We used a derivative of the reported HK-SOX-1 fluorescent indicator of intracellular $O_2^-$ to test if probe 1 could indeed cause the accumulation of superoxide.[9] This fluorescein-based superoxide sensor is a fluorogenic molecule that displays green fluorescence only upon reaction with superoxide, which reacts with the fluorescence-masking triflate moieties to yield a fluorescent reporter (Fig. 4A).

Superoxide formation was monitored in HEK293 cells previously treated with probes 1 or 3. We employed Antimycin A, an inhibitor of complex III that induces mitochondrial $O_2^-$ production,[11] as a positive control, and DMSO as a negative control (Fig. 4B). We observed that the cells treated with 1 displayed significantly higher $O_2^-$ accumulation than cells treated with probe 3 (Fig. 4C). We therefore concluded that PBu$_3$ induces accumulation of cellular superoxide.

PBu$_3$ release should trigger GSH production. To test whether the increase in $O_2^-$ induced by probe 1 depends on the total concentration of GSH, total glutathione levels were lowered by inhibiting the enzyme involved in the rate-limiting step of the GSH biosynthesis using buthionine sulfoximine (BSO).[12]

In cells treated with both BSO and probe 1, a significant decrease in $O_2^-$ levels was observed compared to cells treated only with probe 1 (Fig. 5). In contrast, BSO did not affect the levels of $O_2^-$ in cells treated with either control probe 3 or DMSO. This observation supports the hypothesis that the observed oxidative stress induced by probe 1 results from $O_2^-$ accumulation mediated by excess GSH.

### 2.4 Integrated Stress Response

We next wanted to understand further how the cell reacts to PBu$_3$-induced mitochondrial redox stress. Therefore, we performed global mRNA sequencing and differential gene expression analysis from cells previously treated with 1, 3 and DMSO for 2 h. We compared the differences in expression levels between probe 1 vs. DMSO and probe 3 vs. DMSO. The results of the mRNA sequencing are shown as volcano plots (Fig. 6A,B), with each dot representing a single gene. The significantly upregulated and downregulated genes are depicted in blue and gray, respectively.

This study revealed that cells treated with probe 1 displayed significant upregulation of several genes, particularly γ-glutamyl cyclotransferase (CHAC1), compared to DMSO-treated cells. CHAC1 regulates redox homeostasis through degradation of GSH (Fig. 6C),[13] suggesting that the cell responds to redox stress by promoting the depletion of excess GSH produced by tributylphosphine.
3. Conclusions

Diphenylphosphine is known to reduce GSSG to GSH rapidly and selectively, even over other disulfide bonds in proteins. When activated in mitochondria, however, it induces oxidative stress through the accumulation of O$_2^\cdot$. These increased levels of superoxide are positively correlated with the total GSH present in the cell, supporting previous observations of GSH-mediated O$_2^\cdot$ accumulation.[10] This redox imbalance activates the ATF4-ATF3-CHOP cascade, which upregulates the CHAC1 gene (Scheme 2). These observations are consistent with reports of mitochondrial oxidation triggered by glutathione-dependent reductive stress[10] and activation of ATF4 by O$_2^\cdot$.[12]

Our results indicate that trialkylphosphines, a broad family of compounds that have been largely neglected in chemical biology, can expand the chemical space of small molecules that are used to modulate redox biology, with potential impact in the development of new therapies.

For example, CHAC1 upregulation has been reported to deplete GSH levels in triple-negative breast cancer cells, making them more susceptible to necroptosis and ferroptosis during cysteine starvation.[14]

In this work, we demonstrated that even though trialkylphosphines are highly reducing and often water-insoluble compounds, they can be transformed into chemical probes for biological use by developing strategies to tune their reactivity, mask their reducing power, target them to specific organelles, and release them selectively.

Acknowledgements

We thank the Scientific Center for Optical and Electron Microscopy (ScopeM) at ETHZ and the Bioimaging and Optics Platform (BIOP) of EPFL for access to confocal microscopes. mRNA library generation, sequencing, and preliminary bioinformatic analyses were carried out at the Functional Genomics Center Zurich. We thank Prof. Dr. Pablo Rivera-Fuentes for his guidance and Dr. Alina Tirla for the design of probe 1 and valuable discussions. This work was funded by the Swiss National Science Foundation through grants 200021_165551 and PCEGP2_186862.

Received: January 31, 2022

---


License and Terms

This is an Open Access article under the terms of the Creative Commons Attribution License CC BY 4.0. The material may not be used for commercial purposes.

The license is subject to the CHIMIA terms and conditions: (https://chimia.ch/chimia/about).

The definitive version of this article is the electronic one that can be found at https://doi.org/10.2533/chimia.2022.308