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Ratiometric fluorescence imaging of Golgi H₂O₂ reveals a correlation between Golgi oxidative stress and hypertension†

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Golgi oxidative stress is significantly associated with the occurrence and progression of hypertension. Notably, the concentration of hydrogen peroxide (H₂O₂) is directly proportional to the degree of Golgi oxidative stress. Therefore, based on a novel Golgi-targeting phenylsulfonamide group, we developed a two-photon (TP) fluorescent probe, Np-Golgi, for *in situ* H₂O₂ ratiometric imaging in living systems. The phenylsulfonamide moiety effectively assists Np-Golgi in the precise location of Golgi apparatus. In addition, the raw material of phenylsulfonamide is easily available, and chemical modification is easily implemented. By application of Np-Golgi, we explored the generation of H₂O₂ during Golgi oxidative stress, and also successfully revealed increases on the levels of Golgi H₂O₂ in the kidneys of mice with hypertension. This work provides an ideal tool to monitor Golgi oxidative stress for the first time and novel drug targets for the future treatment of hypertension.

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Introduction

Hypertension is a major risk factor for the development of cardiovascular disease (CVD).¹ It is predicted that the prevalence of hypertension will increase by more than 50% during the next 30 years.^{2,3} Until now, despite receiving antihypertensive treatment, many people with the disorder still cannot adequately control their blood pressure. Thus, novel therapies are urgently needed to address resistant hypertension. A large amount of studies suggest that oxidative stress plays a central role in the pathogenesis of hypertension by perturbing the balance between reactive oxygen species (ROS) and antioxidant defenses.^{4–7} Excess ROS promote hypertension by inducing endothelial dysfunction.^{8,9} Therefore, complete understanding of the mechanisms of oxidative stress could contribute to the development of new therapies.

Since the Golgi complex acts as a key trafficking and sorting station and a vital biosynthetic centre for glycoproteins and lipids,¹⁰ Golgi oxidative stress plays both physiological and pathophysiological roles in cells along with extensive ROS production.^{11–13} Therefore, quantitative detection of various

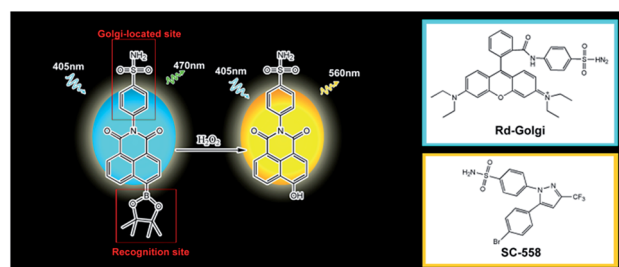
ROS is essential to study the mechanism of Golgi oxidative stress, especially hydrogen peroxide (H₂O₂),^{14,15} an indicator of oxidative stress. However, the concentration and generation of H₂O₂ in the Golgi complex remain poorly understood to date, which is mainly due to a lack of tools for specific measurement of Golgi-located H₂O₂ *in situ*. This ultimately causes difficulties in revealing the direct relevance between H₂O₂ levels and hypertension.

Two-photon (TP) fluorescence imaging is a noninvasive approach for *in situ* detection of various biomolecules.^{16–19} It exhibits increased tissue penetration depth, higher temporal resolution and less specimen photodamage than one-photon fluorescence imaging.²⁰ To date, many TP fluorescent probes have been developed to visualize H₂O₂ in various organelles in cells,^{21–25} but *in situ* bioimaging of Golgi H₂O₂ is still scarce. Developing a TP fluorescent probe for tracing Golgi H₂O₂ in living systems could contribute to defining the relationship between hypertension and Golgi oxidative stress. However, the

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Scheme 1 The structure and response mechanism of Np-Golgi.



inconspicuous cytotoxicity at concentrations less than 20 μM (Fig. S6–S8[†]), which demonstrated that Np-Golgi could be used in living systems. We next applied Np-Golgi for endogenous H_2O_2 detection in human hepatoma cells (SMMC7721). Phorbol 12-myristate 13-acetate (PMA, 1 $\mu\text{g mL}^{-1}$) was used to induce generation of H_2O_2 in cells. The fluorescence of Np-Golgi in the green channel (530–580 nm) increased significantly upon the addition of PMA, while the fluorescence in the blue channel (430–480 nm) was constant. The ratio of $F_{\text{green}}/F_{\text{blue}}$ in PMA-treated cells showed 1.5-fold enhancement than in control cells. To confirm that the fluorescence enhancement was caused by increased H_2O_2 concentrations, we also evaluated the FI ratio $F_{\text{green}}/F_{\text{blue}}$ of Np-Golgi in *N*-acetylcysteine³⁵ (NAC: H_2O_2 eliminator) incubated cells. As shown in Fig. S9,[†] both NAC-stimulated cells and PMA + NAC-stimulated cells presented negligible fluorescence in the green channel with lower $F_{\text{green}}/F_{\text{blue}}$ ratios than control cells. This phenomenon demonstrates that intracellular H_2O_2 has been effectively eliminated. These observations validate that Np-Golgi can be used for fluorescence imaging of H_2O_2 in cells with high selectivity and good sensitivity to quantify the concentrations of H_2O_2 . Next, we explored the intracellular photostability of Np-Golgi (Fig. S10[†]). The fluorescence of Np-Golgi was consistent for 60 min after cells were rinsed thoroughly with PBS. The result implies that Np-Golgi can be applied for long-term imaging of H_2O_2 *in vivo*.³⁶

Subcellular localization of Np-Golgi

To verify the Golgi complex-targeting ability of Np-Golgi, a colocalization experiment was performed to observe the subcellular distribution of the probe. As shown in Fig. 2, the green fluorescence of Np-Golgi overlapped well with the fluorescence of the commercial probe Golgi-Tracker Red (Pearson's colocalization coefficient: 0.94) in human cervical carcinoma cells (HeLa). In the meantime, the fluorescence of Np-Golgi showed poor overlap with that of Mito-Tracker Red (0.10), Lyso-Tracker Red (0.54) and ER-Tracker Red (0.44). To further confirm that Np-Golgi possessed universal Golgi-targeting

capability, colocalization experiments were also performed in SMMC7721 and human hepatic cells (HL-7702). As expected, the Np-Golgi probe also showed excellent Golgi-targeting characteristics in these cells (Fig. S11 and S12[†]). Collectively, Np-Golgi exhibits superior Golgi complex-locating capability in various cell types.

Subsequently, to validate that the targetability of the Np-Golgi to the Golgi complex was dependent on the phenylsulfonamide moiety, we synthesized a new phenylsulfonamide-modified fluorescent probe Rd-Golgi (Fig. S13[†]). Then the Golgi-targeting ability of Rd-Golgi was investigated. Rd-Golgi and Golgi-Tracker Red presented remarkable fluorescence overlap in cells with the Pearson's colocalization coefficient 0.95 (Fig. S14[†]). This result suggests that Rd-Golgi possesses Golgi-targeting ability and phenylsulfonamide is the crucial part of the probe for effective targeting of the Golgi apparatus. These outcomes establish that phenylsulfonamide is a powerful and universal Golgi-targeting group. In addition, compared with the reported Golgi-targeting peptide and the chemical group cysteine, phenylsulfonamide exhibits the following benefits: (1) the raw material is easily available; (2) chemical modification is simple; and (3) long-term accumulation in the Golgi is possible.

The oxidative stress of Golgi apparatus

We next applied the H_2O_2 -responsive, excellently Golgi-targeting TP fluorescent probe Np-Golgi to imaging of the fluctuations in intracellular H_2O_2 associated with Golgi oxidative stress. Monensin^{37,38} was used to induce Golgi oxidative stress by disturbing pH homeostasis to reduce the activity of Golgi proteins. Under NAC-pretreated conditions, monensin-stimulated cells exhibited brighter green fluorescence than NAC-incubated cells, and the F_{560}/F_{470} ratio was enhanced by



Fig. 2 Co-localization cell imaging of Np-Golgi and commercial dyes including Golgi-Red (a–d), Mito-Red (e–h), Lyso-Red (i–l), ER-Red (m–p) in HeLa cells.

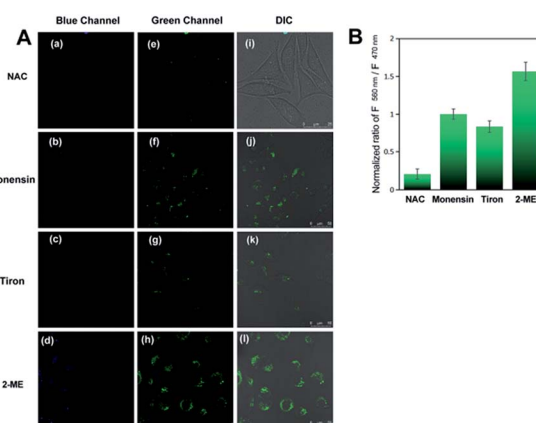


Fig. 3 (A) Fluorescence imaging of Np-Golgi after cells were treated with different stimulants. (a, e and i) NAC cells: The cells were incubated with NAC (20 mM). (b, f and j) Monensin cells: The cells were incubated with NAC and then monensin (10 μM) was added. (c, g and k) Tiron cells: the cells were incubated with NAC, and then monensin and Tiron (10 μM) were added. (d, h and l) 2-ME cells: the cells were incubated with NAC, and then monensin and 2-ME (1 $\mu\text{g mL}^{-1}$) were added. (B) Relative fluorescence intensity of Np-Golgi labelled cells from images. $n = 3$.



4.9-fold (Fig. 3A). This result shows the level of Golgi H_2O_2 is obviously increased in the Golgi oxidative stress process.

Many studies show that a large proportion of intracellular H_2O_2 is generated from superoxide anions (O_2^-) by catalysis reactions of various oxidases, such as superoxide dismutase (SOD). To seek evidence that Golgi H_2O_2 was produced from O_2^- in the Golgi oxidative stress, the fluorescence of Np-Golgi was detected after incubating cells with O_2^- inducer (2-methoxyestradiol,³⁹ $1.0 \mu\text{g mL}^{-1}$) or O_2^- scavenger (Tiron,⁴⁰ $10 \mu\text{M}$). In order to avoid the interference from cytoplasmic H_2O_2 and induce Golgi oxidative stress of the cells, NAC was used to eliminate the intracellular H_2O_2 at first, and then monensin was added to cause higher level of H_2O_2 . In this case, we investigated the relations between O_2^- and H_2O_2 by using 2-ME and Tiron. As illustrated in Fig. 3, stronger green fluorescence was observed in both 2-methoxyestradiol (2-ME) and monensin co-incubated cells, and the F_{560}/F_{470} ratio was enhanced by 1.57-fold compared with monensin incubated cells. After the addition of Tiron to eliminate O_2^- , the FI of Np-Golgi in Tiron and monensin co-incubated cells decayed with a decreasing F_{560}/F_{470} ratio (by 0.83-fold) compared with monensin incubated cells. The above results indicate that the concentration of Golgi H_2O_2 has positive correlation with O_2^- levels. Collectively, these data are in excellent agreement with our prediction that Golgi H_2O_2 is mainly generated from O_2^- in Golgi oxidative stress.

TP fluorescence imaging of HBP mice *in situ*

To prove that the probe could detect H_2O_2 levels *in vivo*, we applied Np-Golgi for TP fluorescence imaging of H_2O_2 in the kidneys of mice. The animal experiments were performed in compliance with the relevant laws and guidelines issued by the Ethical Committee of Shandong University and were in agreement with the guidelines of the Institutional Animal Care and Use Committee. The probe ($10 \mu\text{M}$, $100 \mu\text{L}$) in the LPS-injected area of the abdominal cavity of the mice showed remarkable fluorescence, and the $F_{\text{red}}/F_{\text{green}}$ ratio was increased by 1.9-fold in the probe-injected area compared with the normal saline-injected area (Fig. S15[†]). This suggests that Np-Golgi could quantitatively monitor fluctuations of H_2O_2 *in vivo* through TP fluorescence imaging.

Whether high blood pressure (HBP) is accompanied by Golgi oxidative stress was further investigated. Mice were injected with either 0.9% saline (control mice) or $50 \mu\text{g}$ ouabain per day (HBP mice) for 20 days, and the blood pressure of the mice was measured three times per day. Eventually, the blood pressure of HBP mice exceeded 160 mmHg (Fig. S16[†]), and the results of Masson's trichrome staining of the kidneys showed that there was more collagen deposition in HBP mice than in control mice (Fig. S17[†]), proving that the HBP mouse model was successfully constructed. After incubation of the kidneys of mice with Np-Golgi ($10 \mu\text{M}$, $100 \mu\text{L}$) for 30 min, the $F_{\text{red}}/F_{\text{green}}$ ratio of the probe was compared between HBP mice and control mice combined with two-photon imaging technology. As shown in Fig. 4, the $F_{\text{red}}/F_{\text{green}}$ ratio in HBP mice was significantly increased (by 1.8-fold) compared with that in control mice, indicating increased H_2O_2 levels in the mice with HBP. These

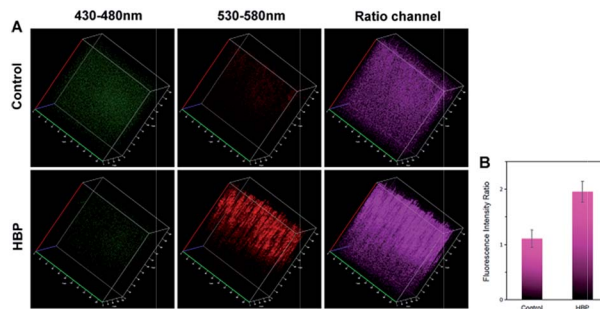


Fig. 4 TP ratiometric fluorescence imaging of kidney tissue in control mice and HBP mice with Np-Golgi. The fluorescence intensity ratio ($R_{\text{red/green}}$) of the kidneys in mice. Two-photon excited wavelength: 810 nm.

data demonstrate that pathological changes in kidney tissue in HBP mice cause severe Golgi oxidative stress and excess H_2O_2 accumulation in the process, which suggests that hypertension is closely related to Golgi oxidative stress.

Conclusion

To discover the role of Golgi oxidative stress in hypertension, we developed a novel TP ratiometric fluorescent sensor, Np-Golgi, to quantitatively detect Golgi H_2O_2 levels *in situ*. By application of Np-Golgi, we successfully detected increased levels of H_2O_2 in the kidneys of mice with hypertension. This work not only reveals the relationship between hypertension and Golgi oxidative stress, but also provides a robust tool for uncovering the connections between Golgi oxidative stress and severe diseases in the future.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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