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## Two-photon fluorescence imaging reveals a Golgi apparatus superoxide anion-mediated hepatic ischaemia-reperfusion signalling pathway†

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Hepatic ischaemia-reperfusion (IR) injury is mainly attributed to a burst of reactive oxygen species (ROS) that attack biological macromolecules and lead to cell death. The superoxide anion ( $O_2^{\cdot-}$ ) is the first ROS to be generated and triggers the production of other ROS; thus, explorations of the role of  $O_2^{\cdot-}$  in the IR process are meaningful. Meanwhile, the Golgi apparatus generates  $O_2^{\cdot-}$  via Golgi-associated proteins, which might play an essential role in IR injury. However, the molecular mechanism by which  $O_2^{\cdot-}$  from the Golgi apparatus regulates hepatic IR injury is unclear. Therefore, to solve this problem, a two-photon (TP) excited fluorescence probe (CCA) was designed and prepared for the reversible detection of  $O_2^{\cdot-}$  in the Golgi apparatus. With the assistance of TP fluorescence microscopy, we observed a substantial increase in the levels of  $O_2^{\cdot-}$  in the Golgi apparatus of an IR mouse liver for the first time, as well as increased caspase-2 activity and apoptosis. Furthermore, we found that the tumour necrosis factor (TNF- $\alpha$ ) functions as a positive mediator of  $O_2^{\cdot-}$  generation. Based on these data, we identified the potential signalling pathway in the Golgi that mediates  $O_2^{\cdot-}$  fluctuations in IR mice and revealed the related molecular mechanisms; we also provide a new target for treating IR injury.

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## Introduction

An interruption in hepatic blood flow is an inherent phenomenon during diverse types of hepatic surgery.<sup>1</sup> Once the blood flow and oxygen supply are restored, the liver is subjected to a further insult, aggravating the injury.<sup>2</sup> Hepatic ischaemia reperfusion (IR) injury is attributed to the damage caused by reactive oxygen species (ROS),<sup>3</sup> such as the superoxide anion ( $O_2^{\cdot-}$ ),<sup>4,5</sup> hydrogen peroxide ( $H_2O_2$ ),<sup>6</sup> and peroxynitrite ( $ONOO^-$ ).<sup>7</sup> Because the superoxide anion ( $O_2^{\cdot-}$ ) is the first ROS to be generated and can trigger and regulate the production of other ROS,<sup>8</sup> explorations of the role of  $O_2^{\cdot-}$  in the IR process are meaningful.

The Golgi apparatus is an important organelle responsible for protein processing, classification and packaging, thus maintaining the normal function and survival of cells.<sup>9</sup> Meanwhile, the Golgi apparatus generates  $O_2^{\cdot-}$  via Golgi-associated proteins such as NOS<sup>10</sup> and releases  $O_2^{\cdot-}$  when it catalyses reactions with substrate molecules. Under normal circumstances, the low levels of  $O_2^{\cdot-}$  in the Golgi body is catalytically transformed to  $H_2O_2$  by SOD, which acts as a second messenger. However, when the body

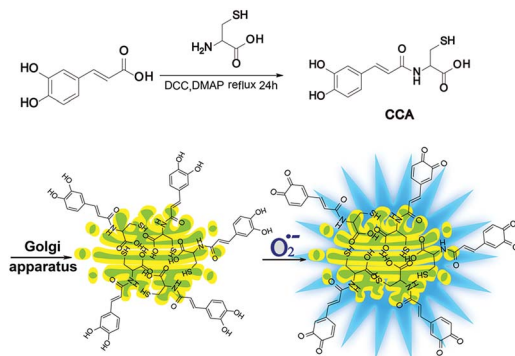
is subjected to IR, the  $O_2^{\cdot-}$  levels fluctuate abnormally, leading to an imbalanced redox state in the Golgi body. More likely, these unbalanced Golgi  $O_2^{\cdot-}$  levels directly participate in IR injury. However, the number of studies focusing on the relationship between  $O_2^{\cdot-}$  levels in the Golgi apparatus and IR injury is limited, although some studies have referred to mitochondrial  $O_2^{\cdot-}$  fluctuations during IR.<sup>11</sup> Therefore, an accurate analysis of the changes in Golgi  $O_2^{\cdot-}$  levels and their effects on the process of IR injury will provide a more comprehensive understanding of pathogenesis, which can assist with the identification of a target for the treatment of this disease. However, the main obstacle is the lack of ideal analytical tools.

Fluorescence microscopy techniques offer many benefits<sup>12–24</sup> and are ideal methods for detecting Golgi  $O_2^{\cdot-}$  levels.<sup>25–29</sup> Fluorescent probes designed to detect  $O_2^{\cdot-}$  levels in the Golgi apparatus are rare. Therefore, we designed and synthesized a new  $O_2^{\cdot-}$  two-photon fluorescence probe that targets the Golgi and dynamically and reversibly detects  $O_2^{\cdot-}$  levels (*cis*-caffeic acid, CCA, Scheme 1). The CCA probe was designed using the following strategy: 1, a caffeic acid group for detecting the dynamic responses of  $O_2^{\cdot-}$  that displays blue fluorescence; and 2, an L-cysteine group for targeting the Golgi apparatus.<sup>30</sup> Because Golgi apparatus contains many receptors for the cysteine residues or cysteine rich region.<sup>32,33</sup> So L-cysteine is more easily to anchor in Golgi apparatus. Furthermore, the Huang group<sup>31</sup> has been proved that probe with L-cysteine could targeting Golgi apparatus. With the aid of two-photon fluorescence microscopy, we detected Golgi  $O_2^{\cdot-}$  fluctuations in IR mice. We also used this approach to study

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Scheme 1 The synthesis and luminescence mechanism of CCA.

related signalling pathways and illuminate the molecular mechanism by which  $O_2^{\bullet-}$  regulates IR.

## Results and discussion

### The synthesis and properties of CCA

CCA was constructed with an amido linkage between the caffeic acid group and L-cysteine (ESI†). According to the probe design strategy, the blue fluorescence (495 nm) was dramatically increased with the addition of  $O_2^{\bullet-}$  (Fig. 1A, fluorescence quantum yield = 0.21). Two-photon excited fluorescence responses to  $O_2^{\bullet-}$  were identical (Fig. S1†, two-photon absorption cross section = 38.7 GM). Fig. 1A and B also illustrate the linear relationship between the fluorescence intensity ratio and

$[O_2^{\bullet-}]$  in a wide range of 0–5  $\mu\text{M}$ . The linear equation was  $F = 1621.514 [O_2^{\bullet-}] (\mu\text{M}) + 631.98$ , with a linear correlation coefficient of 0.991 and a detection limit of 18 nM. Collectively, CCA potentially possessed the ability to detect  $O_2^{\bullet-}$ , as determined by the blue fluorescence intensity.

Next, we examined the selectivity of CCA for  $O_2^{\bullet-}$  under simulated physiological conditions.<sup>33,34</sup> The fluorescence responses of CCA to competing ROS, reactive nitrogen species (RNS) and metal ions are shown in Fig. 1C, S2 and S3†. As expected, CCA exhibited high selectivity for  $O_2^{\bullet-}$ . The pH titration experiment revealed that the CCA fluorescence intensity was basically maintained at a constant value at pH 4.0–9.0. Based on these findings, CCA exclusively captures  $O_2^{\bullet-}$  levels in living cells. Additionally, CCA possesses more advantages, including a fast fluorescence response to  $O_2^{\bullet-}$ , reversible detection of  $O_2^{\bullet-}$  and ascorbic acid (Vc) and no marked cytotoxicity at concentrations less than 5.13 mM (Fig. 1D, S4 and S5†). Thus, CCA can serve as a robust sensor to achieve dynamic fluorescence imaging of  $O_2^{\bullet-}$ .

### Validation of CCA for the imaging of living cells

The utility of CCA for fluorescence imaging in living cells was evaluated. We used 2-methoxyestradiol<sup>35,36</sup> (2-ME, 1.0  $\mu\text{g mL}^{-1}$ ) to inhibit both copper–zinc and manganese superoxide dismutases and determine the resulting increase in  $O_2^{\bullet-}$  concentrations, and Vc was to induce the reduction reaction of the CCA product in hepatocytes. As illustrated in Fig. 2, strong blue fluorescence was observed in hepatocytes treated with 2-ME compared with the control cells, indicating significantly increased  $O_2^{\bullet-}$  concentrations. After the addition of 1.0 mM Vc to these cells, the fluorescence ratio decreased immediately, indicating redox reversibility. Furthermore, other reversible fluorescence changes were observed following successive treatments with 2-ME and Vc. We specifically reduced  $O_2^{\bullet-}$  levels using the  $O_2^{\bullet-}$  scavenger Tiron (10  $\mu\text{M}$ ) to further confirm the selectivity of CCA for  $O_2^{\bullet-}$  in living cells.<sup>37</sup> Fig. 2 shows weak fluorescence in hepatocytes treated with Tiron. Thus, the blue fluorescence of CCA changes according to the  $O_2^{\bullet-}$  fluctuations. Based on these results, CCA selectively and reversibly responds to  $O_2^{\bullet-}$  at the cellular level.

### Validation of CCA for the imaging of mice

Next, we performed *in vivo* imaging of  $O_2^{\bullet-}$  levels in mice based on the favourable two-photon excited fluorescence properties of CCA. Consistent with the design used for cellular imaging, we used 2-ME and Tiron to increase or decrease the  $O_2^{\bullet-}$  concentrations. As shown in Fig. 2B, more intense blue fluorescence was observed in mice treated with 2-ME and weak fluorescence was observed in mice treated with Tiron compared with the normal group, indicating an increase or decrease in  $O_2^{\bullet-}$  concentrations, respectively. Thus, CCA visualized  $O_2^{\bullet-}$  fluctuations in small animals and achieved imaging at relatively deep levels in the tissue (depth = 350  $\mu\text{m}$ ).

### Golgi apparatus localization of CCA

We performed an imaging experiment to determine whether CCA specifically targeted the Golgi apparatus. 2-ME stimulated hepatocytes were co-cultured with CCA and various commercial

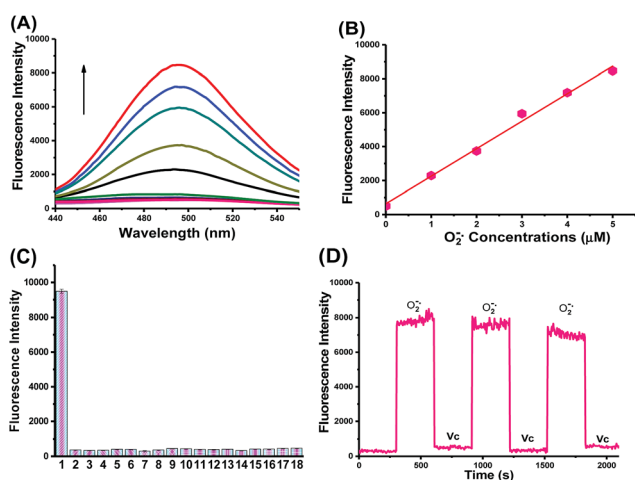


Fig. 1 Fluorescence properties of the CCA probe for  $O_2^{\bullet-}$  detection. (A) One-photon fluorescence spectra of 10  $\mu\text{M}$  CCA after the addition of various concentrations of  $O_2^{\bullet-}$  (0–5  $\mu\text{M}$ ). (B) A linear correlation between the ratio of the fluorescence intensity and  $O_2^{\bullet-}$  concentrations. (C) Fluorescence responses of 10  $\mu\text{M}$  CCA to various reactive oxygen species, reactive nitrogen species and metals (5  $\mu\text{M}$   $O_2^{\bullet-}$ , 20  $\mu\text{M}$  GSH, 100  $\mu\text{M}$  TBHP, 10 mM  $H_2O_2$ , 1  $\mu\text{M}$   $^1O_2$ , 1  $\mu\text{M}$   $\cdot\text{OH}$ , 2  $\mu\text{M}$   $ONOO^-$ , 50  $\mu\text{M}$  NO, 100  $\mu\text{M}$  NaClO, 10 mM  $Na^+$ , 10 mM  $K^+$ , 500  $\mu\text{M}$   $Ca^{2+}$ , 500  $\mu\text{M}$   $Zn^{2+}$ , 500  $\mu\text{M}$   $Fe^{2+}$ , 500  $\mu\text{M}$   $Fe^{3+}$ , 20  $\mu\text{M}$   $Cu^{2+}$  and 20  $\mu\text{M}$   $Cu^+$ ). (D) Reversibility of CCA fluorescence in the presence of alternating treatments with 5  $\mu\text{M}$   $O_2^{\bullet-}$  and 0.5 mM ascorbic acid (Vc). All one-photon spectra were acquired in cell extracts at  $\lambda_{\text{ex}} = 370$  nm and  $\lambda_{\text{em}} = 490$  nm.



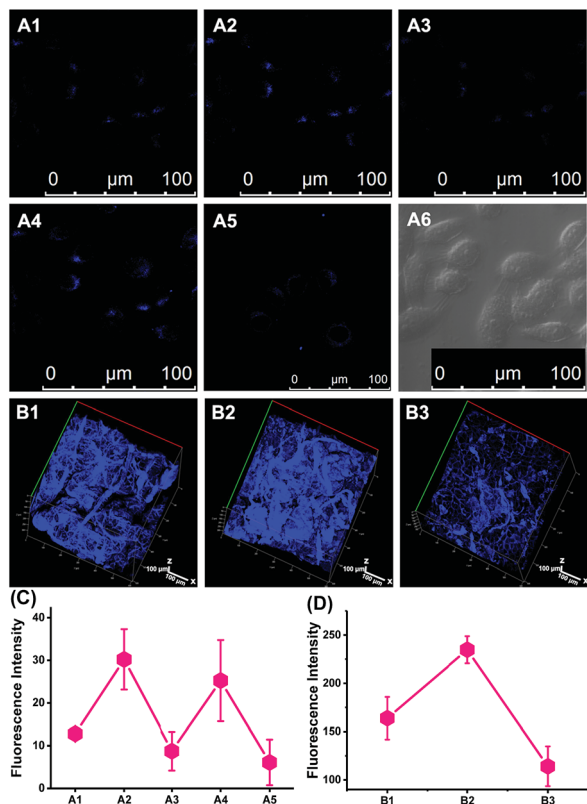


Fig. 2 TP fluorescence imaging of  $O_2^{\bullet-}$  levels in hepatocytes and in mice. (A) Hepatic cells shown in (A1) were incubated with  $10 \mu\text{M}$  CCA for 10 min. (A2) Cells shown in (A1) were stimulated with  $1.0 \mu\text{g mL}^{-1}$  2-methoxyestradiol (2-ME). (A3) Cells shown in (A2) were treated with  $1.0 \text{ mM}$  Vc. (A4) Cells shown in (A3) were stimulated with  $1.0 \mu\text{g mL}^{-1}$  2-ME again. (A5) Cells were loaded with  $10 \mu\text{M}$  Tiron 30 min before the addition of CCA. (A6) Bright field images. (B) *In vivo* 3D images of normal animals (B1), 2-ME stimulated mice (B2) and Tiron-treated mice following an injection of  $10 \mu\text{M}$  CCA. (C) The average fluorescence intensity output of (A). (D) The average fluorescence intensity output of (B). Images were acquired at an excitation wavelength of  $800 \text{ nm}$  and emission wavelengths corresponding to the blue channel of  $430\text{--}530 \text{ nm}$ .

organelle dyes, including Golgi-Track Red,<sup>38</sup> Mito-Tracker Deep Red, Lyso-Tracker Deep Red and ER-Tracker Red. The overlapped fluorescence images in Fig. 3A indicated that CCA fluorescence merged well with that of Golgi-Track Red and Pearson's colocalization coefficient was  $0.93$ .<sup>39</sup> In contrast, CCA displayed almost no fluorescence inside the mitochondria (Fig. 3B, colocalization coefficient  $0.15$ ), lysosomes (Fig. 3C, colocalization coefficient  $0.25$ ) and endoplasmic reticulum (Fig. 3D, colocalization coefficient  $0.24$ ). These data provide strong evidence that the probe predominantly accumulates in the Golgi apparatus and reflects the Golgi  $O_2^{\bullet-}$  level.

### Increased $O_2^{\bullet-}$ levels in the Golgi apparatus of IR cells and mice

We established IR cell and mouse models by simulating liver surgery.<sup>40,41</sup> With the assistance of two-photon fluorescence microscopy, we imaged  $O_2^{\bullet-}$  fluctuations in the Golgi apparatus of IR cells and mice. As shown in Fig. 4, IR groups exhibited

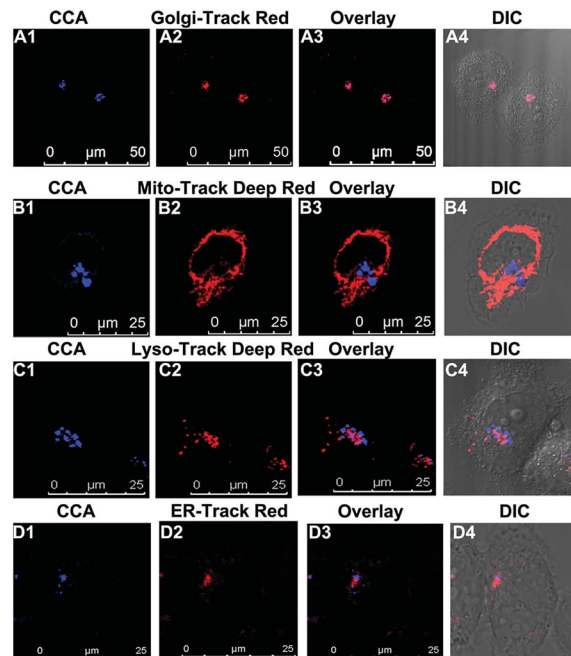


Fig. 3 Images of the intracellular localization and Golgi apparatus-targeting capability of CCA. Hepatic cells were co-cultured with 2-ME ( $1.0 \mu\text{g mL}^{-1}$ ), CCA ( $10 \mu\text{M}$ ,  $\lambda_{\text{ex}} = 405$  and  $\lambda_{\text{em}} = 430\text{--}550 \text{ nm}$ ) and organelle dyes, including Golgi-Track Red ( $50 \text{ nM}$ ,  $\lambda_{\text{ex}} = 561 \text{ nm}$  and  $\lambda_{\text{em}} = 600\text{--}750 \text{ nm}$ ), Mito-Tracker Deep Red ( $100 \text{ nM}$ ,  $\lambda_{\text{ex}} = 633 \text{ nm}$  and  $\lambda_{\text{em}} = 650\text{--}740 \text{ nm}$ ), Lyso-Tracker Deep Red ( $100 \text{ nM}$ ,  $\lambda_{\text{ex}} = 633 \text{ nm}$  and  $\lambda_{\text{em}} = 650\text{--}740 \text{ nm}$ ) and ER-Tracker Red ( $500 \text{ nM}$ ,  $\lambda_{\text{ex}} = 561 \text{ nm}$  and  $\lambda_{\text{em}} = 580\text{--}630 \text{ nm}$ ).

strong blue fluorescence compared with normal groups of both living cells and mice *in vivo*, indicating that IR increased  $O_2^{\bullet-}$  levels in the Golgi apparatus. We are the first to discover the connection between the IR process and Golgi  $O_2^{\bullet-}$  levels in living cells and *in vivo*.

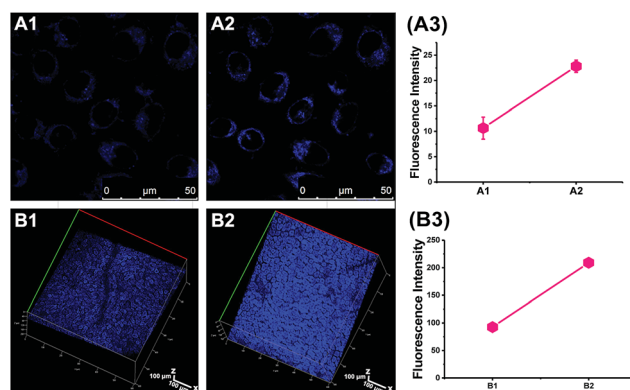


Fig. 4 Increased  $O_2^{\bullet-}$  levels in IR cells and mice. (A) The normal (A1) and IR hepatic (A2) cells were loaded with  $10 \mu\text{M}$  CCA for blue fluorescence imaging, and (A3) shows the average fluorescence intensity output of (A). (B) The *in vivo* 3D images of normal (B1) and IR mice (B2) injected with  $10 \mu\text{M}$  CCA for blue fluorescence imaging are shown, and (B3) shows the average fluorescence intensity output of (B). Images were acquired at an excitation wavelength of  $800 \text{ nm}$  and emission wavelengths corresponding to the blue channel of  $430\text{--}530 \text{ nm}$ .





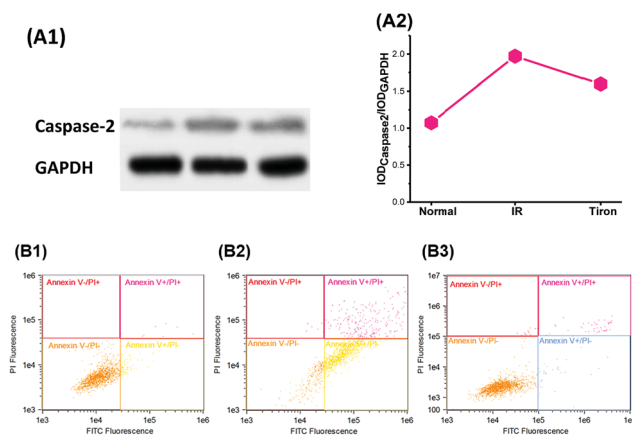


Fig. 5 Increased Golgi  $O_2^{\bullet-}$  levels, caspase-2 levels and apoptosis in IR cells. (A1) Western blot showing caspase-2 levels in normal cells, IR hepatic cells and 10  $\mu$ M Tiron-treated cells. (A2) The average intensity output of the cells shown in (A1). (B) Flow cytometry analysis of normal cells (B1), IR hepatic cells (B2) and IR cells with 10  $\mu$ M Tiron (B3).

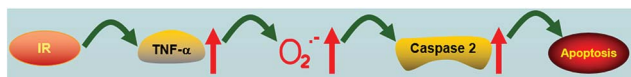


Fig. 6 Model for the signalling role of  $O_2^{\bullet-}$  in IR cells and mice.

### Increased $O_2^{\bullet-}$ levels in the Golgi apparatus induced apoptosis

We next investigated the influence of excess  $O_2^{\bullet-}$  levels in the Golgi apparatus on the levels of the pro-apoptotic protein caspase-2 and cell survival in IR hepatocytes. In Fig. 5A, high levels of  $O_2^{\bullet-}$  in the Golgi increased the caspase-2 level and low Golgi  $O_2^{\bullet-}$  levels decreased the caspase-2 level. Furthermore, excess  $O_2^{\bullet-}$  induced apoptosis, as evidenced by the results of the flow cytometry experiment presented in Fig. 5B. Correspondingly, decreased  $O_2^{\bullet-}$  levels with Tiron (10  $\mu$ M) could inhibit apoptosis. Based on these data, excess  $O_2^{\bullet-}$  levels in the Golgi apparatus provide a signal to increase caspase 2 levels and apoptosis.

### TNF- $\alpha$ induced $O_2^{\bullet-}$ generation in the Golgi apparatus

TNF- $\alpha$  plays an important role in hepatic IR injury.<sup>42</sup> Therefore, we explored the relationship between TNF- $\alpha$  and Golgi  $O_2^{\bullet-}$  levels. In Fig. S6 and S7,<sup>†</sup> we observed higher levels of TNF- $\alpha$  in IR cells compared with normal cells (TNF- $\alpha$  kit) and high Golgi  $O_2^{\bullet-}$  concentrations. After the addition of a TNF- $\alpha$  inhibitor (100  $\mu$ g mL<sup>-1</sup> silymarin),<sup>43</sup> the blue fluorescence of IR cells changed slightly, indicating that TNF- $\alpha$  inhibition decreased Golgi  $O_2^{\bullet-}$  levels in IR cells comparing with cells in which TNF- $\alpha$  was not inhibited. Thus, TNF- $\alpha$  was located upstream of  $O_2^{\bullet-}$  generation in the Golgi apparatus and the signal for IR injury is TNF- $\alpha$ - $O_2^{\bullet-}$ -caspase 2-apoptosis (Fig. 6).

## Conclusions

We developed a two-photon fluorescence probe for the dynamic and reversible detection of  $O_2^{\bullet-}$  levels in the Golgi apparatus

and to determine the relationship between Golgi  $O_2^{\bullet-}$  levels and IR injury. Using two-photon fluorescence microscopy, we imaged excess  $O_2^{\bullet-}$  levels in the Golgi apparatus and studied the connections between TNF- $\alpha$ ,  $O_2^{\bullet-}$  and caspase-2. Furthermore, we provided a potential mechanism of the signal transduction pathway mediated by Golgi  $O_2^{\bullet-}$  during the IR process, which provides new insights into potential treatments for hepatic IR injury.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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## Notes and references

- 1 F. Serracinoinglott, N. A. Habib and R. T. Mathie, *Am. J. Surg.*, 2001, **181**, 160–166.
- 2 R. A. Gottlieb, K. O. Burleson, R. A. Kloner, B. M. Babior and R. L. Engler, *J. Clin. Invest.*, 1994, **94**, 1621–1628.
- 3 T. H. Sanderson, C. A. Reynolds, R. Kumar, K. Przyklenk and M. Huettemann, *Mol. Neurobiol.*, 2013, **47**, 9–23.
- 4 A. Koo, H. Komatsu, G. Tao, M. Inoue, P. H. Guth and N. Kaplowitz, *Hepatology*, 2010, **15**, 507–514.
- 5 W. Zhang, P. Li, F. Yang, X. Hu, C. Sun, W. Zhang, D. Chen and B. Tang, *J. Am. Chem. Soc.*, 2013, **135**, 14956–14959.
- 6 B. González-Flecha, J. C. Cutrin and A. Boveris, *J. Clin. Invest.*, 1993, **91**, 456–464.
- 7 C. Szabo, *Shock*, 1996, **6**, 79–88.
- 8 B. C. Dickinson and C. J. Chang, *Nat. Chem. Biol.*, 2011, **7**, 504–511.
- 9 S. Munro, *Trends Cell Biol.*, 1998, **8**, 11–15.
- 10 T. J. Guzik, R. Korbut and T. Adamekguzik, *J. Physiol. Pharmacol.*, 2003, **54**, 469–487.
- 11 E. T. Chouchani, V. R. Pell, A. M. James, L. M. Work, K. Saeb-Parsy, C. Frezza, T. Krieg and M. P. Murphy, *Cell Metab.*, 2016, **23**, 254–263.
- 12 P. Zhang, X. X. Yang, Y. Wang, N. W. Zhao and C. Z. Huang, *Nanoscale*, 2014, **6**, 2261–2269.
- 13 J. V. Frangioni, *Curr. Opin. Chem. Biol.*, 2003, **7**, 626–634.
- 14 X. Chen, X. Tian, I. Shin and J. Yoon, *Chem. Soc. Rev.*, 2011, **40**, 4783–4804.
- 15 J. Du, M. Hu, J. Fan and X. Peng, *Chem. Soc. Rev.*, 2012, **41**, 4511.



- 16 H.-W. Liu, K. Li, X.-X. Hu, L. Zhu, Q. Rong, Y. Liu, X.-B. Zhang, J. Hasserodt, F.-L. Qu and W. Tan, *Angew. Chem.*, 2017, **129**, 11950–11954.
- 17 H. K. Kim, J. Liu, J. Li, N. Nagraj, M. Li, C. M. Pavot and Y. Lu, *J. Am. Chem. Soc.*, 2007, **129**, 6896–6902.
- 18 F. Helmchen and W. Denk, *Nat. Methods*, 2005, **2**, 932–940.
- 19 W. Zhang, X. Wang, P. Li, H. Xiao, W. Zhang, H. Wang and B. Tang, *Anal. Chem.*, 2017, **89**, 6840–6845.
- 20 L. Zhou, X. Zhang, Q. Wang, Y. Lv, G. Mao, A. Luo, Y. Wu, Y. Wu, J. Zhang and W. Tan, *J. Am. Chem. Soc.*, 2014, **136**, 9838–9841.
- 21 X. Chen, F. Wang, J. Y. Hyun, T. Wei, J. Qiang, X. Ren, I. Shin and J. Yoon, *Chem. Soc. Rev.*, 2016, **45**, 2976–3016.
- 22 H.-W. Liu, L. Chen, C. Xu, Z. Li, H. Zhang, X.-B. Zhang and W. Tan, *Chem. Soc. Rev.*, 2018, **47**, 7140–7180.
- 23 G. Mao, T. Wei, X. Wang, S. Huan, D. Lu, J. Zhang, X. Zhang, W. Tan, G. Shen and R. Yu, *Anal. Chem.*, 2013, **85**, 7875–7881.
- 24 W. Zhang, X. Wang, P. Li, H. Xiao, W. Zhang, H. Wang and B. Tang, *Anal. Chem.*, 2017, **89**, 6840–6845.
- 25 W. Zhang, P. Li, F. Yang, X. F. Hu, C. Z. Sun, W. Zhang, D. Z. Chen and B. Tang, *J. Am. Chem. Soc.*, 2013, **135**, 14956–14959.
- 26 P. Li, W. Zhang, K. Li, X. Liu, H. Xiao, W. Zhang and B. Tang, *Anal. Chem.*, 2013, **85**, 9877–9881.
- 27 P. Li, L. Liu, H. Xiao, W. Zhang, L. Wang and B. Tang, *J. Am. Chem. Soc.*, 2016, **138**, 2893–2896.
- 28 X. Gao, C. Ding, A. Zhu and Y. Tian, *Anal. Chem.*, 2014, **86**, 7071–7078.
- 29 J. J. Hu, N.-K. Wong, S. Ye, X. Chen, M.-Y. Lu, A. Q. Zhao, Y. Guo, A. C.-H. Ma, A. Y.-H. Leung, J. Shen and D. Yang, *J. Am. Chem. Soc.*, 2015, **137**, 6837–6843.
- 30 R. S. Li, P. F. Gao, H. Z. Zhang, L. L. Zheng, C. M. Li, J. Wang, Y. F. Li, F. Liu, N. Li and C. Z. Huang, *Chem. Sci.*, 2017, **8**, 6829–6835.
- 31 D. Aoki, N. Lee, N. Yamaguchi, C. Dubois and M. N. Fukuda, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 4319–4323.
- 32 Y. Maeda, G. V. Beznoussenko, J. Van Lint, A. A. Mironov and V. Malhotra, *EMBO J.*, 2001, **20**, 5982–5990.
- 33 H. Maeda, K. Yamamoto, Y. Nomura, I. Kohno, L. Hafsi, N. Ueda, S. Yoshida, M. Fukuda, Y. Fukuyasu and Y. Yamauchi, *J. Am. Chem. Soc.*, 2005, **127**, 68–69.
- 34 D. Oushiki, H. Kojima, T. Terai, M. Arita, K. Hanaoka, Y. Urano and T. Nagano, *J. Am. Chem. Soc.*, 2010, **132**, 2795–2801.
- 35 P. Huang, L. Feng, E. A. Oldham, M. J. Keating and W. Plunkett, *Nature*, 2000, **407**, 390.
- 36 Y. Zhou, E. O. Hileman, W. Plunkett, M. J. Keating and P. Huang, *Blood*, 2003, **101**, 4098–4104.
- 37 Y. Du, C. M. Miller and T. S. Kern, *Free Radical Biol. Med.*, 2003, **35**, 1491–1499.
- 38 M. Y. Chang, A. L. Shiau, Y. H. Chen, C. J. Chang, H. W. Chen and C. L. Wu, *Cancer Sci.*, 2008, **99**, 1479–1484.
- 39 J. Adler and I. Parmryd, *Cytometry, Part A*, 2010, **77**, 733–742.
- 40 R. Liu, L. Zhang, X. Lan, L. Li, T. T. Zhang, J. H. Sun and G. H. Du, *Neuroscience*, 2011, **176**, 408–419.
- 41 S. Thiberge, S. Blazquez, P. Baldacci, O. Renaud, S. Shorte, R. Ménard and R. Amino, *Nat. Protoc.*, 2007, **2**, 1811–1818.
- 42 M. Serteser, T. Koken, A. Kahraman, K. Yilmaz, G. Akbulut and O. N. Dilek, *J. Surg. Res.*, 2002, **107**, 234–240.
- 43 X. Zi, H. Mukhtar and R. Agarwal, *Biochem. Biophys. Res. Commun.*, 1997, **239**, 334–339.

