様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　５ 年　 ６ 月 ２６ 日現在

機関番号：１４３０１
研究種目：若手研究
研究期間：2021～2022
課題番号：２１Ｋ１４７５２

研究課題名（和文）Revealing oxidative stress in neurodegenerative brain by hydroxyl-radical-focused imaging and proteomics

研究課題名（英文）Revealing oxidative stress in neurodegenerative brain by hydroxyl-radical-focused imaging and proteomics

研究代表者
朱　浩（ZHU, Hao）

京都大学・工学研究科・助教

研究者番号：９０８７４５４５

交付決定額（研究期間全体）：（直接経費）　　3,600,000円

研究成果の概要（和文）：ヒドロキシルラジカルは、生体分子を非選択的に酸化する活性酸素種(ROS)の1つであり、多くのヒト疾患の発症原因となります。本研究は、蛍光イメージングとプロテオミクスによって生体内のヒドロキシルラジカルの局在と周囲/関連タンパク質を解明するためのヒドロキシルラジカル応答性タンパク質標識試薬を開発します。先ず一連の化学プローブが合成され、試験管でのタンパク質標識実験により、ヒドロキシルラジカル選択的プローブを同定しました。生細胞に適用するために、プローブの膜透過性と双直交性をさらに改善しました。最後に、活性化されたマクロファージの内因性ROSへの応答におけるその有効性を示す予備的な結果を得ました。

研究成果の学術的意義や社会的意義
本研究は、生体内のヒドロキシルラジカルの網羅的な分析を目指し、蛍光イメージングおよびプロテオミクス用のヒドロキシルラジカル応答性タンパク質標識試薬の最初の例を開発します。これは、細胞のヒドロキシルラジカルの分子メカニズムを解明するのに役立つだけでなく、神経変性疾患などの関連疾患の治療薬標的を発見するための手がかりを提供する可能性があります。

研究成果の概要（英文）：Hydroxyl radical is one member of reactive oxygen species (ROS) that regulates diverse biological and pathological processes in living systems. Due to its high reactivity, hydroxyl radical non-selectively modifies biomolecules which is the onset of many human diseases. This research is developing a new method to decipher hydroxyl radical homeostasis in living systems by hydroxyl-radical-responsive protein labeling, which can unravel the localization and surrounding/associated proteins of hydroxyl radical via fluorescence imaging and proteomics. Chemical probes that can be selectively activated by hydroxyl radical for efficient protein labeling have been identified. Preliminary results have demonstrated that such probe enables hydroxyl-radical-responsive protein labeling in response to endogenously produced hydroxyl radicals in macrophages during immune response. Such progress confirmed the feasibility of the proposed concept of hydroxyl radical conditional protein labeling.

研究分野：chemical biology

キーワード：reactive oxygen species　hydroxyl radical　conditional proteomics

１．研究開始当初の背景

Neurodegenerative diseases (NDs), such as Alzheimer's and Parkinson's, cause dementia of the elderly and is increasingly suffered in the aging society. Unfortunately, the current drug development has yet delivered effective treatments for NDs, due to our insufficient knowledge on the exact molecular mechanism of its onset and progression. Reactive oxygen species (ROS) are a class of oxygen-containing and reactive molecules, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH), that are continuously produced and consumed in living systems. Uncontrolled production of ROS in the brain contributes to the pathogenesis of NDs, due to the ROS-derived oxidative modifications on biomolecules (e.g., proteins), leading to impaired protein function, oxidative stress, and neuron death. In particular, ·OH is the most deleterious ROS because it exhibits the highest reactivity while cannot be enzymatically detoxified. Indeed, ·OH-mediated lipid peroxidation and protein carbonylation have been identified as oxidative-stress biomarkers in NDs.

Investigation of biological ROS conventionally relies on fluorescent/chemiluminescent sensors and redox proteomics that can detect the production of ROS in live samples and analyze the oxidized proteins by ROS, respectively. Although useful, such methods usually lack selectivity to one specific kind of ROS and are insufficient for the comprehensive understanding of biological ROS regarding their production, localization, and modification, which is fundamental for the development of precise and effective treatment of ROS-related NDs.

２．研究の目的

This research aims at the development of a new method for the study of biological ·OH that allows for comprehensive analysis of ·OH-surrounding proteins by proteomics, namely conditional proteomics that we previously established (T. Miki et al. *Nat. Methods* **2016**, 13, 931; Y. Nishikawa et al. *ACS Chem. Biol.* **2019**, 14, 397; H. Zhu et al. *J. Am. Chem. Soc.* **2020**, 142, 15711), and observation of ·OH production by microscopy imaging (Figure 1). This method relies on designed chemical probes of ·OH-responsive protein labeling reagents that can specifically react with ·OH and subsequently transform to a highly reactive intermediate for immediately labeling surrounding proteins. The labeled proteins can be enriched and identified by mass-spectrometry-based proteomics. This proteomics enables the whole mapping of the proteome environment of ·OH, including its generation sources, modification targets, and other proteins that are spatially close to ·OH. In addition, the ·OH-responsive protein labeling immobilizes the probe at the loci of ·OH production and is compatible with fixation and immunostaining to specifically determine the ·OH localization. When applied in the NDs brain, this method will specifically

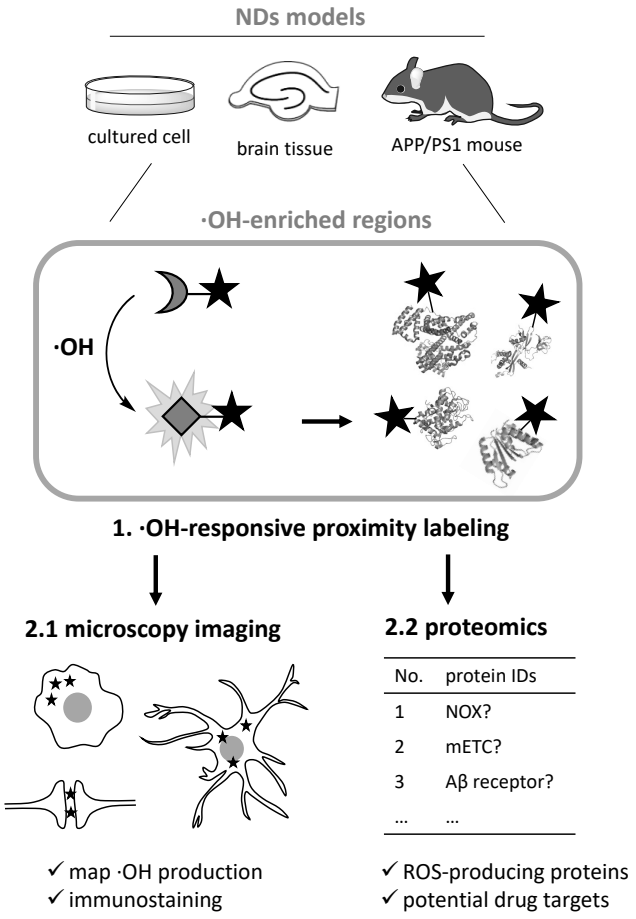

Figure 1 ·OH-responsive protein labeling for microscopy imaging and proteomics.

and comprehensively map the production of ·OH and unravel the ·OH-associated proteins, which will greatly expand our understanding of the pathological role of ·OH and potentially contribute to the development of new treatment of NDs.

３．研究の方法

Firstly, a series of chemical probes for ·OH-responsive protein labeling was designed and synthesized. Such probes contain a biotin tag that enables the chemiluminescence detection of protein labeling with the streptavidin-horseradish peroxidase (SAv-HRP) conjugates. The protein labeling behavior and ROS selectivity were evaluated in cell lysates and analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. The chemical probes that showed high protein labeling efficiency in response to ·OH and high selectivity to ·OH over other ROS were selected for further evaluation.

For the application in living cells, cell permeability of the ·OH probes was improved and determined by confocal laser scanning microscopy (CLSM) imaging. To test whether the ·OH probes enable response to endogenously produced ·OH in living cells, cultured mouse macrophages were stimulated to generate an immune response model, where NADPH oxidase is activated and generates $O_2^{\cdot-}$ and downstream ROS, including ·OH.

４．研究成果

Chemical probes that can be selectively activated by ·OH for efficient protein labeling have been identified. As shown in Figure 2, probe 1 alone was stable in the HeLa cell lysates and showed no detectable protein labeling after incubation at 37 ºC for 60 min (lane 1). Significant protein labeling by probe 1 was induced by ·OH (lane 4), but not by other ROS, including $O_2^{\cdot-}$, H_2O_2, $^1O_2$ (singlet oxygen), and OCl^- (hypochlorite). For the application in living cells, probe 2 having a diacetylfluorescein moiety was synthesized for improved cell permeability and retention. CLSM imaging showed that probe 2 could be rapidly taken up into living cells within minutes and broadly distributed throughout the entire cell, which is favorable for the unbiased detection of ·OH production. Probe 2 was further slightly modified to probe 3, with improved biorthogonality (reduced background labeling by non-specific cellular oxidation). Preliminary results have demonstrated that probe 3 enabled ·OH-responsive protein labeling in response to endogenously produced ·OH in cultured mouse macrophages during immune response. Such progress confirmed the feasibility of the proposed concept of ·OH conditional protein labeling.

In the future, CLSM imaging that can observe the labeled proteins and determine the ·OH localization and proteomics that can comprehensively unravel ·OH surrounding proteins will be pursued. This method appears as the first example of ·OH-responsive protein labeling to decipher ·OH homeostasis by imaging and proteomics. Thanks to its stable covalent protein labeling, this method is compatible with fixation and conventional immunostaining that allows for the determination of ·OH localization *via* specific antibody staining. By contrast, the conventional ROS sensors are only available in live conditions and are not amenable to fixation. Compared with traditional redox proteomics that identify protein oxidation that can be carried out by one or diverse kinds of ROS, this protein labeling is highly specific to ·OH over other ROS. More importantly, the ·OH conditional proteomics enables comprehensively unravel ·OH surrounding proteins, which covers the sources for ·OH production, the ·OH-modified proteins, and other spatially relevant proteins.

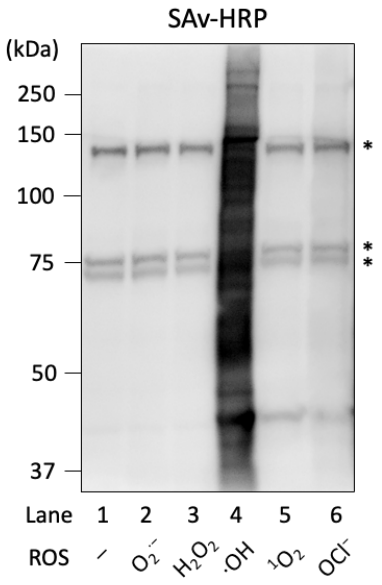

Figure 2 ·OH-responsive protein labeling in cell lysates. * denotes the endogenously biotinylated proteins.

This research aims at the application of the ·OH-responsive protein labeling in neurodegenerative brains, which would provide powerful and integrative means to unravel the pathological roles of ·OH in the NDs, and even discover potential therapeutic drug targets for the treatment of NDs.

During the screening of chemical probes for ROS-responsive protein labeling, probes that can be selectively activated by one specific ROS other than ·OH were also discovered. We will pursue global profiling of ROS production and associated proteins by the combination of multiple ROS-responsive protein labeling, while each of them is specific to individual ROS.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計5件（うち招待講演　0件／うち国際学会　1件）

１．発表者名 Hao Zhu
２．発表標題 H2O2応答性タンパク質修飾剤による酸化環境プロテオームのイメージングとプロファイリング
３．学会等名 第74回日本酸化ストレス学会・第21回日本NO学会合同学術集会
４．発表年 2021年～2022年

１．発表者名 Hao Zhu
２．発表標題 ROS conditional proteomics for identification of H2O2-rich subcellular compartments
３．学会等名 フリーラジカルスクール2021
４．発表年 2021年～2022年

１．発表者名 Hao Zhu
２．発表標題 ROS conditional proteomics (2): identification of H2O2-rich subcellular compartments
３．学会等名 日本化学会 第102春季年会
４．発表年 2021年～2022年

１．発表者名 Hao Zhu
２．発表標題 Tyrosinase-catalyzed proximity labeling in living cells and in vivo
３．学会等名 10th Asian Biological Inorganic Chemistry Conference（国際学会）
４．発表年 2022年～2023年

１．発表者名
Hao Zhu

２．発表標題
Proximity protein labeling with tyrosinase

３．学会等名
生命金属科学シンポジウム2022

４．発表年
2022年～2023年

〔図書〕　計0件

〔産業財産権〕

〔その他〕
Hamachi Laboratory
http://www.sbchem.kyoto-u.ac.jp/hamachi-lab/index.php?hamachi-lab

６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関