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　３ 年　 ５ 月 ２７ 日現在

機関番号：１３３０１
研究種目：若手研究
研究期間：2019～2020
課題番号：１９Ｋ１６３４８

研究課題名（和文）Characterization of human glutathione S-transferase P1-1-catalyzed glutathionylation of proteins: as a clue to understand the inter-individual difference in drug-induced toxicity

研究課題名（英文）Characterization of human glutathione S-transferase P1-1-catalyzed glutathionylation of proteins: as a clue to understand the inter-individual difference in drug-induced toxicity

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交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：グルタチオン転移酵素GSTP1は、グルタチオン付加を触媒し生体内のタンパク質の機能を変化させうる。Keap1は通常Nrf2と相互作用し、その核内への移行を抑制しているが、酸化ストレス時にはNrf2はKeap1から離れ核内に移行し、解毒遺伝子を発現誘導する。この系における酸化ストレス時のGSTP1の役割を明らかにすることを目的とした。Keap1は実際にグルタチオン付加を受けることを明らかにし、さらに、Keap1とGSTP1がタンパク間相互作用することを高速電子間力顕微鏡(HS-AFM)で近接・解離をリアルタイムで観察することにより初めて明らかにした。Nrf2もHS-AFMで初めて観察成功した。

研究成果の学術的意義や社会的意義
The elucidation of dynamic structures of Keap1 and Nrf2 is helpful for the understanding of their physiological roles and regulatory roles in oxidative stress status. The regulation of Keap1 glutathionylation on interaction with Nrf2 provides new insights into mechanisms of drug-induced toxicity.

研究成果の概要（英文）：GSTP1 catalyzes glutathionylation of proteins to alter their functions. Under normal condition, Keap1 protein interacts with Nrf2 protein, which induces detoxification-related genes, to interfere the translocation into nucleus. Upon oxidative stress, Keap1 is suggested to be glutathionylated, which may affect the interaction with Nrf2 protein. Several experiments were conducted to understand molecular mechanisms, and the following results were obtained. 1. Purified human GSTP1, Keap1, and Nrf2 were successfully obtained from E.coli heterologous expression system. 2. HS-AFM imaging conditions for these proteins were established and optimized. This is the first observation of these dynamic structure at single molecule level. 3. Clear oxidants-dependent glutathionylation of Keap1 were observed, which also indicating an oxidative status-dependency of Keap1 molecular structure. With application of this method, glutathionylated protein concentrations were determined from in vitro samples.

研究分野：薬系衛生および生物化学関連

キーワード：Glutathionylation　Keap1　GSTP1　drug-induced toxicity

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１．研究開始当初の背景

Identification of risk factors is important for understanding the complex mechanisms of drug-induced toxicity. Recently glutathionylation of proteins has been recognized to play essential roles in several cases of drug-induced toxicity. Glutathionylation is a post-translational modification of thiol groups (e.g. cysteine residues) of proteins by conjugating a GSH molecule to form disulfide conjugates. By glutathionylation, proteins can be shielded from oxidative insults, which normally ends up with irreversible oxidation of protein thiol groups to sulfonic acid and triggering proteotoxicity. Many proteins involving in important physiological and pathological processes, such as cytoskeletal proteins, signaling proteins, transcriptional factors, proteins involved in endogenous and exogenous metabolism, have been reported to be prone to glutathionylation (Pastore and Piemonte. Eur J Pharm Sci. 2012; Zamaraev et al. Trends Cell Biol. 2017). Glutathionylation often results in alterations in protein functionality and activity. Besides, with the corresponding de-glutathionylation process, which refers to the removal of glutathione from the glutathionylated substrate, protein is restored to the native status. Therefore, glutathionylation is considered as a regulatory process together with deglutathionylation, which manipulates various cellular redox signaling pathways.

２．研究の目的

Glutathionylation occurs at low levels under physiological situations, while is significantly induced under electrophilic or oxidative stress stimuli. Glutathionylation reaction occurs both spontaneously and enzymatically. GSTP1 is a multi-functional phase II drug-metabolizing enzyme, with the major role of conjugating reduced GSH to electrophilic compounds. Besides, GSTP1-1 has been reported to be the major enzyme catalyzing glutathionylation of proteins (Tew et al., Free Radical Bio Med. 2011). Nevertheless, GSTP1's role in catalyzing glutathionylation of protein is to be elucidated compared with its role in catalyzing GSH conjugation of small molecules. Until now, only a few studies reported that GSTP1-mediated glutathionylation influenced cellular metabolic function (Zhang et al., J Biol Chem. 2018) or sensitivity to drug-induced stress response (Ye et al., Antioxid Redox Signal. 2017) and to drug-induced toxicity (McGarry et al., J Pharmacol Exp Ther. 2015). Whereas vital information which will help understanding the regulatory role of glutathionylation, such as the sites of glutathionylated cysteines and the dynamics of GSTP1-1 interaction with protein substrates, are limited.

My previous research showed a large interindividual variation of GSTs in population (den Braver-Sewradi et al., Curr Drug Metab. 2018). Moreover, this interindividual variability of GST expression is suggested to be a risk factor for amodiaquine- and diclofenac-induced toxicity (Zhang et al., Toxicol Lett. 2017; den Braver et al., Toxicol Lett. 2016). As for human *GSTP1* gene, three variants, **B* (Val105/Ala114), **C* (Val105/Val114), and **D* (Ile105/Val114), have been subjected to most studies so far, since significant differences in catalytic activity of conjugating electrophilic compounds with the wild-type **A* (Ile105/Ala114) have been reported. Until now, the effects of polymorphisms of *GSTP1* on glutathionylation and glutathione-regulated subsequent cell signaling pathways are unknown.

Collectively, I hypothesized that the GSTP1-catalyzed glutathionylation of proteins involving in cell death/survival signaling pathways might play an important role in drug-induced toxicity. Moreover, variation of glutathionylation, which is manipulated by *GSTP1* gene polymorphisms, might be a risk factor for interindividual susceptibility to drug-induced toxicity. To validate these hypotheses, research questions "(1) How GSTP1 catalyzes the glutathionylation of essential proteins regulating cell death/survival pathways at molecular level?" (2) How, and to what extent, will the functions/activities of the target proteins change after being glutathionylated? (3) What will be the impact of glutathionylation in cell fate-determining signaling pathways upon exposure to reactive drug metabolites? (4) Will the polymorphisms of human *GSTP1* have an impact on the glutathionylation and being a risk factor for drug-induced toxicity?" were explored.

３．研究の方法

In this project, several key proteins regulating important cell death/survival signaling pathways and known to be glutathionylated were planned to be selected as model substrates. Finally, I focused on Kealch-like ECH-associated protein 1 (Keap1, oxidative stress pathway). To investigate the dynamics of GSTP1-catalyzed glutathionylation of Keap1, and to examine whether variation in glutathionylation of Keap1 and genetic polymorphisms of *GSTP1* would be determining factors for drug-induced toxicity, following experiments were planned.

(1) Real-space and real-time characterization of GSTP1-mediated glutathionylation using high-speed atomic force microscopy (HS-AFM).

Firstly, the recombinant proteins including GSTP1, Keap1, and its related protein NF-E2-related factor 2 (Nrf2) were heterologously expressed and purified. The dynamics of interaction between GSTP1 and substrate proteins were investigated by using HS-AFM. Conditions mimicking physiological and stressed scenarios, such as varying concentration of GSTP1, substrate proteins, GSH and GSSG were planned to be reconstituted and loaded to HS-AFM to obtain the real-space and real-time information on GSTP1-catalyzed glutathionylation.

(2) Evaluation of GSTP1-mediated glutathionylation in cellular systems and the impact on cell death/survival-related signaling pathways.

Cell lines with different GSTP1 expression levels were planned to be utilized to investigate GSTP1's role in glutathionylation upon chemical-induced toxicity.

４．研究成果

(1) Observation of human Keap1 protein

Keap1 protein tagged with histidine hexamer expressed in *E. coli* expression system was purified with Ni^{2+}-column, and was observed with HS-AFM. It has been reported that Keap1 protein forms dimer, and the shape looks like cherry-bob as reported previously (Ogura et al., Proc Natl Acad Sci USA, 2010). Conditions for observation of Keap1 protein were optimized with several kinds of buffers and bare mica.

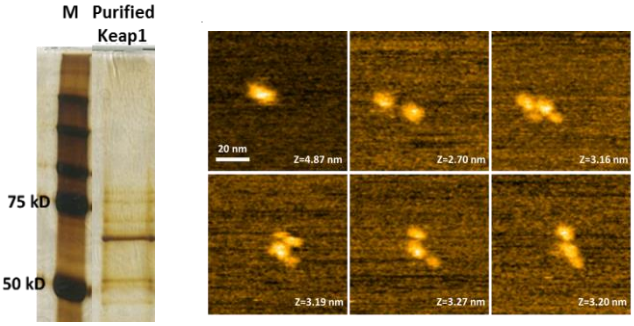

Figure 1. Visualization of human Keap1 protein by HS-AFM. (Left) Silver staining of the purified His-tagged human Keap1 protein. Molecular weight of Keap1 protein is approximately 70 kDa. (Right) HS-AFM images showing the molecular features of Keap1 dimer.

As a result, Keap1 dimer was observed in 20 mM sodium phosphate buffer, pH 7.4, on bare mica. Imaging time range was 8.4 s. In this condition, Keap1 dimer association in physiological relevant environment was observed for the first time.

(2) Observation of the interaction between Keap1 and GSTP1

As described above, Keap1 is likely glutathionylated by GSTP1. In this study, the interaction between Keap1 and GSTP1 was tried to be observed with HS-AFM. To this end, human GSTP1 protein expressed in *E.coli* was purified with GSH-affinity chromatography, and was observed with HS-AFM. GSTP1 visualization using HS-AFM was tried under various conditions, and eventually a potential interaction between GSTP1 and Keap1 monomer was observed in oxidative environment (Figure 2), which still needs to be confirmed in the future.

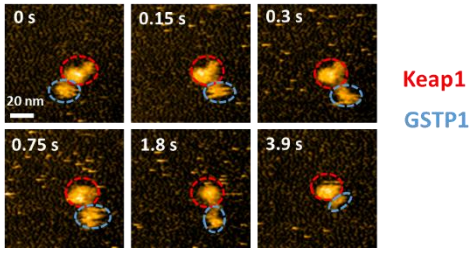

Figure 2. Visualization of potential Keap1-GSTP1 interaction by HS-AFM. In this condition, Keap1 monomer, which was indicated in red circle was interacted with GSTP1, which was indicated in blue circle, within a time range of 3.9 s. 1 mM oxidized glutathione was present in the imaging buffer as an oxidant.

(3) Observation of human Nrf2 protein

Under normal condition, Nrf2 protein is interacted with Keap1 protein, leading to the degradation of Nrf2. Upon oxidative stress, Nrf2 is released from Keap1 protein, resulting in the translocalization into nucleus. To understand the role of the glutathionylation of Keap1 protein upon oxidative stress, the interaction between Keap1 and Nrf2 should be also observed.

Similar to the observation of Keap1 protein, Nrf2 protein tagged with histidine hexamer expressed in *E. coli* expression system was purified with Ni^{2+}-column and several ion-exchange columns, and was observed by HS-AFM. In contrast to Keap1 protein, Nrf2 protein could not be obtained with a high purity. It has

been recognized that it is difficult to observe proteins with a low purity. However, I tried to observe Nrf2 protein by HS-AFM (Figure 3).

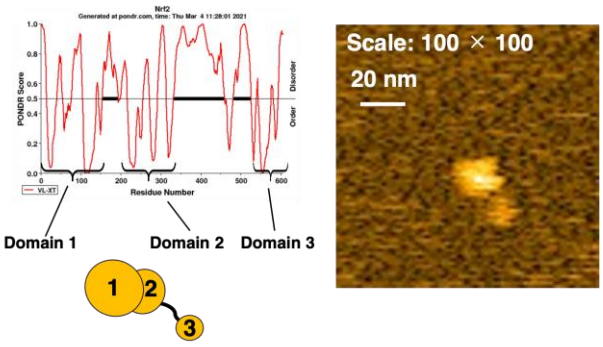

Figure 3. Visualization of Nrf2 protein with HS-AFM. (Left) Prediction of structure of Nrf2 protein by PONDR. Order and disorder in Y-axis represent folded and unfolded regions, respectively. (Right) Visualization of human Nrf2 by HS-AFM. His-tagged human Keap1 protein (1 nM) was loaded on bare mice with 10 mM HEPES buffer (pH 7.4).

First, since protein structure has not been determined in any reports, it was predicted using Predictor of Natural Disordered Regions (www.pondr.com), resulting Nrf2 protein likely two large domains and one small domain. In fact, the molecules similar to the predicted shape could be detected with 10 mM HEPES and bare mica. Large two domains at N-terminus were observed to be overlapped; therefore, two objects were observed. Thus, I firstly succeeded to visualize Nrf2 protein with HS-AF. However, due to the impurity of Nrf2 protein, it was difficult to conduct the subsequent study, observation of the effect of glutathionylation of Keap1 by GSTP1 on the interaction with Nrf2. I have to find the best way to purify Nrf2 protein for HS-AFM.

(4) Evaluation of GSTP1-mediated glutathionylation in cellular systems and the impact on cell death/survival-related signaling pathways.

In preliminary experiments, biochemical assay based on WB and analytical assay based on LC-MS/MS were established for the assessment of glutathionylation of total cytosol hepatic proteins and Keap1 from human. Biochemical assay showed clear oxidants-dependent glutathionylation of Keap1 and soluble hepatic proteins, which also indicating an oxidative status-dependency of Keap1 molecular structure. Analytical assay was used for the determination of glutathionylated protein concentrations from in vitro and cellular samples. Later research will focus on the effect of GSTP1 on biological significance of Keap1 glutathionylation on the cell death/survival-related signaling pathways.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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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