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Glutathione-mediated reversibility of covalent modification of ubiquitin carboxyl-terminal hydrolase L1 by 1,2-naphthoquinone through Cys152, but not Lys4

Takashi Toyama [a,b,1], Yasuhiro Shinkai [a,c,1], Aki Yazawa [d,1], Hidenao Kakehashi [c], Toshiyuki Kaji [e], Yoshito Kumagai [a,c,*]

[a] Doctoral Program in Biomedical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
[b] Research Fellow of the Japan Society for the Promotion of Science, 1-8 Chiyoda, Tokyo 102-8472, Japan
[c] Master's Program in Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
[d] College of Biological Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
[e] Department of Environmental Health, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Noda, Chiba 278-8510, Japan

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ABSTRACT

Covalent modification of cellular proteins by electrophiles affects electrophilic signal transduction and the dysfunction of enzymes that is involved in cytotoxicity. We have recently found a unique reaction which restores glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that has been modified by 1,2-naphthoquinone (1,2-NQ) through a glutathione (GSH)-dependent *S*-transarylation reaction. We report here that ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) undergoes the same reaction. Exposure of human neuroblastoma SH-SY5Y cells to 1,2-NQ after pretreatment with buthionine sulfoximine (BSO) to deplete GSH resulted in an enhancement of covalent modification of UCH-L1 by 1,2-NQ. With recombinant human UCH-L1, we demonstrated that UCH-L1 underwent arylation by 1,2-NQ through Cys152 and Lys4, thereby decreasing its catalytic activity. Addition of GSH to an incubation mixture of 1,2-NQ-UCH-L1 adduct partially restored this decline in enzyme activity which was accompanied by decreased covalent attachment of 1,2-NQ, together with production of 1,2-NQ-GSH adduct. UCH-L1 in which Lys4 was mutated exhibited a lower level of covalent modification and enzyme inhibition, but completely recovered after addition of GSH. Taken together, these results suggest that Cys152 modification in UCH-L1 by 1,2-NQ is reversible via GSH-mediated *S*-transarylation reaction whereas Lys4 modification by 1,2-NQ is irreversible by GSH. Because UCH-L1 dysfunction has been associated with neurodegeneration, the electrophilic modification of Lys rather than Cys in UCH-L1 may be implicated in such neurodegenerative diseases.

1. Introduction

Numerous endogenous electrophiles such as 8-nitro-cGMP, prostaglandin J_2 (PGJ_2), acrolein, 4-hydroxy-2-nonenal (HNE) and estrogen 3,4-quinones are produced in situations such as inflammation and oxidative stress [1–4]. Electrophiles are highly reactive chemicals capable of covalently modifying nucleophilic functions such as thiols and amino groups on proteins. Once proteins are covalently modified by electrophiles, the enzyme function, three-dimensional structure and redox status is altered and, electrophilic signaling [5] and cellular damage can result. Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is a deubiquitination enzyme specifically expressed in neuronal cells [6]. It has been reported that covalent modification of UCH-L1 by endogenous electrophiles such as PGJ_2 and HNE causes disruption of UCH-L1 function and is implicated in neurodegeneration [7,8]. However, the cellular fate of UCH-L1 modified by electrophiles remained unclear.

1,2-Naphthoquinone (1,2-NQ) is a metabolite of naphthalene produced by enzymes of xenobiotic metabolism and photooxidation [9,10]. We reported previously that 1,2-NQ was identified as an atmospheric electrophile from ambient sample collected at Riverside, California [11]. It was subsequently shown that 1,2-NQ is covalently bound to a variety of sensor proteins such as protein tyrosine phosphatese 1B (PTP1B) [12], cAMP response element-binding protein [13], thioredoxin (Trx) [14] and Kelch-like

* Corresponding author. Tel.: +81 29 853 3133.
E-mail address: yk-em-tu@md.tsukuba.ac.jp (Y. Kumagai).
[1] These authors contributed equally to this study.

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ECH-associated protein 1 (Keap1) [15], thereby affecting signal transduction and gene expression regulated by these proteins. We have recently found that a glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is easily modified by 1,2-NQ at low concentration, leading to a decline of its catalytic activity in a cell-free system, whereas minimal covalent modification and inhibition of enzyme activity of GAPDH were seen during exposure of human pulmonary epithelial A549 cells to 1,2-NQ even at 20 μM [16]. Additional studies suggested that GAPDH can protect itself from 1,2-NQ-based electrophilic attack of by cellular glutathione (GSH) through a *S*-transarylation reaction [16]. An analogous reaction by GSH was also involved in the removal of 2-*tert*-butyl-1,4-benzoquinone from Keap1 [17]. We therefore thought that GSH-mediated *S*-transarylation could also diminish the covalent modification of UCH-L1 by electrophiles. To address this issue, we performed a buthionine sulfoximine (BSO) differential 2-demensional SDS–PAGE and prepared recombinant UCH-L1 to clarify the capability of GSH-mediated *S*-transarylation by using 1,2-NQ as a model electrophile.

2. Materials and methods

2.1. Materials

1,2-NQ was obtained from Tokyo Chemical Industries Co. (Tokyo, Japan). 1,2-NQ-GSH adduct (1,2-NQ-SG) was synthesized as reported previously [16]. *Escherichia coli* BL21 cells and trypsin were purchased from Promega Co. (Madison, WI). Reduced GSH was obtained from MP Biomedicals (Irvine, CA). BSO and Coomassie brilliant blue (CBB) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Anti-UCH-L1 antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal antibody against 1,2-NQ was prepared as reported previously [18]. All other reagents used were of the highest purity available.

2.2. Cell culture

SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium/nutrient F-12 Ham supplemented, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM GlutaMAX-I (Invitrogen), and 10% (v/v) fetal bovine serum. The cells were cultured at 37 °C with 5% CO_2 and subconfluent cultures were used for all experiments. Before treatment, cells were serum-starved overnight and then exposed to 1,2-NQ (dissolved in dimethylsulfoxide (DMSO)) in serum-free medium to avoid the loss of 1,2-NQ effect through binding to serum components.

2.3. Two-dimensional SDS–PAGE

Cells were dissolved in lysis buffer containing 9.8 M urea, 4% CHAPS, 2% IPG buffer and bromophenol blue. Protein concentrations of cell lysates were determined by Bradford method. Cell lysates (50 μg of proteins) were applied to an Immobiline DryStrip pH 3–10, 7 cm (GE Healthcare) for 10 h under silicon oil. Isoelectric focusing was performed for 1 min at 200 V, 90 min at 3500 V, and 65 min at 3500 V by using a Multiphor II (GE Healthcare). Proteins separated by isoelectric focusing were further separated by SDS–PAGE according to the method of Laemmli [19].

2.4. Western blotting

Proteins separated by SDS–PAGE [19] were electro-transferred onto PVDF membranes (Bio-Rad Laboratories) at 2 mA/cm^2 for 1 h, according to the method of Kyhse-Anderson [20]. After blocking with 5% skim milk, the membrane-bound proteins were incubated with primary antibodies as described in Section 2.1. Secondary antibodies coupled to horseradish peroxidase were used to detect primary antibodies on the membrane. Proteins were detected with an ECL system (Nacalai Tesque Inc., Kyoto, Japan) and exposed to X-ray film (Konica Minolta Health Care Co., Tokyo, Japan). The bands were quantified by using ImageJ software.

2.5. Identification of UCH-L1

Identification of UCH-L1 was performed using a nanoAcquity UPLC system (Waters, Milford, MA, USA) coupled to a Synapt High Definition mass spectrometer (HDMS; Waters). The extracted protein from SDS-gel was digested with 5 μL of MS grade modified trypsin (100 ng) for 16 h at 37 °C. The eluted peptides (2 μL) were loaded on a BEH130 nanoAcquity C_{18} column (100 mm × 75 μm i.d., 1.7 μm) held at 35 °C. Mobile phases A [0.1% formic acid] and B [Acetonitrile containing 0.1% formic acid] at a flow rate of 0.3 μL/min were linearly mixed using a gradient system as follows: 3% B for 1 min; linear increase over 30 min to 40% B; linear increase over 2 min to 95% B; maintain at 95% B for 5 min before returning linearly to 3% B over 2 min. The total running time, including the conditioning of the column to the initial conditions, was 70 min. The eluted peptides were then transferred to the nanoElectroSpray source of a quadrupole time-of-flight (Q-TOF) mass spectrometer (Synapt High Definition Mass Spectrometry (HDMS) system, Waters) through a Teflon capillary union and a pre-cut PicoTip (Waters). The initial parameters of the Synapt HDMS were set as follows: capillary voltage 3.0 kV, sampling cone voltage 35 V and source temperature 100 °C. Low (6 eV) or elevated (step from 15 to 30 eV) collision energy was used to generate either intact peptide precursor ions (low energy) or peptide product ions (elevated energy). The MS survey scan was *m*/*z* 200–2000. The data were acquired using an independent reference spray using the nanoLockSpray interference procedure in which Glu-1-fibrinopeptide B (*m*/*z* 785.8426), infused via the nanolockspray ion source, was sampled every 10 s as the external mass calibration.

2.6. Purification of UCH-L1

RNA was extracted from the human neuroblastoma SH-SY5Y cells with Sepasol-RNA I super reagent (Nacalai Tesque) and complementary DNA (cDNA) synthesis was performed using Prime Script RT (Takara Bio, Shiga, Japan). Amplification of ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) gene was performed by PCR with Prim Script (Takara Bio, Shiga, Japan). cDNA corresponding to human UCH-L1 was cloned by the StrataClone Bluent PCR Cloning Kit (Stratagene, La Jolla, CA). The cloned UCH-L1 cDNA was placed downstream of the phage T7 RNA polymerase promoter at the XhoI and BamHI sites of the pET15b vector (Novagen, Madison, WI). The plasmid sequence was verified on an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA). Lys4-to-Ala and Cys152-to-Ser mutations were introduced using a PCR-based approach using QuikChange Site-Directed Mutagenesis Kit (Agilent technologies, Palo Alto, CA) according to the manufacturer's instruction. The resulting plasmids were transformed into *E. coli* BL21 (DE3) pLysS cells for protein expression. The bacterial culture was grown in LB broth at 37 °C with shaking at 120 rpm (Taitec Co., Saitama, Japan). UCH-L1 expression was induced by the 1 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C. After 24 h incubation, the cells were collected by centrifugation, and the protein expressions were analyzed by Western blotting. Following purification steps were performed at 4 °C. UCH-L1 expressed BL21 cells were suspended in lysis buffer consisting of 50 mM Tris–HCl (pH 7.5), 0.1 mM sodium chloride, 10 mM 2-mercaptoethanol, and 5% glycerol. Sonicated-cell lysates were centrifuged at 100,000×*g* for 1 h and the supernatant was applied to a ProBond Nickel-Chelating