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Letter

S-Mercuration of ubiquitin carboxyl-terminal hydrolase L1 through Cys152 by methylmercury causes inhibition of its catalytic activity and reduction of monoubiquitin levels in SH-SY5Y cells

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ABSTRACT — Methylmercury (MeHg) is an environmental electrophile that covalently modifies cellular proteins. In this study, we identified proteins that undergo *S*-mercuration by MeHg. By combining two-dimensional SDS-PAGE, atomic absorption spectrometry and ultra performance liquid chromatography mass spectrometry (UPLC/MS/MS), we revealed that ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is a target for *S*-mercuration in human neuroblastoma SH-SY5Y cells exposed to MeHg (1 μM, 9 hr). The modification site of UCH-L1 by MeHg was Cys152, as determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. MeHg was shown to inhibit the catalytic activity of recombinant human UCH-L1 in a concentration-dependent manner. Knockdown of UCH-L1 indicated that this enzyme plays a critical role in regulating mono-ubiquitin (monoUb) levels in SH-SY5Y cells and exposure of SH-SY5Y cells to MeHg caused a reduction in the level of monoUb in these cells. These observations suggest that UCH-L1 readily undergoes *S*-mercuration by MeHg through Cys152 and this covalent modification inhibits UCH-L1, leading to the potential disruption of the maintenance of cellular monoUb levels.

Key words: Electrophile, Methylmercury, *S*-Mercuration, UCH-L1

INTRODUCTION

Methylmercury (MeHg) is a global organometal contaminant. Under certain conditions, MeHg-mediated toxicity involves the modification of cellular proteins through the *S*-mercuration reaction (Rabenstein and Saetre, 1977). Once MeHg invades cells, this environmental electrophile undergoes glutathione (GSH) conjugation, resulting in the excretion of a polar metabolite into the extracellular spaces through multidrug resistance-associated proteins (MRPs) (Toyama *et al.*, 2007; Kumagai *et al.*, 2013; Ballatori, 2002; Madejczyk *et al.*, 2007). We reported previously that MeHg activates transcription factor Nrf2 through *S*-mercuration of its negative regulator Keap1 and up-regulates its downstream genes, such as glutamate-cysteine ligase (GCL), a late-limiting enzyme for GSH synthesis, GSH *S*-transferases and MRPs, resulting in detoxification of the organomercury compound (Toyama *et al.*, 2007, 2011; Kumagai *et al.*, 2013; Itoh *et al.*, 1997; Vollrath *et al.*, 2006). However, MeHg exposure at higher concentrations causes dysfunction of central nervous systems (Harada, 1978, 1995) and daily intake of MeHg through a large predatory fish diet may represent a health risk (Grandjean *et al.*, 2010). High levels of MeHg target proteins such as tubulin and a disintegrin and metalloproteinases (Imura *et al.*, 1980; Wasteneys *et al.*, 1988; Tamm *et al.*, 2008). We also found that MeHg inactivates neuronal nitric oxide synthase, sorbitol dehydrogenase and arginase-I through *S*-mercuration of these proteins (Shinyashiki *et al.*, 1998;

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Kanda *et al.*, 2012, 2008). We therefore hypothesize that an approach to detect electrophilic modification of proteins by MeHg is required for understanding the toxicity or health effects of MeHg.

To determine post-transcriptional modifications of proteins, western blotting with antibodies against the modifier or radiolabeled compounds are used (Garcia *et al.*, 1974; Zheng and Hammock, 1996). We have developed previously an assay to detect chemical modifications using biotin-$PEAC_5$-maleimide (BPM), which can label free thiols (Toyama *et al.*, 2013; Abiko *et al.*, 2015). However, global analysis for detection of *S*-mercurated proteins is challenging to perform with these assays. In this study, we have identified molecular targets that undergo *S*-mercuration by MeHg in SH-SY5Y cells using a combination of two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D SDS-PAGE), atomic absorption spectrometry and ultra performance liquid chromatography mass spectrometry (UPLC/MS/MS).

MATERIALS AND METHODS

Materials

MeHg, avidin-HRP and avidin-agarose were obtained from Sigma-Aldrich (St. Louis, MO, USA). *Escherichia coli* BL21 cells and trypsin were purchased from Promega Co. (Madison, WI, USA). Anti-ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) and anti-GAPDH antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). BPM was obtained from Dojindo (Kumamoto, Japan).

Cells and cell culture

Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 with 10% fetal bovine serum, 2 mM L-alanyl-L-glutamine and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Cultured cells were seeded at a density of 1×10^5 cells/cm^2 on a culture plate and maintained at 37°C in a humidified incubator under an atmosphere of CO_2 (5%) and ambient air (95%). Before treatment, cells were cultured in serum-free medium overnight, and then were exposed to MeHg.

2D SDS-PAGE following thermal decomposition gold amalgamation atomic absorption spectrophotometry (AAS) analysis

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 the Bradford method. Cell lysates (50 μg of proteins) were applied to an Immobiline DryStrip pH 3-10, 7 cm (GE Healthcare, Buckinghamshire, UK) for 10 hr under silicon oil. Isoelectric focusing was performed for 1 min at 200 V, 90 min at 3500 V and 65 min at 3500 V using a Multiphor II (GE Healthcare). Proteins separated by isoelectric focusing were further separated by SDS-PAGE. The gel was stained with a CBB solution [0.1% Coomassie brilliant blue (w/v), 50% methanol (v/v), 7.5% acetic acid (v/v)] for 1 hr, and bleached for an additional 5 hr in bleaching solution [5% methanol (v/v) and 7.5% acetic acid (v/v)]. The gel was cut into pieces (3 × 3 × 1 mm) by a microtome blade and mercury concentrations in the obtained gels were analyzed by thermal decomposition gold amalgamation atomic absorption spectrophotometry (Mercury analyzer MA-3000; Nippon Instruments Corporation, Tokyo, Japan). Detection limit for Hg was 0.012 ng under the conditions used. The concentrations of Hg in the gels were expressed in Hg ng/segment.

Western blotting

Proteins separated by SDS-PAGE (Laemmli, 1970) were electro-transferred onto PVDF membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 2 mA/cm^2 for 1 hr, according to the method of Kyhse-Andersen (Kyhse-Andersen, 1984). After blocking with 5% skim milk, the membrane-bound proteins were incubated with primary antibodies. 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).

UPLC/MS/MS analysis

Identification of target proteins for MeHg was performed using a nanoAcquity UPLC system (Waters, Milford, MA, USA) coupled to a Synapt High Definition Mass Spectrometry (Synapt HDMS; Waters). The extracted proteins from the gel were digested with 5 μL of MS grade modified trypsin (100 ng) for 16 hr at 37°C. The eluted peptides (2 μL) were loaded onto 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 HDMS system, Waters) through a Teflon capillary union and a precut 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 to 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 sec as the external mass calibration.

Purification of recombinant human UCH-L1

RNA was extracted from the human neuroblastoma SH-SY5Y cells with Sepasol-RNA I super reagent (Nacalai Tesque Inc.) and complementary DNA (cDNA) synthesis was performed using Prime Script RT (Takara Bio, Shiga, Japan). Amplification of the UCH-L1 gene was performed by PCR with Prim Script (Takara Bio). cDNA corresponding to human UCH-L1 was cloned by the StrataClone Bluent PCR Cloning Kit (Stratagene, La Jolla, CA, USA). 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, USA). The plasmid sequence was verified on an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA). 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 addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM and the *E. coli* were grown at 30°C. After 24 hr incubation, the cells were harvested by centrifugation, and protein expression was analyzed by western blotting. The following purification steps were performed at 4°C. UCH-L1 expressed BL21 cells were suspended in lysis buffer consisting of 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (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 hr and the supernatant was applied to a ProBond Nickel-Chelating Resin (Invitrogen Co., Carlsbad, CA, USA) column (40 × 10 mm i.d.) that had been equilibrated with a 50 mM Tris-HCl (pH 7.5), 0.1 mM sodium chloride and 10 mM 2-mercaptoethanol solution. The affinity column was extensively washed with the equilibration buffer, and each protein was eluted by a linear gradient with this buffer containing 0-150 mM imidazole. Thiol groups oxidized during purification were reduced by incubation with 2 mM DTT for 1 hr; the DTT was removed in an Econo-Pac 10 DG column (Bio-Rad Laboratories Inc.).

BPM labeling assay

To detect the *S*-modification of recombinant UCH-L1, the BPM labeling assay was performed as described previously (Toyama *et al.*, 2013; Abiko *et al.*, 2015). Briefly UCH-L1 (0.3 μg) was reacted with MeHg in 20 mM Tris-HCl (pH 7.5) at 37°C for 30 min. The samples were further incubated with BPM (15 μM) at 37°C for 30 min. Aliquot of samples (12 μL) were mixed with 6 μL of SDS-PAGE loading buffer without 2-mercaptoethanol and subjected to SDS-PAGE. The BPM modifications were detected by Western blotting.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS)

The recombinant UCH-L1 protein (20 μM) was incubated with or without MeHg for 30 min at 37°C in buffer containing 20 mM Tris-HCl (pH 7.5). The UCH-L1 was digested by incubation with trypsin at 37°C for 16 hr. The peptides were mixed with α-cyano-4-hydroxycinnamic acid (2.5 mg/mL) containing 50% acetonitrile and 0.1% trifluoroacetic acid, and dried on stainless steel targets at room temperature. Analyses were performed using an AXIMA-TOF^2 (Shimadzu, Kyoto, Japan) with a nitrogen laser. All analyses were performed in the positive ion mode and the instrument was calibrated prior to each series of studies.

Short interfering RNA (siRNA) transfection

Transient transfection of siUCH-L1-1, -2 (GenixTalk, Osaka, Japan) and control siRNA (Qiagen, Valencia, CA, USA) was performed using the HiPerFect transfection reagent (Qiagen), according to the manufacturer's protocol. Briefly, siRNA duplex (0.6 μL) and the HiPerFect reagent (12 μL) were incubated with OPTI-MEM (Invitrogen) for 5 min at room temperature to encourage the formation of complexes. The complex was added into 35-mm dishes, in which 1×10^5 cells were seeded. The cells were incubated 36 hr in the CO_2 incubator and the medium was changed into serum-free medium for 12 hr before the experiment.

Statistical analysis

The band intensities were quantified by the ImageJ software, version 1.37. Statistical significance was assessed by the *t*-test. All *p* values are two tailed.

RESULTS AND DISCUSSION

Exposure of SH-SY5Y cells to 1 μM of MeHg for 9 hr led to the modification of a large number of cellular proteins, as detected by 2D SDS-PAGE/AAS (Fig. 1). Consistent with this, we indirectly detected *S*-modified proteins by the BPM labeling assay (Toyama *et al.*, 2013). To identify *S*-merculated proteins in SH-SY5Y cells exposed to MeHg, we selected five MeHg bound proteins with isometric points of 5-6 and higher covalent modification of MeHg (> 0.06 ng) (Fig. 1), and then identified these proteins by UPLC/MS/MS analysis. As shown in Table 1 and Fig. 1, spots 1, 2, 3, 4 and 5 were 150 kDa oxygen regulated protein, 78 kDa glucose regulated protein, nucleolin isoform CRA c, UCH-L1, UCH-L1 and heat shock protein beta 1, respectively. The 150 kDa oxygen regulated protein and 78 kDa glucose regulated protein, which are known to be chaperone proteins at the endoplasmic reticulum, have four and two cysteine residues, respectively (Bando *et al.*, 2000; Hendershot *et al.*, 1994; Wisniewska *et al.*, 2010; Ikeda *et al.*, 1997). Nucleolin isoform CRA c and heat shock protein beta 1 have a cysteine residue in the structure (Srivastava *et al.*, 1989; Sinsimer *et al.*, 2008). Of interest, UCH-L1 contained the highest amount of MeHg (0.116 ng), indicating that this deubiquitinating enzyme mediating stabilization of monoubiquitin (monoUb), is a key target of *S*-mercuration in SH-SH5Y cells.

To confirm that UCH-L1 is modified by MeHg, purified recombinant human UCH-L1 protein was incubated with MeHg for 30 min and the modification was detected by the BPM labeling assay. UCH-L1 was covalently modified by MeHg and thus its catalytic activity was markedly suppressed by MeHg in a concentration-dependent manner (Fig. 2A and Toyama *et al.*, unpublished observation). Under these conditions, MALDI-TOF/MS revealed that the modification site was Cys152 in UCH-L1 (Fig. 2C and Table 2) among the six cysteine residues in the protein (Fig. 2B). While Cys90 and His161 are found to be active site residues for UCH activity (Larsen *et al.*, 1996), Cys90 has been reported to not play an important role in the stabilization of monoUb (Osaka *et al.*, 2003). Interestingly, endogenous electrophiles such as 4-hydroxyl-2-nonenal and 15-deoxy-Δ12,14-prostaglandin J2 covalently bind to Cys152, resulting in aggregation and/or disruption of UCH-L1 enzyme activity (Koharudin *et al.*, 2010; Kabuta *et al.*, 2008; Li *et al.*, 2004). Consistent with this notion, we previously found that an endogenous dopamine derivative covalently modifies Cys152 in UCH-L1 and an atmospheric electrophile, 1,2-naphthoquinone, also modifies the Cys and inhibits the deu-

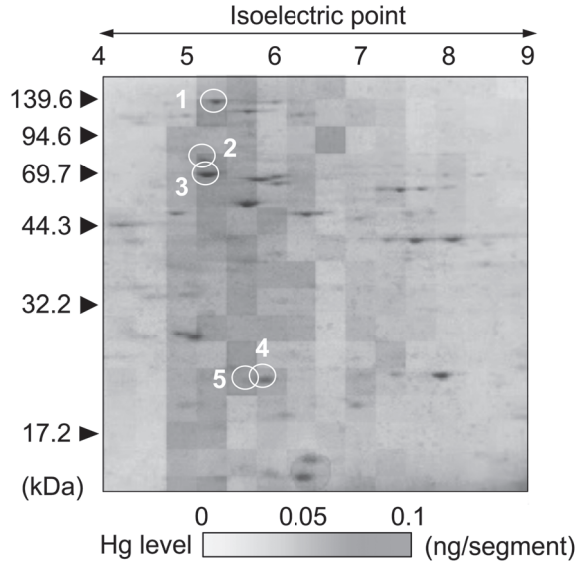

Fig. 1. Proteomics analysis of the target proteins for MeHg. SH-SY5Y cells were exposed to 1 μM of MeHg for 9 hr, and the cell lysates were then subjected to 2D SDS-PAGE following AAS analysis. The results of 2D SDS-PAGE and AAS analysis were merged and proteins in gels containing more than 0.06 ng of mercury were further digested with trypsin and analyzed by UPLC/MS/MS. The identified proteins in the circles are listed in Table 1.

Table 1. *S*-Mercurated proteins identified by 2D SDS-PAGE-AAS following UPLC/MS/MS.

Spot No.	Accession	Description	MW (Da)	pI (pH)	Coverage (%)
1	A8C1Z0	150 kDa oxygen regulated protein	111,266	4.97	11.71
2	P11021	78 kDa glucose regulated protein	72,288	4.87	52.91
3	B3KM80	Nucleolin isoform CRA c	58,519	4.36	32.84
4	P09936	Ubiquitin carboxyl terminal hydrolase isozyme L1	24,808	5.18	53.36
5	P09936	Ubiquitin carboxyl terminal hydrolase isozyme L1	24,808	5.18	59.64
5	P04792	Heat shock protein beta 1	22,768	5.96	57.07

"Coverage" corresponds to the percentage of identified amino acid sequence in the protein.

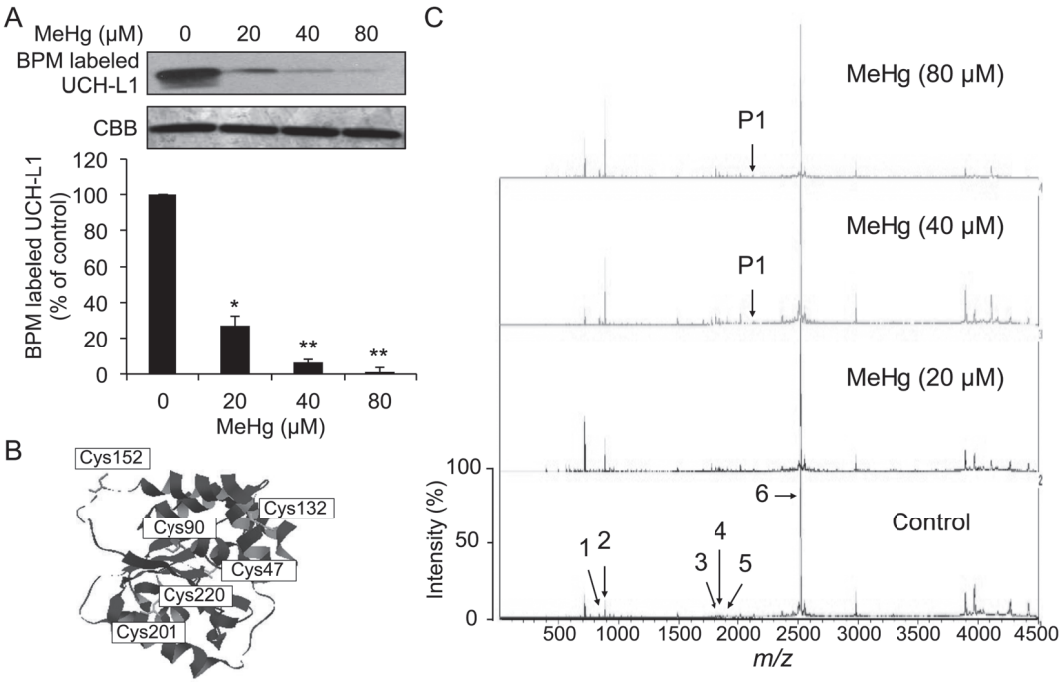

Fig. 2. *S*-Mercuration of UCH-L1 by MeHg. Recombinant UCH-L1 (20 μM) was incubated with indicated concentrations of MeHg for 30 min at 37°C and the BPM labeling assay was performed (A). Cysteine residues of UCH-L1 (B). The samples were digested with trypsin and analyzed by MALDI-TOF/MS (C). The corresponding peptide sequences of peaks marked with an arrow are shown in Table 2.

Table 2. Site of MeHg modification in UCH-L1.

Peak No.	Position	Peptide sequence	Calculated MS	Observed MS
1	214-221	FSAVALCK	838.45	838.5
2	20-27	LGVAGQWR	886.48	886.5
3	1-15	MQLKPMEINPEMLNK	1,815.9	1,815.7
4	179-195	MPFPVNHGASSEDTLLK	1,842.9	1,842.6
5	136-153	NEAIQAAHDAVAQEGQCR	1,910.87	1,910.6
6	158-178	NFHFILFNNVDGHLYELDGR	2,519.24	2,519.6
P1	136-153	NEAIQAAHDAVAQEGQ**C***R +MeHg	2,127.6	2,127.4

*MeHg-modified Cys. "Position" corresponds to the portion of the amino acid sequence of human UCH-L1.

biquitination activity of this enzyme in SH-SY5Y cells (Contu *et al*., 2014; Toyama *et al.*, 2014). Taken together, we suggest that residue Cys152 of UCH-L1 is a common target for not only endogenous but also exogenous electrophiles.

As shown in Fig. 3A, knockdown of UCH-L1 by siRNA significantly diminished the monoUb level. This result indicates that UCH-L1 predominantly stabilizes monoUb in SH-SY5Y cells. In addition to this, MeHg significantly inhibited UCH activity (Toyama *et al.*, unpublished observation) and presumably decreased cellular monoUb levels in a concentration-dependent manner (Fig. 3B).

In the present study, we found that UCH-L1 undergoes *S*-mercuration by MeHg through Cys152, and such a reaction reduces the catalytic activity, i.e., mono-ubiquitin levels, of UCH-L1 in SH-SY5Y cells. Loss of UCH-L1 activity, which is an abundant protein in neurons, causes neurodegeneration and UCH-L1 is linked to Parkinson's disease (Wilkinson *et al.*, 1989; Saigoh *et al.*, 1999; Kurihara *et al.*, 2001; Leroy *et al.*, 1998). Exposure of MeHg could be one of the risk factors associated with neurodegenerative disorders such as Parkinson's disease and the disruption of neuronal cells (Petersen *et al.*, 2008; Annau and Cuomo, 1988). Our study therefore indicates

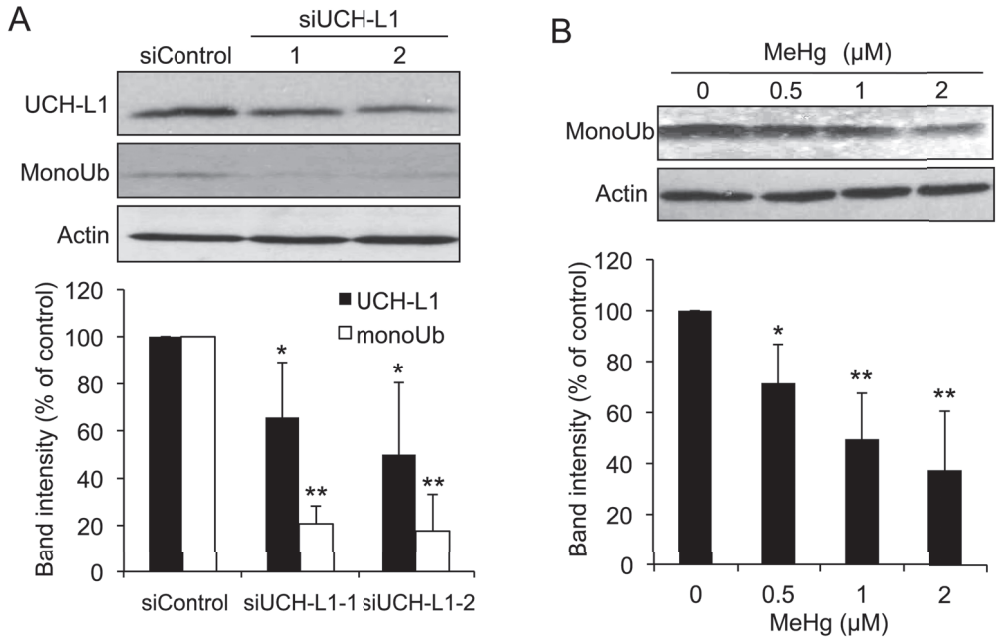

Fig. 3. Cellular mono-ubiquitin (monoUb) levels in SH-SY5Y cells. Control siRNA, UCH-L1 siRNA-1 or UCH-L1 siRNA-2 transfected SH-SY5Y cells were collected in 2% SDS and western blotting analysis was performed with the indicated antibodies (A). SH-SY5Y cells were treated with MeHg (0, 0.5, 1, or 2 μM) for 9 hr and western blotting analysis performed with the indicated antibodies (B). *$p < 0.05$, ** $p < 0.01$ *vs*. control. Each value is the mean ± S.E. of three determinations.

that inactivation of UCH-L1 by *S*-mercuration, resulting in a reduction of cellular mono-ubiquitin levels, might be, at least in part, involved in MeHg-mediated neurotoxicity.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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