Toxicogenomics/proteomics Report

siRNA-mediated AMPK α 1 subunit gene PRKAA1 silencing enhances methylmercury toxicity in HEK293 cells

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(Received February 27, 2010; Accepted April 3, 2010)

ABSTRACT — The environmental pollutant methylmercury is a potent neurotoxin. The mechanisms of toxicity and biological defense remain largely unknown. We found that inhibiting the expression of PRKAA1 (AMPK α 1), an activated subunit of AMP-activated protein kinase (AMPK), increased susceptibility of HEK293 cells to methylmercury toxicity. Treatment of the cells with AICAR (5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside), an AMPK activator, reduced the methylmercury toxicity. Here, we suggest for the first time that the activation (phosphorylation) of AMPK may play an important role in reducing the toxicity of methylmercury.

Key words: Methylmercury, AMP-activated protein kinase, PRKAA1 (AMPK α 1), AICAR

INTRODUCTION

Methylmercury causes serious disorders of the central nervous system (Clarkson and Magos, 2006). Antioxidative enzymes and antioxidants, for example, reduce the toxicity of methylmercury (Naganuma *et al.*, 1998; Usuki *et al.*, 2001; Watanabe *et al.*, 2009). According to several reports, the ubiquitin proteasome system, a selective proteolysis pathway, plays an important role in reducing the toxicity of methylmercury (Furuchi *et al.*, 2002; Hwang *et al.*, 2002, 2005, 2007 and 2009). However, other defense mechanisms might exist against the toxicity of methylmercury (Hwang *et al.*, 2007; Lee *et al.*, 2009). Hence, we introduced an siRNA library into HEK293 cells to investigate factors that make cells highly susceptible to methylmercury toxicity via decreased gene expression.

MATERIALS AND METHODS

Transduction of the siRNA library into HEK293 cells and microarray analysis

HEK293 cells, a human embryonic kidney cell line,

were maintained in Dulbecco's modified eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin in a humidified 5% CO₂ atmosphere at 37°C. A GeneNetTM human 50 K siRNA library (System Biosciences, Mountain View, CA, USA) that contained about 50,000 human genes was prepackaged in pseudoviral particles using the FIV lentiviral expression system. The detailed protocol for the transduction of the siRNA library is available from System Biosciences (Hwang *et al.*, 2007). Briefly, HEK293 cells (2 × 10⁶ cells/plate) were plated on five 10-cm plates and cultured in 10-ml aliquots of medium. After incubation for 24 hr, the culture medium was removed and the cells were infected with the pseudoviral particles (4 × 10⁶ ifu/plate) in plates containing 5 ml of DMEM/3% FBS with polybrene (final: 6 μ g/ml). After incubation for 12 hr, the culture medium was removed from each plate and replaced by 10 ml of DMEM/3% FBS without polybrene and then cultured for an additional 60 hr. The siRNA library-transduced cells (5 × 10⁶ cells) were plated into seven 10-cm plates containing 10 ml of DMEM/10% FBS medium. After incubation

for 24 hr, 3 μ M methylmercury chloride was added and the cells were cultured for an additional 48 hr. Total RNA was isolated with TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then, the purified RNA was reverse transcribed by M-MLV reverse transcriptase (Invitrogen) using a cDNA synthesis primer (5'-TG CAT GTC GCT ATG TGT TCT GGG A-3') specific to a sequence in the pFIV-H1 siRNA expression vector (System Bioscience). For microarray hybridization, the amplified siRNA targets were labeled with biotin and used as hybridization targets with GeneChip® arrays (HG_U133 set; Affymetrix, Santa Clara, CA, USA) employing standard protocols.

Construction of an siRNA expression plasmid

For cloning into the pFIV-H1 siRNA expression vector, two DNA oligonucleotide pairs were employed: (PRKAA1-1: 5'-GATCCGTCACGA-TAACTTATGAGAGAAGTAAAGCTTCCTGTCA-GACTTTGCTTCTCTTATAAGTTATTGTGATTTTTG-3' AND 5'-AATTCAAAAATCACAATAACT-TATAAGAGAAGCAAAGTCTGACAGGAAGCTT-TACTTCTCTCATAAGTTATCGTGACG-3'; AND PRKAA1-2: 5'-GATCCGCCTGTGATAAGCGCT-TACTCCAGAATGCTTCCTGTCAGACATTTT-GGAGTAAGTGCTTGTACAGGTTTTTTG-3' and 5'-AATTCAAAAACCTGTGACAAGCACTTACTC-CAAAATGCTCTGACAGGAAGCATTCTGGAG-TAAGCGCTTATCACAGGCG-3'), which encoded siRNAs that targeted the 4,095-4,121 and 1,354-1,380 regions of the gene PRKAA1, respectively. The oligonucleotides were annealed, phosphorylated by T4 DNA polynucleotide kinase (Takara, Shiga, Japan), and then ligated into the pFIV-H1 siRNA expression vector. The sequences of oligonucleotide inserts were verified by using an automated sequencer. Plasmid DNA was transfected with Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's protocols.

Confirmation of knockdown by real-time PCR or immunoblotting

For confirmation of the knockdown with the siRNA expression vectors expressing PRKAA1-1 and PRKAA1-2 siRNA, cells were lysed in TRIzol® reagent in order to isolate total RNA. First strand cDNA synthesis was performed using the PrimeScrip™ RT reagent kit (Takara). We performed real-time PCR reactions (Bio-Rad, iCycler™ thermal cycler) with the following primers: PRKAA1-F, 5'-CCAACTATGCTGCACCAGAA-3' and PRKAA1-R, 5'-TGGCACATGGTCATCATCA-3' for the PRKAA1 gene; and GAPDH-F, 5'-CGGGAAGCTT-

GTCATCAATGG-3' and GAPDH-R, 5'-GGCAGTGAT-GGCATGGACTG-3' for the GAPDH gene. The fold decreases in PRKAA1 mRNA levels were determined from standard curves after calibration of the assay. For immunoblotting, cells were fixed in 20% trichloroacetic acid on ice for 30 min and then washed five times with cold PBS. The cell pellet was solubilized with lysis buffer (1% SDS, 45 mM HEPES-Na pH 7.5). Cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA), and visualized using PRKAA1 (Upstate, Lake Placid, NY, USA)- or PRKAA2 (Cell Signaling, Danvers, MA, USA)-specific antibodies.

RESULTS AND DISCUSSION

An siRNA expression plasmid library against approximately 50,000 human genes was introduced into HEK293 cells using lentivirus. These siRNA-expressing cells were cultured for 48 hr in the presence of 3 μ M methylmercury chloride, a sufficient concentration to inhibit the growth of normal cells by about 10%. Subsequently, total RNA was extracted for microarray analysis using, as a probe, the PCR product specifically amplified from the sense strand of the siRNA sequence. The signal intensity of the probe obtained in this analysis indicates the percentage of living cells into which each siRNA was introduced. The results of the microarray analysis demonstrated that the signal intensity of the probe targeting the PRKAA1 gene was markedly reduced by treatment with methylmercury (data not shown). Then, two kinds of siRNA targeting different base sequences were synthesized and separately introduced into HEK293 cells, resulting in a susceptibility to methylmercury toxicity higher than that for the control cells (Fig. 1A). Both siRNAs markedly reduced the mRNA (data not shown) and protein levels of PRKAA1 compared with those of the control cells (Fig. 1B). These results suggest that inhibition of expression of PRKAA1 caused increased susceptibility of cells to methylmercury toxicity.

PRKAA1 is a catalytic subunit of AMPK (AMP-activated protein kinase) involved mainly in the maintenance of the homeostasis of intracellular energy metabolism, etc. (Hardie, 2005; Kahn *et al.*, 2005). AMPK is a heterotrimeric protein kinase consisting of α subunits (α 1 and α 2) exhibiting kinase activity and β subunits (β 1 and β 2) and γ subunits (γ 1, γ 2, and γ 3) involved in the control of the α subunits. PRKAA1 corresponds to the α 1 subunit of AMPK (Carling, 2004). AMPK is activated (phosphorylated) by an increased ratio of intracellular AMP/ATP and various stresses (Marsin *et al.*, 2002; Hardie, 2004;

Fig. 1. Effects of knockdown of PRKAA1 on HEK293 cell sensitivity to methylmercury toxicity. (A) HEK293 cells (2×10^4 cells), that had been stably transfected with PRKAA1 siRNA, were plated on 96-well plates and cultured in 100 μ l aliquots of medium. After incubation for 24 hr, methylmercury chloride (MeHg) was added and cells were cultured for a further 24 hr. Cell viability was determined by treating cells with Alamer Blue (Biosource, Camarillo, CA, USA). Each point and bar represents the mean value and S.D. of the results from three cultures, respectively. Data represents the mean $\pm$ S.D. of three replicates. * $P < 0.001$ as compared to control cells; ** $P < 0.005$ as compared to control cells; n.s., not significant. Data were analyzed using the Student's *t*-test. (B) Levels of PRKAA1 protein in HEK293 cells transfected with PRKAA1 siRNA were analyzed by immunoblotting. Levels of PRKAA2 protein, the other component of AMPK, served as a loading control.

Fig. 2. Effects of AMPK activator (AICAR) on HEK293 cell sensitivity to methylmercury toxicity. HEK293 cells (2×10^4 cells) were pretreated or not with AICAR (100 μ M or 200 μ M), which had been dissolved in DW (distilled water), and 3 hr later exposed to methylmercury chloride (MeHg) at the indicated concentrations. Data represents the mean $\pm$ S.D. of three replicates. * $P < 0.005$ as compared to control cells; ** $P < 0.01$ as compared to control cells; n.s., not significant. For further details, see the legend to Fig. 1A.

Sanders *et al.*, 2007). In cells carrying PRKAA1 siRNA, AMPK activity is presumed to be suppressed. Hence, decreased AMPK activity may make cells highly susceptible to methylmercury toxicity. The effects of treatment with AICAR (5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside), an activator of AMPK, on the susceptibility to methylmercury toxicity were examined. The toxicity of methylmercury (at the higher levels tested: 10 and 15 μ M MeHgCl) was significantly reduced depending on AICAR concentration (Fig. 2). Thus, the signaling, including AMPK activation, may function *in vivo* as a novel defense mechanism against the toxicity of methylmercury. However, there has been no report that AMPK functions as an energy sensor to reduce environmental pollutants including methylmercury. In the future, detailed studies on the relationship between AMPK phosphorylation and methylmercury should elucidate the mechanism of toxicity of methylmercury and the biological defense mechanism.

ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid for Scientific Research (S) from the Japan Society for the Promotion of Science.

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