Letter

Methylmercury induces CCL2 expression through activation of NF-κB in human 1321N1 astrocytes

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ABSTRACT — Methylmercury is an environmental pollutant that is toxic to the central nervous system; however, the molecular mechanisms underlying its toxicity remain unclear. Methylmercury increases expression of several chemokines in the cerebellum of mice treated with methylmercury. The present study analyzes the mechanism underlying methylmercury-induced chemokine expression using human 1321N1 astrocytes, and shows that methylmercury increases CCL2 expression in these cells. The transcription factor NF-κB is involved in the induction of chemokine expression. Methylmercury increased the level of the NF-κB p65 subunit in the nuclei of 1321N1 cells. The methylmercury-induced increase in CCL2 expression was significantly decreased by suppression of p65 expression by RNA interference. These results suggest that methylmercury induces chemokine expression through activation of NF-κB in human astrocytes.

Key words: Methylmercury, Chemokine, CCL2, NF-κB

INTRODUCTION

Methylmercury is the causative agent of Minamata disease and other severe central nervous system (CNS) disorders, such as senile paralysis and language disorder (Castoldi et al., 2003; Hirooka et al., 2010); however, the mechanisms underlying its toxicity remain unclear.

We analyzed gene expression changes in the cerebellum of mice treated with methylmercury and demonstrated increased expression of several chemokines in the cerebellum of mice treated with methylmercury. The present study analyzes the mechanism underlying methylmercury-induced chemokine expression using human 1321N1 astrocytes, and shows that methylmercury increases CCL2 expression in these cells. The transcription factor NF-κB is involved in the induction of chemokine expression. Methylmercury increased the level of the NF-κB p65 subunit in the nuclei of 1321N1 cells. The methylmercury-induced increase in CCL2 expression was significantly decreased by suppression of p65 expression by RNA interference. These results suggest that methylmercury induces chemokine expression through activation of NF-κB in human astrocytes.

MATERIALS AND METHODS

Cell culture and treatment

The human astrocytoma cell line 1321N1 was purchased from ICI Pharmaceutical Ltd (London, UK). Cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C. 1321N1 cells were plated at 5 × 10⁴ cells/well in 6-well plates and cultured for 24 hr, followed by treatment with 10 μM methylmercury chloride (MeHgCl) for 0, 3, 6, 12, and 24 hr.

Knockdown of the NF-κB p65 subunit using shRNA

NF-κB subunit p65/RelA (p65) in 1321N1 cells was knocked down by expression of shRNA using the Trans-Lentiviral Packaging System (Thermo Fisher Scientific, Waltham, MA, USA) according to the manu-
facturer’s protocol. Lentivirus was produced in HEK293T cells by co-transfection of the lentiviral vector pGIPZ encoding p65-shRNA and the Trans-Lentiviral Packaging Plasmid Mix (Thermo Fisher Scientific). 1321N1 cells were infected with lentivirus in the presence of polybrene (5 μg/ml) and selected in medium containing puromycin (1 μg/ml). The target sequence of p65 shRNA used was 5'-ACCATCAAGATCAATGGCT-3'.

**Quantitative real-time PCR (qPCR)**

Total RNA was isolated from cells using the Isogen II kit (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. First-strand cDNA synthesis was carried out using the PrimeScript™ RT reagent kit (Takara, Shiga, Japan). qPCR was performed using SYBR Premix EX Taq (Takara) (Hwang et al., 2011a; Takahashi et al., 2011). PCR primers used were GAPDH, 5'-GGGGAAGCTTGTCAATGG-3' (sense) and 5'-GGCAGTGATGGCATGGACTC-3' (antisense); p65, 5'-CTGCAGTTTGATGATGAAGA-3' (sense) and 5'-TAGGCGAGTTATAGCCTCAG-3' (antisense); and CCL2, 5'-CATTGTGGCCAAGGAGATCTG-3' (sense) and 5'-CTTCGGAGTTTGGGTTTGCTT-3' (antisense). Levels of P65 and CCL2 mRNA were normalized to that of GAPDH.

**Enzyme-linked immunosorbent assay (ELISA)**

CCL2 secreted into culture medium was quantified using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

**Preparation of nuclear and post-nuclear fractions**

1321N1 cells were lysed in ice-cold hypotonic buffer (10 mM HEPES-KOH [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 1 mM Dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) containing protease inhibitors (Roche Applied Science, Indianapolis, IN, USA) and 10% Nonidet P-40, and centrifuged at 15,000 × g at 4°C. The supernatant (post-nuclear fraction) was removed, and the pellet was resuspended in nuclear lysis buffer (10 mM HEPES-KOH [pH 7.9], 400 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 1 mM DTT, 5% glycerol) containing protease inhibitors (Roche Applied Science). The suspension was sonicated and centrifuged at 15,000 × g at 4°C to obtain the nuclear fraction. Protein concentration of each fraction was determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

**Immunoblotting**

Proteins in nuclear and post-nuclear fractions were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA). Immunoblotting was carried out using primary antibodies against p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), lamin A/C (Cell Signaling, Danvers, MA, USA), GAPDH (Santa Cruz Biotechnology), and horseradish peroxidase- (HRP-) coupled secondary antibodies (Dako A/S, Glostrup, Denmark). Immunoreactive proteins were detected by enhanced chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

**RESULTS AND DISCUSSION**

In the brain, chemokines are secreted primarily from glial cells (Giraud et al., 2010; Ito et al., 2006). We examined the effect of methylmercury on expression of the chemokine CCL2 in 1321N1 astrocytes, a type of human glial cell. CCL2 mRNA expression was not detectable in 1321N1 cells at steady state, but was observed after methylmercury treatment (Fig. 1A). CCL2 protein was undetectable in culture medium by ELISA at steady state, but increased in a time-dependent manner after treatment of cells with methylmercury (Fig. 1B).

To investigate the involvement of NF-κB in methylmercury-induced expression of CCL2, we knocked down the NF-κB p65 subunit in 1321N1 cells (Fig. 2A). Knockdown cells showed significantly smaller increases in CCL2 mRNA and extracellular CCL2 after methylmercury treatment than control cells (Figs. 2B and 2C). These results suggest that NF-κB is involved, at least in part, in CCL2 expression induced by methylmercury. Transcription factors other than NF-κB are also involved in modulating the expression of chemokines, including CCL2 (Fujimura et al., 2009; Kuper et al., 2012), and these may be involved in chemokine induction by methylmercury.

Nuclear transport of NF-κB is inhibited by binding of NF-κB to IkBα in the cytoplasm. IkBα is degraded by oxidative stress and as a result of the activities of TNFα and other cytokines, allowing NF-κB transport into the nucleus to promote transcription of target genes (Hoffmann and Baltimore, 2006; Sumi et al., 2010). When we investigated the effect of methylmercury on NF-κB activation, we found that the level of p65 in the nuclear fraction increased in a time-dependent manner after methylmercury treatment, whereas that in the cytoplasmic fraction decreased (Fig. 3). These results suggest that methylmercury induces CCL2 expression through activation of NF-κB. Recent-
Fig. 1. Effect of methylmercury on expression of CCL2 in human astrocytes. 1321N1 cells were treated with 10 μM MeHgCl for the indicated times. CCL2 mRNA level was measured by qPCR (A). CCL2 protein secreted into the culture medium was analyzed by ELISA (B). Values represent mean ± S.D. (n = 3). ND, not detected.

Fig. 2. Effect of knockdown of NF-κB subunit p65 on methylmercury-induced CCL2 expression in human astrocytes. Lysates were prepared from 1321N1 cells stably transfected with non-silencing (control) shRNA or p65-targeted shRNA. The level of p65 was measured by immunoblotting (A). The p65-knockdown cells were treated with 10 μM MeHgCl for the indicated times. CCL2 mRNA level was measured by qPCR (B). CCL2 protein secreted into the culture medium was analyzed by ELISA (C). Values represent mean ± S.D. (n = 3). ND, not detected.
Godefroy et al. (2012) reported that CCL2 functions defensively against methylmercury toxicity, although the underlying molecular mechanism is unknown. In future research, the detailed mechanisms underlying methylmercury induction of NF-κB activation and chemokine expression will be further explored, and perhaps reveal a novel mechanism of methylmercury neurotoxicity.

REFERENCES


