Attenuation of Macrophage Migration Inhibitory Factor-Stimulated Signaling via $\text{S}$-Nitrosylation

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Nitric oxide (NO) is a key signaling molecule that has various effects via $\text{S}$-nitrosylation, a reversible post-translational modification that affects the enzymatic activity, localization, and metabolism of target proteins. As chronic nitrosative stress correlates with neurodegeneration, the targets have received focused attention. Macrophage migration inhibitory factor (MIF) plays a pivotal role in the induction of gene expression to control inflammatory responses. MIF acts as a ligand for CD74 receptor and activates the Src-p38 mitogen-activated protein kinase (MAPK) cascade. MIF also elevates the expression of brain-derived neurotrophic factor (BDNF), which contributes to the viability of neurons. Here, we show that MIF is $\text{S}$-nitrosylated by a physiological NO donor. Interestingly, the induction of $\text{S}$-nitrosylation resulted in a loss of MIF activity following stimulation of the Src and p38 MAPK signaling pathways and the induction of BDNF expression. Our results shed light on the pathogenic mechanisms of neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease.

Key words nitric oxide; $\text{S}$-nitrosylation; macrophage migration inhibitory factor; brain

INTRODUCTION

Nitric oxide (NO) is a short-lived gaseous molecule that can be produced by three isoforms of NO synthase (NOS) (endothelial, neuronal, and inducible), generating L-citrulline from L-arginine. Under physiological conditions, a low concentration of NO regulates signaling pathways involved in neurotransmission, vasodilation, inflammation, and memory formation. However, chronic or excessive NO stress disrupts intracellular homeostasis, causing neurodegenerative diseases. One action of NO is to promote protein $\text{S}$-nitrosylation, a reversible post-translational modification conserved in plants, bacteria, and mammals. $\text{S}$-Nitrosylation is the covalent binding of NO to free cysteine residues to generate $\text{S}$-nitrosothiols. This modification may result in changes in the enzymatic activity, localizations, and/or metabolism of target proteins. We previously reported that the $\text{S}$-nitrosylation of protein disulfide isomerase and inositol-requiring enzyme 1α resulted in a loss of enzymatic function of these targets, and is related to the pathogenesis of neurodegenerative diseases, including Alzheimer’s disease (AD) and Parkinson’s disease (PD).

Macrophage migration inhibitory factor (MIF) is an inflammatory cytokine that induces the expression of various inflammatory mediators, such as tumor necrosis factor $\alpha$, Interleukin-1β and inducible NOS, and plays essential roles in inflammation and innate immunity. MIF also has a variety of biological properties, functioning as a tautomerase and oxidoreductase and acting as a chaperone. MIF acts as a ligand for the type II transmembrane receptor, the CD74/CD44 complex, to activate the phosphatidylinositol 3-kinase (PI3K)/Akt and p38 mitogen-activated protein kinase (MAPK) signaling pathways, which induce the expressions of brain-derived neurotrophic factor (BDNF). Here, we show that MIF is $\text{S}$-nitrosylated by a physiological NO donor. We also determined that the induction of $\text{S}$-nitrosylation resulted in a loss of MIF activities, such as the stimulation of PI3K/Akt and p38 MAPK signaling pathways, and the induction of BDNF expression.

MATERIALS AND METHODS

Materials and Antibodies Human recombinant MIF (rMIF) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Antibodies against MIF (ab187064; Abcam, Cambridge, U.K.), Src (ab109381; Abcam), phospho-Src (ab185617; Abcam), p38 (PM1381; ECM Biosciences, Versailles, KY, U.S.A.) and phospho-p38 (PM1391; ECM Biosciences) were purchased from the indicated vendors.

Cell Culture Human embryonic kidney (HEK) 293T, murine neuroblastoma Neuro-2a (N2a), and murine microglioma BV-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) heat-inactivated fetal calf serum at 37°C in a humidified atmosphere of 5% CO$_2$/95% air.

Induction of the $\text{S}$-Nitrosylation of rMIF Human rMIF was incubated with an NO donor, S-nitrosocysteine (SNOC; 100 µM), at room temperature for 30 min in the dark condition. The mixture of rMIF and decayed (old) SNOC was used as a negative control. After incubation, the mixtures were added to N2a or BV-2 cells.

Western Blot Analysis Cells were lysed in RIPA buffer (50 mM Tris–HCl pH 7.5, 0.15 M NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton-X and 1% sodium deoxycholate containing protease inhibitor cocktail (Roche, Basel, Switzerland).
After protein quantification with a BCA Protein Assay Kit (TaKaRa, Tokyo, Japan), cell lysates were boiled in 1x Laemmli buffer (62.5 mM Tris–HCl (pH 6.8), 5% 2-ME, 2% SDS, and 10% glycerol). Samples were subjected to Western blot analysis as previously described. SDS, and 10% glycerol. Samples were subjected to Western blot analysis as previously described. The quantification analysis of target proteins was performed using ImageJ software (NIS, Bethesda, MD, U.S.A.).

**Biotin-Switch Assay for S-Nitrosylated MIF (SNO-MIF)**

Total cell lysates from SNOC-exposed HEK293T or N2a cells were prepared in ice-cold RIPA buffer. Whole cell lysates (800 μg) were subjected to biotin-switch assay. Samples were mixed with blocking buffer (2.5% SDS and 10mM methylmethane thiosulfonate [MMTS] in HEN buffer) and incubated for 15 min at 50°C to block free cysteine residues. After removing excess MMTS by acetone precipitation, nitrosothiols were reduced by 12.5 mM ascorbic acid and then linked to the sulfhydryl-specific biotinylation reagent, HPDP-biotin (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Biotinylated proteins were pulled down using streptavidin agarose beads (Invitrogen, Carlsbad, CA, U.S.A.). Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed to identify S-nitrosylated proteins. Samples were also subjected to immunoblotting to confirm SNO modification of MIF. Before pull-down, 1/10 volume of samples was removed to determine total MIF (input).

**RNA Extraction and RT-PCR**

Total RNA was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO, U.S.A.). cDNA was synthesized using a First Strand cDNA Synthesis Kit (TaKaRa). Target cDNA was amplified using PCR (Emerald Amp PCR Mix; TaKaRa). The following primers were used: BDNF sense, 5'-GGT CGA GAG GTG CTG AGC-3'; BDNF antisense, 5'-GAC ATG TTT TGC GGC CCG-3'; β-actin sense, 5'-CCT GAC GGC CAG CTG ATC-3'; and β-actin antisense, 5'-GGA CTC GTCATA CTC CTG-3'. PCR products were separated on 2.0% agarose gels.

**Statistical Analysis**

All experiments were repeated at least three times independently. All data are expressed as the mean ± standard error of the mean (S.E.M.) Statistical comparisons were made using ANOVA with post hoc Bonferroni’s test in GraphPad Prism 7 software. The significance levels were set at *p < 0.05, **p < 0.01, ***p < 0.001.

**RESULTS AND DISCUSSION**

To identify S-nitrosylated proteins, SNOC-treated HEK293T cell lysates were subjected to the biotin-switch assay and LC-MS/MS analysis. In the course of our experiments, we found MIF peptide-containing biotinylated Cys81 (data not shown), as previously reported. In the previous report, SNO-MIF produced by a physiological NO donor was not confirmed; therefore, we attempted to detect it using Western blotting. Since murine neuroblastoma N2a cells generate MIF under normal conditions, we examined if NO results in SNO formation of endogenous MIF. As shown in Figs. 1A and B, the level of SNO-MIF increased in a dose-dependent manner following SNOC treatment in N2a cells. Our analysis confirmed that MIF is a target of NO.

Next, we analyzed the effects of SNO-MIF on p38 MAPK signaling in murine microglia BV-2 cells, since this cell line expresses MIF receptors. MIF works as a ligand for the CD74/CD44 complex and activates the Src kinase-p38 MAPK cascade. MIF was incubated with vehicle or SNOC to induce S-nitrosylation (see Materials and Methods). After incubation, the reaction mixture was added to BV-2 cells, and cell lysates were subjected to Western blotting. As expected, rMIF effectively induced the phosphorylation of Src kinase and p38 MAPK. However, SNOC-treated MIF failed to activate this kinase cascade (Figs. 2A–D). The activation of p38 MAPK signaling by MIF enhances the expression of target genes such as BDNF. To examine the effect of the SNO modification of MIF on gene expression, RT-PCR analysis was performed. Whereas untreated MIF upregulated BDNF transcription, SNOC-treated MIF failed to effect BDNF expression (Fig. 2E).

The S-nitrosylation site of MIF, Cys81, is located in a region that interacts with another MIF to form a homotrimer. Our analysis suggests that the S-nitrosylation of MIF mediates the conformational change to impair trimer formation and binding to the CD74 receptor. It is also possible that the transnitrosylation of CD74 results from SNO-MIF suppressing CD74 activities. Indeed, other groups have reported that protein interactions such as DJ-1–PTEN or caspase3–XIAP cause transnitrosylation.

We also revealed that SNO-MIF lost its ability to upregulate BDNF expression. In the central nervous system, MIF upregulates the expression of BDNF to encourage survival...
and synapse formation in neurons. Interestingly, the brain BDNF level is decreased in AD and PD patients. In this study, we found that SNO-MIF suppressed Src and p38 MAPK signaling and following BDNF induction. It is widely recognized that BDNF plays a critical role of neuronal survival and is involved in the protective function against several stresses. Dysfunction of MIF induced by NO may be induce the loss of neuronal viability related to neurodegenerative disorders. Moreover, many studies have suggested that nitrosative stress contributes to the pathogenesis of these diseases.

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Conflict of Interest The authors declare no conflict of interest.

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