Enhancement of Nuclear Localization of Metallothionein by Nitric Oxide

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Nitric oxide (NO) enhanced the nuclear localization of metallothionein (MT) in digitonin-permeabilized semi-intact HeLa cells. Although 1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC5), an NO donor, enhanced the nuclear localization of MT in a manner similar to hydrogen peroxide (H_2O_2), a tenfold higher concentration of NOC5 was required to achieve the same effect as H_2O_2, suggesting that an endogenous NO scavenger existed in the reaction mixture of the nuclear import assay system that we used. We also evaluated the effect of NO on the nuclear localization of MT in intact HeLa cells. Treatment with NOC5 induced the nuclear localization of MT pre-induced with zinc, and its effect was greater than that of H_2O_2. The induced MT was localized in the nucleus and the cytoplasm. The results suggest that MT can scavenge NO using the sulfhydryl groups of cysteines in its molecule to form nitrosothiol, thereby reducing nuclear and cytoplasmic damage by NO.

Key words —— metallothionein, nitric oxide, nucleus, nitrative stress, digitonin

INTRODUCTION

Metallothionein (MT) is a low molecular weight (6–7 kDa) protein that is rich in cysteinyl residues. Its characteristic structure, *i.e.*, high cysteine content, has inspired researchers to propose the following roles for MT, *e.g.*, detoxification of non-essential and excess essential metals, homeostatic regulation of essential metals, intracellular transport of metals, and scavenging of free radicals, such as reactive oxygen species (ROS).[1–4] Although MT is primarily localized in cytoplasm, it becomes karyophilic due to several pathological and physiological stimuli.[5–7] It has been reported that hydrogen peroxide (H_2O_2) induces the nuclear localization of MT in digitonin-permeabilized semi-intact cells and intact cells depending on the cell cycle.[8,9] Karyophilic MT induced by H_2O_2 treatment is suggested to play the role of a nuclear anti-oxidant.[10]

Nitric oxide (NO) is endogenously produced by three nitric oxide synthases (NOSs) and has several physiological roles, including neurotransmission and vasorelaxation.[11] NO induces stress in cells by disturbing the thiol/disulfide balance to form a nitrosothiol via interactions with intracellular reductants, such as glutathione (GSH) and MT.[12,13] Thus, although a cell may have protective mechanisms against this negative effect of NO, *i.e.*, nitrative stress, it remains unclear what those mechanisms are. Since MT can act as a nuclear anti-oxidant against oxidative stress induced by H_2O_2, it is also expected to exhibit a protective role against nitrative stress due to its high cysteine content.

In this letter, we present the results of evaluation of the effect of NO on the nuclear localization of MT in semi-intact and intact cells and clarify the involvement of MT in nitrative stress.

MATERIALS AND METHODS

Chemicals —— Digitonin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rabbit MT and Fluorolink™ Cy3 monofunctional dye were purchased from Sigma (St. Louis, MO, U.S.A.) and GE Health Science (Tokyo, Japan), respectively. 1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC5), an NO donor, was purchased from DOJINDO (Kumamoto, Japan). Deionized water (18.3 MΩ/cm) was used throughout. All reagents were of the highest or analytical grade.

Cell Culture —— HeLa S3 (human cervical carcinoma) cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS; Wako), 100 U/ml penicillin (Invitrogen Corporation, Carlsbad, CA, U.S.A.), and 100 μg/ml streptomycin

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(Invitrogen Corporation) at 37°C under 5% CO_2 atmosphere.

Preparation of Fluorescence-labeled MT —— Rabbit MT (1 mg/ml) was conjugated with Cy3 dye according to the manufacturer's instructions. Unconjugated MT and excess dye were separated from Cy3-labeled MT with a Sephadex G-50 column (GE Health Science) while monitoring the absorbance of Cy3 at 552 nm.

Nuclear Transport Assays with Digitonin-permeabilized Cells —— Nuclear transport assays were performed according to a previous report,[10] using digitonin-permeabilized cells and mouse red blood cell (RBC) lysate as the cytosolic source because of the absence of nuclear proteins. Treatment of cells with low concentrations of digitonin selectively permeabilizes the plasma membrane due to its relatively high cholesterol content, while most other cellular membranes, including nuclear envelope (which is cholesterol poor), remain intact, and the cytoskeleton is largely undisturbed. Thus, this technique is very useful to evaluate the nuclear localization system.[14] Briefly, subconfluent HeLa cells growing on 25-mm collagen-coated cover glasses in 6-well plates were incubated in an ice-water bath with 30 μg/ml digitonin in import buffer (containing 20 mM HEPES-KOH, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, and 3 μg/ml aprotinin) for 10 min. Following three washes with the import buffer, the cover glasses were incubated with 100 μl of the import mixture (containing 1 mM ATP, 1 mM GTP, 5 mM creatine phosphate, 20 U/ml creatine phosphokinase, 5 μg/ml Cy3-MT, 0.2 mM NaN_3, and 1.7 mg/ml RBC lysate with or without 10 mM NOC5 or 1.0 mM H_2O_2 in the import buffer) at 30°C for 30 min in a humidified plastic box. After washing the cells with the import buffer, they were fixed with 10% formaldehyde in phosphate buffered saline (PBS) for 3 min, and the cover glasses were each mounted on a slide glass. The slides were observed under a Zeiss LSM 510 confocal laser scanning microscope.

Nuclear Localization of MT in Intact Cells Induced by NO Donor —— Nuclear localization induced by NO in intact cells was evaluated. Subconfluent HeLa S3 cells growing on 25-mm collagen-coated cover glasses were incubated with 10 μM $ZnCl_2$ for 24 hr in MEM. After washing twice, the cells were incubated with 0, 0.5 or 1.0 μM NOC5 or 1.0 μM H_2O_2 for 30 min in MEM. Then, the cells were fixed with 10% formaldehyde in PBS for 30 min and permeabilized with 0.1% Triton X-100 for 30 min at room temperature. The slide was blocked with 1% skim milk in PBS for 30 min and then incubated with anti-MT monoclonal antibody (E9; Zymed, San Francisco, CA, U.S.A.) at 37°C for 4 hr. The cells were washed three times for 1 min each with PBS containing 0.1% Tween 20 and 0.5% skim milk, and then incubated with fluorescent dye (Alexa Fluor® 488)-conjugated anti-mouse IgG (Molecular Probe/Invitrogen Japan K.K., Tokyo, Japan) at 37°C for 1 hr. After washing three times in PBS containing 0.5% skim milk for 5 min each, fluorescence microscopy was performed with the Zeiss LSM 510 confocal laser scanning microscope.

RESULTS AND DISCUSSION

Enhancement of MT Nuclear Localization with NO Donor in Digitonin-permeabilized Semi-intact Cells

Although macromolecules larger than 20 kDa are required to form the nuclear pore complex that enters a nucleus, MT easily permeates the nuclear pore due to its molecular size (< 7 kDa).[10,15] Indeed, Cy3-labeled MT was detected in the nuclei of digitonin-permeabilized semi-intact cells (Fig. 1A). Treatment with 10 mM NOC5, an NO donor, enhanced the nuclear accumulation of MT in the same manner as treatment with 1.0 mM H_2O_2, suggesting that NO, as well as H_2O_2, promoted the nuclear localization of MT (Fig. 1B and 1C). One molecule of NOC5 liberates two molecules of NO, that is, 10 mM NOC5 is equivalent to 20 mM NO. However, a 20-fold higher concentration of NO was required to achieve the same effect as H_2O_2 on the enhancement of MT localization. The import mixture consisted of RBC lysate as cytosolic source to construct the nuclear import machinery, and RBC lysate contained catalase that catalyzes the decomposition of H_2O_2. Thus, the inhibitor of catalase, NaN_3, was simultaneously added to the import mixture to prevent the decomposition of H_2O_2.[10] In addition, RBC lysate might contain not only H_2O_2 scavengers but also NO scavengers. For instance, it has been reported that hemoglobin, one of the components of RBC lysate, binds NO to form *S*-nitrosohemoglobin, indicating that hemoglobin acts as an endogenous NO scavenger.[16] Although the import mixture contained the catalase inhibitor, NaN_3, no NO inhibitors were added. Therefore, the effect of NO on the enhancement of MT localization

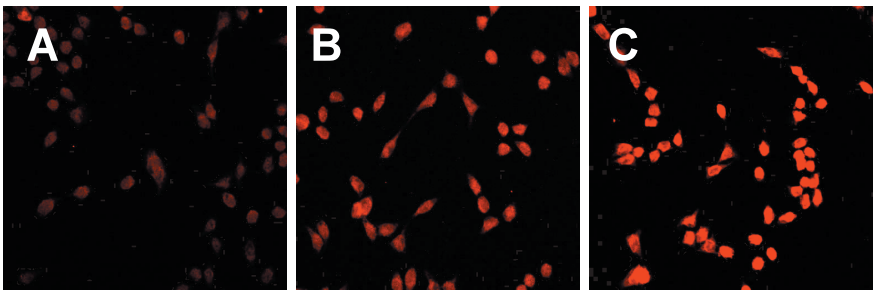

Fig. 1. Nuclear Localization of MT is Enhanced by NO Donor in Digitonin-permeabilized Cells

The complete system contained mouse RBC lysate, sodium azide, an ATP-regenerating system, and Cy3-labeled MT. A: Control, B: 10 mM NOC5, C: 1.0 mM H_2O_2.

was one-twentieth lower than that of H_2O_2. The enhancement effect of NO was actually observed; however, the required concentration was so high compared with the physiological concentration of NO detected *in vivo*. The effect of NO on the enhancement of MT localization should be also evaluated in intact cells in addition to semi-intact cells.

Effect of NO Donor on MT Nuclear Localization in Intact Cells

MT pre-induced with Zn was preferably localized in nuclei (Fig. 2A). Treatment with 0.5 or 1.0 μM NOC5 enhanced MT induction, and induced MT was distributed to not only the nuclei but also the cytoplasm (Fig. 2B and 2C). It was already reported that NO donors, such as sodium nitroprusside, *S*-nitroso-*N*-acetyl-DL-penicillamine, and spermine NONOate, induced one of the MT isoforms at concentrations higher than 0.1 mM.[17] The effect of NOC5 on MT induction appeared at the concentration of 0.5 μM. Thus, considering the concentration of NOC5 for MT induction, it seems to exert a synergistic effect with Zn on MT induction. It was reported that NO induced the nuclear localization of MTF-1, a Zn-dependent transcription factor of MT.[18] Therefore, the combined treatment of Zn and NOC5 effectively induced MT at the transcriptional level, and the induced MT seemed to distribute to the cytoplasm after filling the nucleus. On the other hand, 1.0 μM H_2O_2 also synergistically induced MT with Zn, which was preferably distributed to the nuclei despite the fact that the effect of H_2O_2 was weaker than that of NOC5. It is suggested that MT induced with H_2O_2 acts as a nuclear anti-oxidant to scavenge H_2O_2.[8] MT can also bind NO using the sulfhydryl groups of cysteines in its molecule to form nitrosothiol, thereby reducing nuclear and cytoplasmic damage by NO.

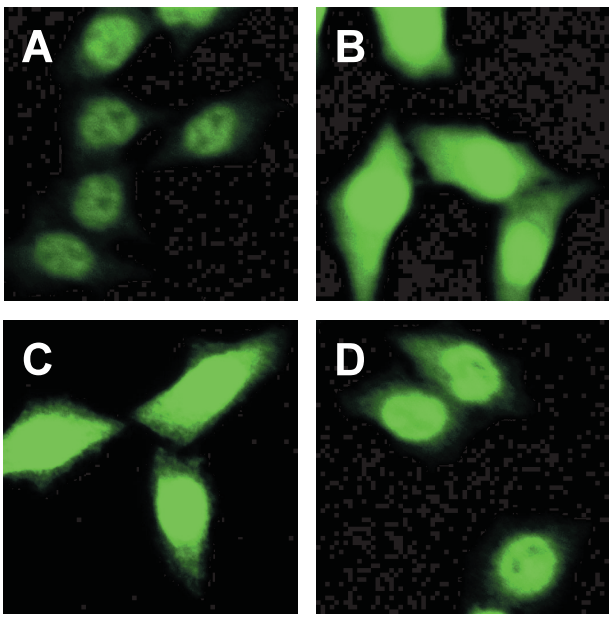

Fig. 2. Nuclear Localization of MT in Intact Cells Following Treatment with NO Donor

HeLa S3 cells, together with MT pre-induced with Zn, were incubated with NOC5 and H_2O_2 in MEM for 30 min. Cells were stained with anti-MT monoclonal antibody. A: Control, B: 0.5 μM NOC5, C: 1.0 μM NOC5, D: 1.0 μM H_2O_2.

Although the mechanism underlying the effect of H_2O_2 on the nuclear localization of MT is still unclear, several mechanisms, such as the presence of a nuclear retention factor or a cargo protein (chaperone) for MT in the nucleus, have been suggested.[15, 19, 20] In those mechanisms, the most characteristic feature of MT, *i.e.*, the abundance of sulfhydryl groups, may play a key role in the interaction between the factors and MT. As mentioned above, NO is bound to MT via a sulfhydryl group; thus, the enhancement effect of NO on the nuclear localization of MT was suggested to have the same mechanism as that of H_2O_2.

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