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Activation of NUDT5, an ADP-ribose pyrophosphatase, by nitric oxide-mediated ADP-ribosylation

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Abstract

The ADP-ribose (ADPR) pyrophosphatase (ADPRase) NUDT5, a member of a superfamily of Nudix hydrolases, hydrolyzes ADP-ribose (ADPR) to AMP and ribose 5′-phosphate. Nitric oxide (NO) enhances nonenzymatic ADP-ribosylation of proteins such as β-actin and glyceraldehydes 3-phosphate dehydrogenase in the presence of free ADPR, suggesting a possibility that NUDT5 could also be ADP-ribosylated by its substrate, ADPR. Here, we show that NO stimulates nonenzymatic ADP-ribosylation of NUDT5 using ADP-ribose and consequently activates its ADPRase activity. We found that ADPRase activity in J774 macrophage cells is increased by the treatment with SNP, an exogenous NO generator or TNF-α/IFN-γ, endogenous NO inducers. Anti-NUDT5 antibody pulled down most of the ADPRase activity increased by NO, indicating that the ADPRase regulated by NO is NUDT5. Using recombinant human NUDT5, we also demonstrated that the increase of ADPRase activity is mediated via ADP-ribosylation at cysteine residue(s) in the presence of reductant. This result suggests that NO activates NUDT5 through ADP-ribosylation at cysteine residues of the enzyme in macrophages.

Keywords: NUDT5; ADP-ribose pyrophosphatase; ADP-ribosylation; Nitric oxide; Macrophage

ADP-ribose (ADPR) is formed by NAD glycohydrolase-mediated hydrolysis of NAD, ADPR hydrolase-mediated hydrolysis of poly-ADPR and protein-bound mono-ADPR, and cADPR hydrolase-mediated hydrolysis of cADPR [1,2]. ADPR is further metabolized by ADP-ribose pyrophosphatase (ADPRase) into AMP and ribose-5-phosphate [3]. Although the biological function of ADPRase has been suggested as a cleaner for potentially toxic ADPR or a regulator for certain ion channels [4,5], the exact function of the enzyme remains to be elucidated.

ADPRases have been identified both by their purification from tissue extracts and by heterologous expression of cDNA sequences containing the so-called Nudix box which all members of the family of Nudix hydrolases contain [6,7]. In rat liver extract, four distinct ADPRases have been identified: three cytosolic ADPRases (ADPRase-I, -II, and –Mn) and one mitochondrial ADPRase (ADPRase-m). They are known to have diverse substrate specificities for ADPR and other nucleoside-X compounds that are partly dependent on the nature of necessary activating cation (Mg^{2+} or Mn^{2+}) used for the assays. ADPRase-I and ADPRase-m share many characteristics, such as low K_m and high specificity for ADP-ribose and IDP-ribose. Their enzymatic features are comparable to those of recombinant NUDT9 [5,8,9]. Cytosolic ADPRase II has a high K_m and low specificity for ADPR. It may correspond to human NUDT5 [3,10]. ADPRase–Mn degrades dinucleotides and CDP–alcohols in the presence of Mn^{2+} and is inactive in the presence of Mg^{2+}.

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ADPR, the substrate for ADPRase, has a reactive aldehyde group that may induce nonenzymatic ADP-ribosylation of protein. Nitric oxide (NO) has been known to stimulate nonenzymatic ADP-ribosylation [11]. Therefore, it is likely that NO could modulate ADPRase activity. Indeed, activity of ADPRase I from rat liver comparable to that of human NUDT9 is inhibited by exogenous NO generating system [12]. However, it has not been known whether NO affects activity of NUDT5 comparable to ADPRase II [3,10]. In this study, we revealed an unexpected data that NO activates NUDT5 activity unlike ADPRase I (NUDT9), which is mediated via NO-induced ADP-ribosylation of cysteine residue on the enzyme.

Materials and methods

Materials. The murine monocyte/macrophage cell line J774A.1 was obtained from the American Type Culture Collection. *N*-1-Naphthylethylene diamine, sulfanilamide, LPS, N(G)-nitro-L-arginine methyl ester (L-NAME), TNF-α, and IFN-γ were from Sigma chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was from Hyclone Laboratories (Logan UT).

Cell culture. The J774 cells were cultured at 37 °C in a humidified 5% CO_2 incubator in DMEM containing 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cells were plated in six culture wells at a density of 1.5×10^6 cells per well.

Preparation of recombinant NUDT5. Briefly, *NUDT5*, the human gene for ADPRase was amplified by reverse transcriptase-polymerase chain reaction. The oligonucleotide primers containing restriction enzyme sites were synthesized (Bioneer, Korea). The primers were 5′-GAGG TAGAATTCATGGAGAGCCAAGAACCAA-3′ and 5′-AAAATTGT CGACTTAAAATTTCAAGAAGGGCACT-3′. The resulting PCR product was ligated with pET vector (Novagen, Darmstadt, Germany). This plasmid was transformed into BL21 cells for expression. A transformant was cultured in LB media at 37 °C to an A_{600} of about 0.7 and 1 mM IPTG was added for 3 h to induce the expression of *NUDT5*. Bacterial lysates were prepared by sonication twice for 10 s on ice and centrifuged at 12000*g* for 15 min. The supernatant was purified using Ni^{2+}/NTA agarose (Qiagen, CA). The purified NUDT5 was analyzed by SDS–PAGE (12% w/v gel).

Preparation of anti-human NUDT5. Recombinant human NUDT5 was purified as described above. A rabbit serum antibody against the recombinant human NUDT5 was prepared after three intramuscular injections of the purified protein (performed by Takara Korea).

Fluorometric assay for ADPRase activity. ADPRase activity was assayed as described elsewhere [13] by measuring the fluorescence of 1,N^6-etheno-adenosine formed by alkaline phosphatase from ε-AMP, a product generated through 1,N^6-etheno-ADPR hydrolysis by ADPRase. ε-Adenosine was separated from the reaction mixture through the anionic exchange resin, AG MP-1. The reaction mixture contained 500 μM ε-ADPR, 4 mM $MgCl_2$, 125 mM glycine–NaOH, pH 9.0, bovine serum albumin 1 mg/ml, 10 U of alkaline phosphatase and cell lysate in a total volume of 200 μl. After incubation for 30 min at 37 °C, the reaction was terminated by adding 2 mM EDTA, and the mixture was added to 500 μl of 20% AG-MP1 (Bio-Rad, CA) in 10 mM Tris–HCl, pH 10.0. After shaking for 10 min and centrifugation at 12,000*g* for 3 min, the supernatant containing ε-adenosine was diluted 2-fold with 0.1 M sodium phosphate buffer (pH 7.2), and fluorescence was measured with Hitachi F-2000 fluorometer. The relative fluorescence intensity was determined at excitation and emission wavelengths of 297 and 410 nm, respectively. Fluorescence change was calculated by subtracting the measured fluorescence from the blank value obtained with a reaction mixture without sample.

Western blotting. Cells (2×10^5 cells) were lysed with cell-lysis buffer (20 mM Hepes, pH 7.2, 0.1 M NaCl, 50 mM NaF, 1 mM $NaVO_4$, 1 mM EGTA, 0.1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 30 min at 4 °C. The 20 μg of cell lysate described above was mixed with 2× SDS sample buffer and boiled for 3 min. The samples were electrophoresed in a 12% polyacrylamide minigel at 100 V for 1 h. The proteins were transferred onto nitrocellulose membranes, according to the manufacturer's instructions (Bio-Rad, CA). The nitrocellulose paper was blocked with 5% BSA and then incubated with rabbit anti-human ADPRase (1:1000) at 4 °C overnight. The membranes were washed five times with 1% Triton 100-X in Tris-buffered saline and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG Ab (1:500) for 1 h. The immunoblots were visualized by an alkaline phosphatase substrate kit.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from J774 cells using Trizol® Reagent (Invitrogen, CA) following the manufacturer's instructions. The extracted RNA was then reverse transcribed to cDNA at 42 °C for 1 h by adding 1 μg RNA per sample to a reaction mixture that contained random primer 0.5 μg, Im Prom-II reaction buffer, 5 mM $MgCl_2$, 0.5 mM dNTP, 100 U reverse transcriptase. The reverse-transcribed cDNA was amplified using PCR core system (Promega, WI). Two microliters of cDNA were added to reaction mixture that contained reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, Taq polymerase (2 U) and primers. Primers for NUDT5 were 5′-TAA AAC CAG AAC TTG GGA AA-3′and 5′-TTC ACC TTT GTA GCC AGT TT-3′. 18S RNA primer was used as internal standard (Ambion, TX). The samples were cycled as follows: 94 °C for 1 min, 49 °C for 30 s and 72 °C for 40 s (30 cycles). The PCR products were run on a 1.5% agarose gel containing ethidium bromide at 100 V for 1 h. The bands were visualized under UV light.

ADP-ribosylation of ADPRase. To identify ADP-ribosylation in NUDT5, 100 μM [^{32}P]ADPR and 100 ng of NUDT5 were incubated at 37 °C for 15 min with or without 1 mM DTT and/or 1 mM SNP. The samples were boiled with 2× SDS sample buffer for 3 min. The samples were electrophoresed in a 12% polyacrylamide minigel at 100 V for 1 h. The proteins were transferred onto PVDF membrane and autoradiographed. And to examine ADP-ribosylation in cysteine residue, PVDF membrane was incubated at 45 °C for 3 h under 10 mM $HgCl_2$ which cleaves ADP-ribosyl cysteine bond and autoradiographed.

The effect of endogenous NO on ADPRase activity in J774 cells. The cells were cultured in six culture plates at a density of 5×10^5 cells per well. After an overnight incubation, the medium was replaced with a fresh medium, and cells were treated with TNF-α 1 ng/ml and IFN-γ 1 ng/ml (0, 1, 3, 6, 12, 24 h) with or without 1 mM L-NAME, a nitric oxide synthetase (NOS) inhibitor. The supernatants were sampled for the measurement of NO concentration. The cell lysates were obtained as described above and subjected to Western blot analysis and ADPRase activity assay.

Nitrite determination. NO was measured as nitrite accumulated in media after the treatment with TNF-α plus IFN-γ. Briefly, Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in phosphoric acid) was added to an equal volume of cell supernatant and the absorbance at 550 nm was measured after 10 min. Sodium nitrite was used as a standard.

Statistical analysis. All experimental data are means ± standard deviation (SD). Statistical analysis was performed using Student's *t* test, and $P < 0.005$ was considered to be significant.

Results

The increase of ADPRase activity by the treatment with SNP in J774 macrophage cells

ADPRase-I, which is comparable to NUDT9, is nonenzymatically ADP-ribosylated by its substrate, ADPR and the ADP-ribosylation is stimulated by NO, resulting in inhibition of ADPRase activity of ADPRase-I [12]. We therefore questioned whether NO could also affect the ADPRase activity of NUDT5 that possesses a different enzymatic characteristic than that of NUDT9. By immunoblot analysis, we observed that J774 murine macrophage