Effect of Nitric Oxide on ADP-ribose Pyrophosphatase Activity

Jong-Hyun Kim

Department of Obstetrics & Gynecology, Chonbuk National University Medical School, Jeonju, Korea

ABSTRACT

Background: ADP-ribosyl pyrophosphatases (ADPRase) has been known to catalyze the hydrolysis of ADP-ribose to ribose-5-phosphate and AMP. The role of ADPRase has been suggested to sanitize the cell by removing potentially toxic ADP-ribose. In this study, we examined the effect of nitric oxide on ADPRase activity in macrophages. **Methods:** ADPRase activity was measured in NO-inducing J774 cells. For in vitro experiments, recombinant human ADPRase was prepared in bacteria. **Results:** ADPRase activity was increased by the treatment of exogenous NO generating reagent, sodium nitroprusside (SNP), in J774 cells. The increased ADPRase activity was mediated by the post-translational modification, likely to cause cADP-ribosylation via nitrosylation of cysteine residue on the enzyme. The stimulation with endogeneous NO inducers, TNF-α/IFN-γ, also increased ADPRase activity through NO synthesis. Futhermore, ADPRase activity may be mediated by the post-translational modification of ADPRase, ADP-ribosylation. **Conclusion:** These results indicate that NO synthesized by macrophage activation plays a critical role in the increase in ADPRase activity following ADP-ribose metabolism. **(Immune Network 2005;5(4):199-204)**

Key Words: Nitric oxide, ADP-ribose pyrophosphatase, macrophage, ribosylation

Introduction

ADP-ribose (ADPR) has been known to be produced from the hydrolysis of cyclic ADPR by NAD^+ glycohydrolase and to be the second messenger of calcium-induced calcium release (1-3). It is further metabolized by ADP-ribose pyrophosphatase (ADPRase), which catalyzes hydrolysis of ADPR to AMP and ribose-5-phosphate. ADPRase is widely distributed in various organisms including *Artemia franciscana*, embryonic cysts, rat mitochondria and human erythrocytes (4-6). So far, eleven ADPRases have been cloned and characterized from archaea, eubacteria and eukaryotes (7-11). All cloned ADPRases belong to the family of nucleoside diphosphate linked to another moiety X (Nudix) hydrolases, a group of phosphoanhydrases that catalyze the hydrolysis of Nudix (12). Members of Nudix family contain consensus sequence $GX_5EX_7REUXEEXGU$ (where U represents Ile, Leu or Val, and X represents any amino acid), which forms part of the versatile catalytic site for diphosphate hydrolysis.

Several enzymes possessing ADPRase activity have also been purified from animal tissues. Upon the traditional protein purification approach, four distinct ADPRases including three cytosolic ADPRases (ADPRase-I, -II, and -Mn) and a mitochondrial ADPRase-m have been found in the rat liver extracts (13). The cytosolic ADPRase-II has a high K_m and low specificity for ADP-ribose. It may correspond to human erythrocyte ADPRase and human NUDT5. The cytosolic rat liver ADPRase-I and the rat liver mitochondrial ADPRase have very similar characteristics to each other, including a low K_m and high specificity for ADP-ribose and IDP-ribose, while ADPRase-Mn is inactive with Mg^{2+} and degrades dinucleotides and CDP-alcohols in the presence of Mn^{2+} (14). Recently, it has been shown that NUDT9 gene encodes a Mg^{2+}-dependent ADPRase that exists as two splice variants, NUDT5 and NUDT9 (15). It has been suggested that the mitochondrial ADPRase is likely to be the protein product encoded by NUDT9.

ADPRases have various K_m value for ADP-ribose ranging from 0.1 μM to 200 μM, indicating the

Correspondence to: Jong-Hyun Kim, Department of Obstetrics & Gynecology, Chonbuk National University Medical School, Jeonju 561-180, Korea. (Tel) 82-63-250-2290, (Fax) 82-63-254-4833, (E-mail) hyeon69@chonbuk.ac.kr

presence of endogenous modulator for ADPRase activity. Several modulators for ADPRase including fluoride, nitric oxide (NO), Mg^{2+}, ADP and dithiothreitol have been identified (5). ADPR, the substrate of ADPRase, has a reactive aldehyde group and induces nonenzymatic ADP-ribosylation of protein. Nonenzymatic ADP-ribosylation has been known to be stimulated by NO. Thus it is likely that the role of ADPRase might be modulated by NO. Indeed, activity of ADPRase-I from rat liver is inhibited in the exogenous NO generating system (16). To elucidate the cellular role of NO mediating the modulation of ADPRase activity, it was determined how ADPRase activity was influenced in the exogenous or endogenous NO generating system in J774 cells. In this study, I found evidence that the increase of ADPRase activity could be mediated via NO-induced nitrosylaion of cysteine residue on the enzyme and the macrophage activation might be accompanied by ADP-ribose metabolism.

Materials and Methods

Cell and Materials. The murine monocyte/macrophage cell line J774A.1 was obtained from Korean Cell Line Bank (Seoul, Korea). Dulbecos modified Eagle medium (DMEM), sodium nitroprusside (SNP), N-1-naphthylethylene diamine, sulfanilamide, lipopolysaccharide (LPS), and recombinant mouse IFN-γ were from Sigma chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT). Horseradish peroxidase- conjugated goat anti-rabbit IgG Ab was from ABI Co. (Jeonju, Korea). Trizol® Reagent was from Invitrogen (Carlsbad, CA). All the other chemicals were first grade reagents. Rabbit anti-human ADPRase antibody was prepared in ABI Co. by the immunization of recombinant human NUDT5 into rabbit (4).

Cell Stimulation. The J774 cells were cultured in DMEM media containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in humidified 5% CO_2. Cells were plated in 6 wells at a density of 1.5×10^6 cells per well. After overnight, the medium was replaced with fresh medium and cells were treated with SNP (0, 0.01, 0.05, 0.1, 0.5, or 1 mM) for 6 h. Cells were scraped and centrifuged at 12,000 g for 5 min. The pellets were washed three times with ice-cold PBS and lysed in 100 μl of cold lysis buffer (20 mM HEPES, 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail) on ice for 30 min. Cell lysates were obtained after centrifugation and used for western blot analysis and ADPRase activity assay. Protein concentration was determined by BCA protein assay kit (Sigma). Futhermore, to examine the effect of endogeneous NO on ADPRase activity, cells were treated with TNF-α (1 ng/ml) and IFN-γ (1 ng/ml) for 0, 1, 3, 6, 12, or 24 h with or without 1 mM NMMA, a NOS inhibitor. The supernatant was collected for the measurement of NO amount. Cell lysates also were used for western blot analysis and ADPRase activity assay.

Fluorometric assay for ADPRase activity. ADPRase activity was assayed by measuring the fluorescence of 1, N6-etheno-adenosine formed by alkaline phosphatase from ε-AMP, a product generated through 1,N6-etheno-ADPR hydrolysis by ADPRase (17). 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 MgCl2, 125 mM glycine-NaOH, pH 9.0, 1 mg/ml BSA, 10 unit of alkaline phosphatase and cell lysate in a total volume of 200 μl. After incubation for 30min at 37°C, the reaction was terminated by adding 2 mM EDTA, and the mixture was added to 500 μl of 20% AG-MP1 in 10 mM Tris-HCl (pH 10). 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 fluoroscence intensity was determined at excitation and emission wavelengths of 297 nm 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. Cell lysate (20 μg) described above was mixed with 2×SDS sample buffer and boiled for 5 min. The samples were electrophoresed in a 12% polyacrylamide gel at 100 V for 1 h and transferred onto nitrocellulose membrane. Membrane was blocked with 5% BSA, incubated with rabbit anti-human ADPRase (1 : 1,000) at 4°C overnight, and then washed five times with PBS containing 0.1% Triton X-100. Membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG Ab (1 : 500) for 2 h. The immunoblot was visualized by CN-DAB solution containing 0.03% H_2O_2.

Reverse transcriptase-polymerase chain reaction (RT- PCR). Total RNA was isolated using Trizol® Reagent following the manufacturer's instructions. The extracted RNA (1 μg) was then reverse transcibed to cDNA at 42°C for 1 hour in the reaction mixture containing random primer (0.5 μg) and 100 U reverse transcriptase. The reverse-transcribed cDNA was amplified using PCR system (Promega). cDNA (2 μl) was added to PCR reaction mixture containing Taq polymerase (2 U) and primers. Primers for ADPRase were sense 5'-TAA AAC CAG AAC TTG GGA AA-3' and antisense 5'-TTC ACC TTT GTA GCC AGT TT-3'. 18S RNA primer was used as internal standard (Ambion). The samples were cycled as

follow: 94°C for 1 min, 49°C for 30 sec and 72°C for 40 sec (30 cycles). PCR products were run on a 1.5% agarose gel containing ethidium bromide for 1 h. The bands were visualized under UV light.

Preparation of recombinant human ADPRase (NUDT5). Briefly, human NUDT5 was amplified by RT-PCR. The specific oligonucleotide primers containing restriction enzyme sites (*Eco*R I or *Sal* I) were synthesized (Bioneer, Korea); sense, 5'-GAGGTAGA ATTCATGGAGAGCCAAGAACCAA-3' and antisense, 5'-AAAATTGTCGACTTAAAATTTCAAGA AGGGCACT-3'. The resulting PCR product was cloned into pET vector (Novagen) and transformed into BL21 cells for expression. A transformant was cultured in LB media at 37°C and then treated with 1 mM IPTG for 3 h to induce the expression of NUDT5. Bacterial lysates were prepared by sonication twice for 10 sec on ice and centrifuged at 12,000 g for 15 min. The supernatant was purified using Ni^{2+}/NTA agarose (Quiagen). The purified NUDT5 was separated by SDS/PAGE (12% w/v gel) and analyzed using rabbit anti-human ADPRase antibody.

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

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

Statistical analysis. The results were expressed as mean ±S.E. for the number of experiments. Statistical significance was compared between each treated sample and control by the Student's *t* test. Each experiment was repeated at least three times and yielded comparable results.

Results and Discussion

The increase of ADPRase activity by the treatment of SNP in J774 macrophage cells. The current reports have suggested that ADP-ribosylaion of proteins was induced by the stimulation of NO. Such post-translational modification of protein could influence the various functions of cells. In this study, we examined the effects of NO on the ADPRase activity in macrophage cells. The ADPRase activity were measured 6 h after treatment of SNP, a NO generating agent. As shown in Fig. 1, the relative fluorescence intensity indicating ADPRase activity was increased by the treatment of SNP in a dose- dependent manner. After the treatment of 1 mM SNP, ADPRase activity was 64% higher than that of untreated cells as the negative control. However, ADPRase activity was diminished at 2.5 mM SNP. It was comparable to our additional data that the cell viability was unaffected at 1 mM SNP, but damaged a little at 2.5 mM SNP (data not shown). Next, I examined the protein and mRNA level of ADPRase changed by SNP treatment in J774 cells. As shown in Fig. 2, the expression of ADPRase was not changed by SNP. Therefore, These results supported that The increase of ADPRase activity by stimulation of NO could be induced without the increase in the protein and mRNA expression level of ADPRase and ascribed to post-translational modification of this enzyme through nitrosylation.

ADPRase activation through NO-induced modification of cysteine residue. I examined whether ADPRase activity is modulated by NO-mediated post-translational modification such as ADP-ribosylation. To prepare recombinant human ADPRase, human NUDT5 cDNA tagged 6×His was constructed and introduced into E. coli to induce recombinant protein as described in "Materials and Methods". NUDT5 purified with Ni^{2+}/NTA agarose was identified by western blot analysis with anti-ADPRase antibody and the size of

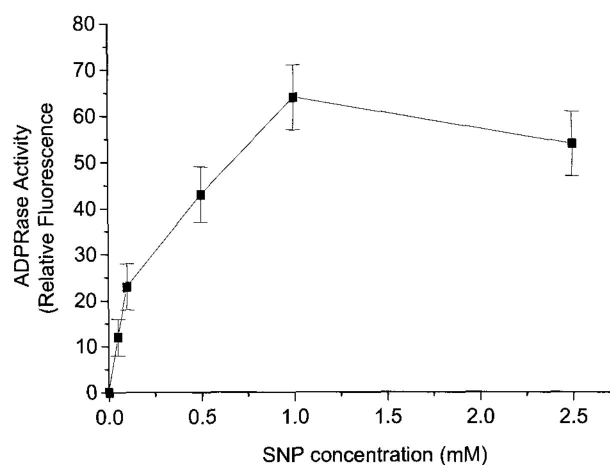

Figure 1. The increased ADPRase activity by the treatment of SNP in J774 macrophage cells. J774 cells was treated with indicated doses of SNP for 6 h. After cell lysis, the relative fluorescence intensity indicating ADPRase activity was measured as described in "Materials and Methods". Values are the mean± S.E. of three separate experiments ($p<0.05$).

pure protein was about 40 kDa (Fig. 3).

Purified NUDT5 was incubated with various doses of SNP 30 min before the ADPRase activity assay. Unexpectedly, ADPRase activity of NUDT5 was not affected directly by the pre-incubation with SNP (Fig. 4). It suggested that the additional factor in cells could be involved in the increase of enzyme activity by NO. Recently, it has been known that NO induced nitrosylation of cysteine residue of protein. Posttranslational modification of cysteine residue protein might be blocked by oxidation. In addition, the treatment of DTT may affect nitrosylation of cysteine residue of protein by creating a reducing environment (16), supporting that cysteine oxidation might be over-

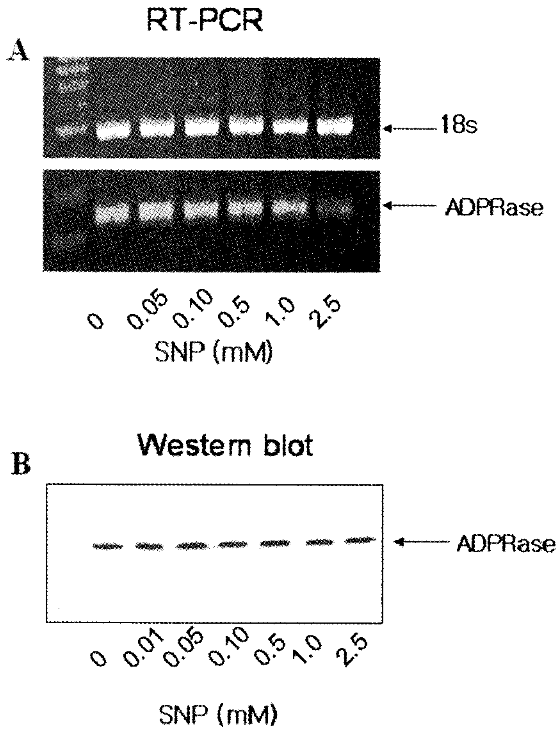

Figure 2. The effect of SNP on mRNA (A) and protein (B) level of ADPRase in J774 cells. J774 cells was treated with indicated doses of SNP for 6 h. After cell lysis, RT-PCR and Western blot were performed as described in "Materials and Methods".

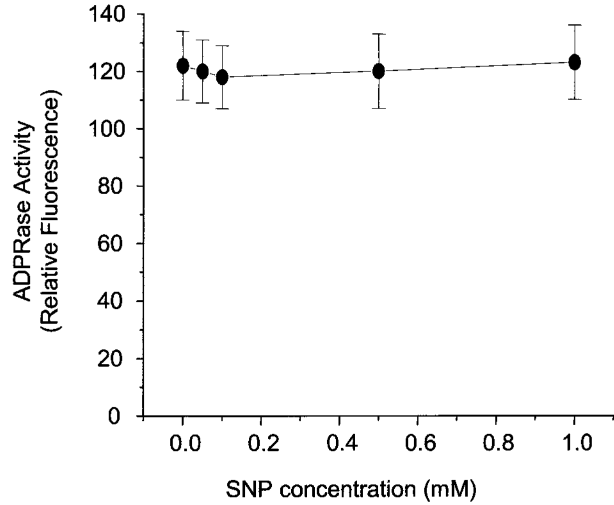

Figure 4. Effect of SNP on the ADPRase activity of NUDT5. His-tagged NUDT (100 ng) was preincubated with indicated doses of SNP for 30 min. ADPRase activity was measured as described in "Materials and Methods". Values are the mean±S.E. of three separate experiments.

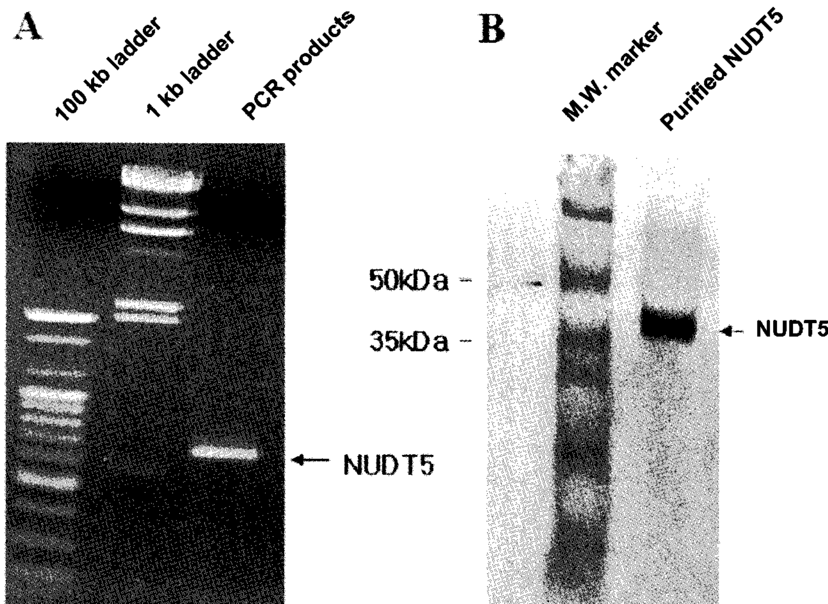

Figure 3. Preparation of recombinant human ADPRase. (A) NUDT5 the human gene for ADPRase was amplified by RT-PCR using total RNA extracted from human lymphocyte. (B) Recombinant human ADPRase (NUDT5) tagged 6xHis were purified using Ni^{2+}/NTA agarose and identified by western blot analysis with rabbit anti-human ADPRase antibody after SDS-PAGE.

comed by DTT. Therefore, ADPRase activity was measured after the preincubation of NUDT5 with 1 mM DTT and/or 1 mM SNP. As shown in Fig. 5, the treatment of both DTT and SNP could increase 40% ADPRase activity compared to the untreated sample. This result indicates that cysteine residue in ADPRase might be modified by NO. Moreover, NO has been known to stimulate nonenzymatic ADP-ribosylation of cysteine in target protein (16). To confirm the ribosylation of NUDT5 by NO, [^{32}P]ADPR and NUDT5 were incubated with or without 1 mM DTT and/or 1 mM SNP for 15 min. The samples were then electrophoresed and ADP-ribosylation was identified by the autoradiography. ADPRase was nonenzymatically ADP-ribosylated by NO and the ADP-ribosylation of ADPRase was increased by combination of NO to DTT (Fig. 6). The radioactivity of ADP-ribosylated ADPRase was removed by the addition of 10 mM $HgCl_2$, which is a condition enable to cleave ADP-ribosylcysteine. These results support that ADPRase activity might be mediated via NO-induced ADP-ribosylation of cysteine residue on the enzyme.

Effect of endogeneous NO on ADPRase activity in J774 cells. As ADPRase activity was affected in the exogenous NO generating system, I demonstrated whether the endogenous NO system, TNF-α and IFN-γ, can increase ADPRase activity in macrophages. J774 cells was stimulated with TNF-α and IFN-γ to measure NO concentration and ADPRase activity in the

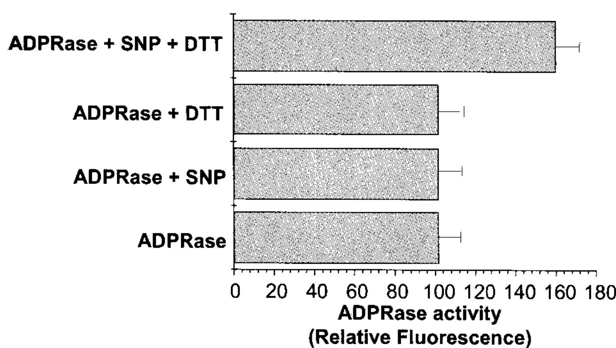

Figure 5. The increase of ADPRase activity by the treatment of DTT and SNP. Purified NUDT5 (100 ng) was preincubated with SNP (1 mM) and/or DTT (1 mM) for 30 min. ADPRase activity was measured as described in "Materials and Methods". Values are the mean±S.E. of three separate experiments ($p<0.05$).

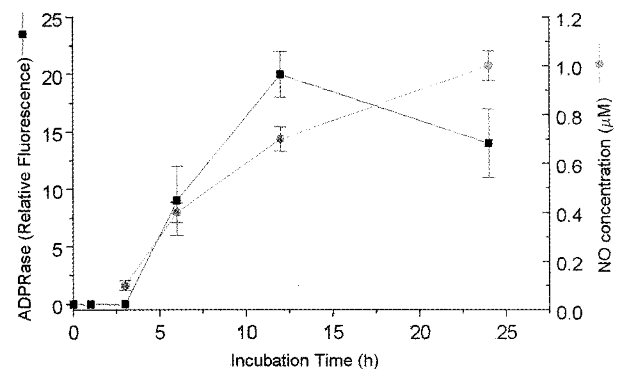

Figure 7. The changes of ADPRase activity and NO synthesis by the treatment of TNF-α/IFN-γ in J774 cells. Cells (1.5×10^6 cells/well) were treated with TNF-α and IFN-γ for the indicated time. NO level and ADPRase activity were measured as described in "Materials and Methods". Values are the mean± S.E. of three separate experiments ($p<0.05$).

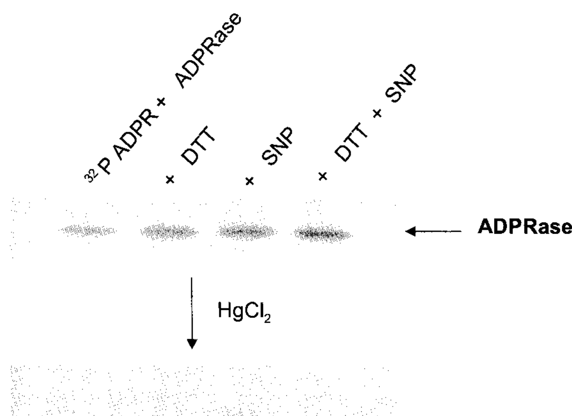

Figure 6. ADP-ribosylation of ADPRase by the treatment of DTT and SNP. [^{32}P]ADPR and NUDT5 were incubated with or without DTT (1 mM) and/or SNP (1 mM) for 15 min. The samples were then electrophoresed and autoradiographed. The radioactivity of [^{32}P]ADP-ribosylated ADPRase was removed under 10 mM $HgCl_2$ at 45°C for 3 h which is a condition able to cleave ADP-ribosylcysteine (lower panel).

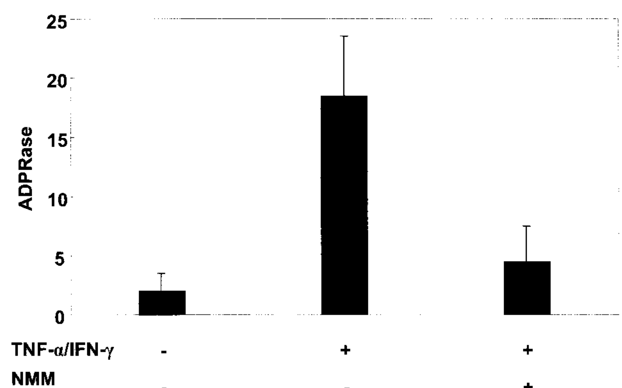

Figure 8. Effect of NOS inhibitor on the ADPRase activity increased in TNF-α/IFN-γ-treated J774 cells. Cells (1.5×10^6 cells/well) were treated with TNF-α and IFN-γ for 10h in the presence or in the absence of NOS inhibitor, NMMA. After cell lysis, ADPRase activity was measured as described in "Materials and Methods". Values are the mean±S.E. of three separate experiments.

supernatants and cells, respectively. The treatment of TNF-α/IFN-γ in J774 cells induced NO synthesis and the increased ADPRase activity in the time dependent manner (Fig. 7). The promotion of ADPRase activity after TNF-α/IFN-γ treatment was comparable to that of NO concentration, showing that the increase of ADPRase activity were accompanied by NO synthesis. In addition, the increased ADPRase activity was abolished by NMMA, a NOS inhibitor (Fig. 8), while protein level of ADPRase was not changed by the treatment of TNF-α/IFN-γ (data not shown). These results indicate that increase of ADPRase activity may be mediated by NO endogenously generated in activated macrophages and the post- translational modification of ADPRase, ADP-ribosylation by NO.

Moreover, the increased ADPRase activity could regulate ADPR metabolism including the synthesis and hydrolysis of ADPR. NAD is hydrolyzed to cyclic ADP-ribose (cADPR) and nicotinamide, and then ADPR is produced from cADPR by NAD glycohydrolase. Thus the increased ADPRase activity may require more ADPR through hydrolysis of NAD, which is released from membarne by NO. The recent study has demonstrated that TNF-α and IFN-γ could mediate transmembrane fluxes of nucleotides such as NAD, ADPR and cADPR (18). Therefore the increase of ADPRase activity may be also mediated by a system enhancing ADPR metabolism in activated macrophages.

Taken together, I observed that ADPRase activity was increased by the treatment of SNP, a exogenous NO generating reagent, as well as TNF-α and IFN-γ, endogeneous NO inducers, in J774 macrophage cells. The increase of ADPRase activity was mediated via posttranslational modification, which is ADP-ribosylation via nitrosylation of cysteine residue on the enzyme. Futhermore, NO synthesis in the activated macrophages might be accompanied with the increase in ADP-ribose metabolism.

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