In vivo inactivation of phosphotyrosine protein phosphatases by nitric oxide

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Abstract The effect of NO on phosphotyrosine protein phosphatases (PTPases) has been investigated in vivo. NO production is induced in interferon-γ and lipopolyaccharide stimulated RAW-264.7 macrophages as indicated by the increase of NO_2^- in the medium. Our results demonstrate an inhibition of *p*-nitrophenylphosphatase activity as a consequence of macrophages activation. Under the described experimental conditions, most of the hydrolysis of *p*-nitrophenylphosphate can be ascribed to the action of cellular PTPases. The presence of N^G-monomethyl-L-arginine, a specific inhibitor of NO synthase decreases the inactivation rate of both membrane-bound and soluble PTPases. This evidence further confirms the ability of NO to inactivate PTPases and suggests a possible role of NO in the regulation of cellular processes involving this class of phosphatases.

Key words: Nitric oxide; Phosphotyrosine protein phosphatase; Macrophage activation

1. Introduction

Nitric oxide (NO) is rapidly emerging as one of the main cellular regulators. It is produced from L-arginine in macrophages, endothelial cells and other cell types, and has been implicated in several physiological processes including vascular smooth muscle relaxation, inhibition of platelet aggregation, neurotransmission, immune regulation and penile erection [1,2]. Girard and Potier suggested also that the NO-mediated *S*-nitrosation could play a role in interconversion of thiol groups and disulfides in chain radical or oxidation-reduction processes that improve protein structural stability, ensure enzymatic catalysis, transport of reducing equivalents, and metabolic regulation [3]. The biological functions of NO are probably mediated by an array of interrelated redox forms such as the nitrosonium cation (NO^+), nitric oxide ($NO^\bullet$), and nitroxyl anion (NO^-). These forms may be interconverted through redox reactions [2]. Several authors reported that the *S*-nitrosation of enzymes and membrane receptors is involved in the regulation of these systems [4–7]. Clearly, the *S*-nitrosation of proteins and other thiol-containing compounds is caused by the nitrosonium ion [2,8].

Recently, we demonstrated that NO causes in vitro inactivation of the low M_r and *Yersinia enterocolitica* PTPases [9]. The inactivation of the enzymes is determined by the reaction of NO with the highly reactive cysteine residue present in the active site of all PTPase family members [10]. In fact, all PTPases possess the evolutionary conserved active site motif CXXXXXR forming a three-dimensional phosphate binding loop that is essential for catalysis [11,12]. The structural analysis performed on the modified low Mr PTPase showed that a disulphide bridge between Cys-12 and Cys-17 (both induced in the conserved loop) was formed during the action of NO on the enzyme [9]. We also demonstrated that the inactivation is reverted by incubation of the modified enzyme with low molecular weight thiols, suggesting that nitric oxide can cause a transient inactivation of PTPases. The aim of this paper is to investigate the possible in vivo involvement of NO in the regulation of PTPase activity in cells. It is well known that macrophage activation by interferon-γ and bacterial lipopolysaccharides, induces the NO synthase activity [13]. Thus, this work describes the effect of murine macrophage activation on the activity of cellular PTPases.

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2. Materials and methods

2.1. Reagents

N-Monomethyl-L-arginine was obtained from Sigma. Interferon-γ was from Amgen, Thousand Oaks, CA. All other reagents were the purest commercially available.

2.2. RAW 264.7 cell culture stimulation, and fractionation

RAW 264.7, an Abelson virus-transformed murine macrophage cell line (American Type Culture Collection, ATCC TIB71), was cultured in Dulbecco's modified Eagles medium containing 4.5 g/l glucose, 2 mM glutamine, supplemented with 10% fetal calf serum (PBI) and antibiotics (50 units/ml penicillin-G, 50 μg/ml streptomycin). Activation experiments were performed in Phenol red-free medium by incubating the cells (70–80%) confluence) with bacterial lipopolysaccharide (LPS from *E. coli* subtype 0127:B8, SIGMA) 1 μg/ml and recombinant mouse interferon-γ 50 units/ml.

The cytosolic and detergent-dissolved membrane fractions were prepared from cells; after removal of the medium, cells were washed twice with 1 ml of ice-cold phosphate buffered saline, then collected in 0.3 ml of 20 mM Tris-HCl buffer, pH 7.2, containing 0.1% 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, 20 mM leupeptine, 5 μg/ml aprotinin.

Cell suspensions were sonicated with two bursts of 10 s with a Vibra-Cell (Sonics and Materials Inc, Danbury, CT, USA). The lysates were centrifuged for 45 min at 100,000 × *g* and 4°C. Supernatants were assayed for protein concentration and enzymatic activity. The pellets were suspended in 0.3 ml of the same buffer containing 1% Triton X-100 and centrifuged for 10 min at 12,550 × *g*. Portions of the supernatant were assayed for protein concentration and activity.

2.3. Cell viability test

Cellular viability was tested by Trypan-blue staining. About 1×10^5 cells were resuspended in 1 ml of PBS (phosphate buffer saline) and trypan-blue reagent (1% Trypan-blue in PBS) was added at the 1:1 ratio. After 10 min incubation on ice, cells were screened for viability by visible-light microscopy. Dead cells were blue-stained, while alive cells remained white and light-refracting.

2.4. Nitrite ion determination

NO release in the culture media was determined as nitrite production using the Greiss reagent that contains 0.5% sulphanylamide and 0.05% *N*-(1-naphthyl-ethylendiamine dihydrochloride in 2.5% phosphoric acid [14]. The Greiss reagent (150 μl) was added to 150 μl of medium,

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and the absorbance was read after 10 min at 492 nm using a 96-well microplate reader.

2.5. Protein determination

Protein concentration was determined by the BCA method (Pierce).

2.6. Determination of PTPase activity

Phosphatase activity was determined using *p*-nitrophenyl phosphate as substrate (this substrate is hydrolysed by all PTPases). The substrate (4 mM) was dissolved in 25 mM Tris-HCl buffer, pH 7.2, containing 1 mM EDTA, 0.1% 2-mercaptoethanol, 20 mM sodium-potassium DL-tartrate and 1 μM okadaic acid. The reaction was stopped with 0.1 M KOH and the released *p*-nitrophenolate ion was measured by reading the absorbance at 400 nm (ε = 18,000 $M^{-1} \cdot cm^{-1}$). The activity measured in these conditions was completely inhibited by 100 μM orthovanadate, a specific and strong inhibitor of all PTPases [15].

3. Results

3.1. NO-mediated inactivation of PTPases in the murine macrophage cell line RAW 264.7 stimulated with lipopolysaccharide and interferon-γ

The murine macrophages were stimulated with lipopolysaccharide (LPS, 1 μg/ml) and interferon-γ (50 U/ml) in order to induce NO-synthase followed by the formation of NO in the cells [13]. At varying time intervals the NO_2^- ion was assayed in the cell medium in order to test NO release from macrophages. At the same time, the cultured cells were lysed with 0.3 ml of 20 mM Tris-HCl buffer, pH 7.2, containing 0.1% 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, 20 mM leupeptin and 5 μg/ml aprotinine. The mixture was centrifuged at 12,550 × *g*, and the phosphatase activity was assayed with *p*-nitrophenyl phosphate as substrate in the presence of tartrate, EDTA, and okadaic acid in order to inhibit lysosomal acid phosphatases and non-specific phosphatases. Under these conditions we were sure of the assay specificity for PTPases. In fact, all the assays were repeated in the presence of 100 μM orthovanadate, a well known specific strong inhibitor of all PTPases, leaving a negligible residual *p*-nitrophenylphosphatase activity. Fig. 1A shows the increase in NO_2^- concentration in the cell culture medium as a function of incubation time. As can be seen, the concentration of this ion increased continuously starting from 3 h until 24 h of incubation time, reaching the maximum concentration of about 60 μM in the LPS and interferon-γ stimulated cells, whereas in the non- stimulated cells the NO_2^- ion concentration showed only a slight increase. With respect to PTPase activity, we observed an initial increase in the 0–3 h period both in the unstimulated and stimulated cells. Nevertheless, at 15 and 24 h of incubation time the activity found in the LPS and interferon-γ stimulated cells was strongly decreased, whereas that of unstimulated cells remained quite constant (Fig. 1B). We selected the 24 h incubation time in order to perform further tests on the RAW-264.7 macrophages. Particularly, we studied the effect of cell stimulation in the presence of N^G-monomethyl-L-arginine (NMA), a powerful specific inhibitor of NO-synthase. The inhibitor was added to the culture medium at the final concentration of 1 mM. Furthermore, the PTPase activity was assayed both in the soluble and particulate fractions, since PTPases are located both in the cell membrane and in the cytosol. Fig. 2A and 2B show the results obtained by assaying the membrane-bound and the soluble PTPase activities respectively. We found similar results for membrane-bound and soluble PTPase activities. In fact, both figures show a strong decrease in PTPase activity and NMA substantially reduced the observed PTPase inactivation. This is in agreement with the reduced production of NO in the NMA-treated cells as demonstrated by the reduced release of the NO_2^- ion in the culture medium (Fig. 2C).

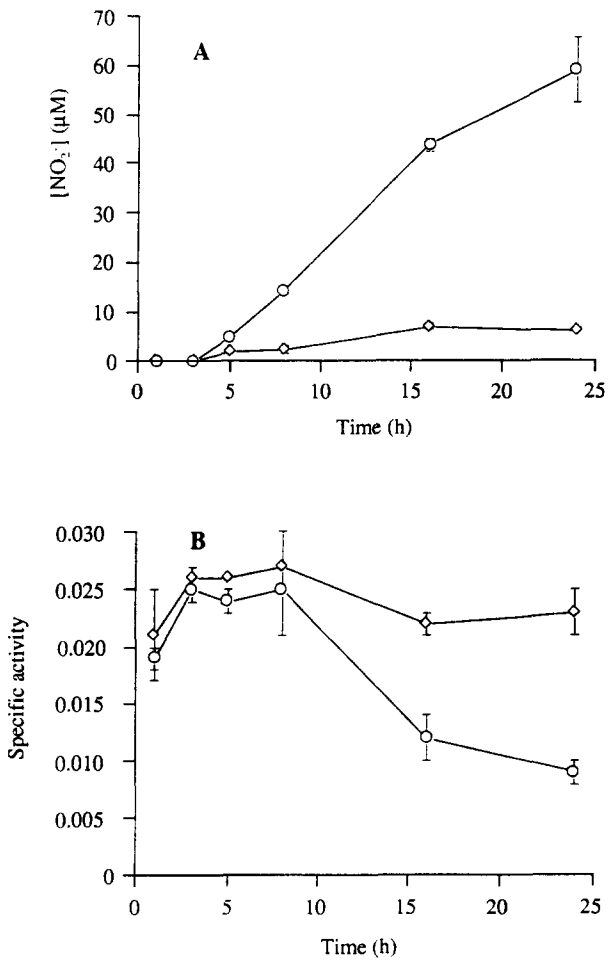

Fig. 1. Inactivation of PTPases in macrophage cell line RAW 264.7 by NO. Cells were incubated for the indicated times either in DMEM alone (◇) or in the same medium supplemented with 50 U/ml interferon-γ and 1 μg/ml bacterial lipopolysaccharide (○). The latter treatment increased NO release, measured as nitrite using Greiss reagent (A). A concomitant decrease in PTPase specific activity (mU/mg of proteins) was observed (B).

4. Discussion

Previous in vitro experiments performed in our laboratory demonstrated that both the isoenzymes of low M_r PTPases (AcP1 and AcP2 from rat liver) and *Yersinia enterocolitica* PTPase are inactivated by NO-producing compounds and NO gas [9]. The low M_r PTPase inactivation is caused by a specific reaction of NO with active site cysteine(s) to form a *S*-nitrosothiol; this is followed by the formation of an S–S bridge between Cys-12 and Cys-17. These residues are included in the active site signature motif CXXXXXR (residues 12–18 in the low Mr PTPase) present in all PTPase family members; Cys-12 and Arg-18 are essential for catalysis [10]. We suggested that the low M_r PTPase inactivation, which can be reverted by low molecu-

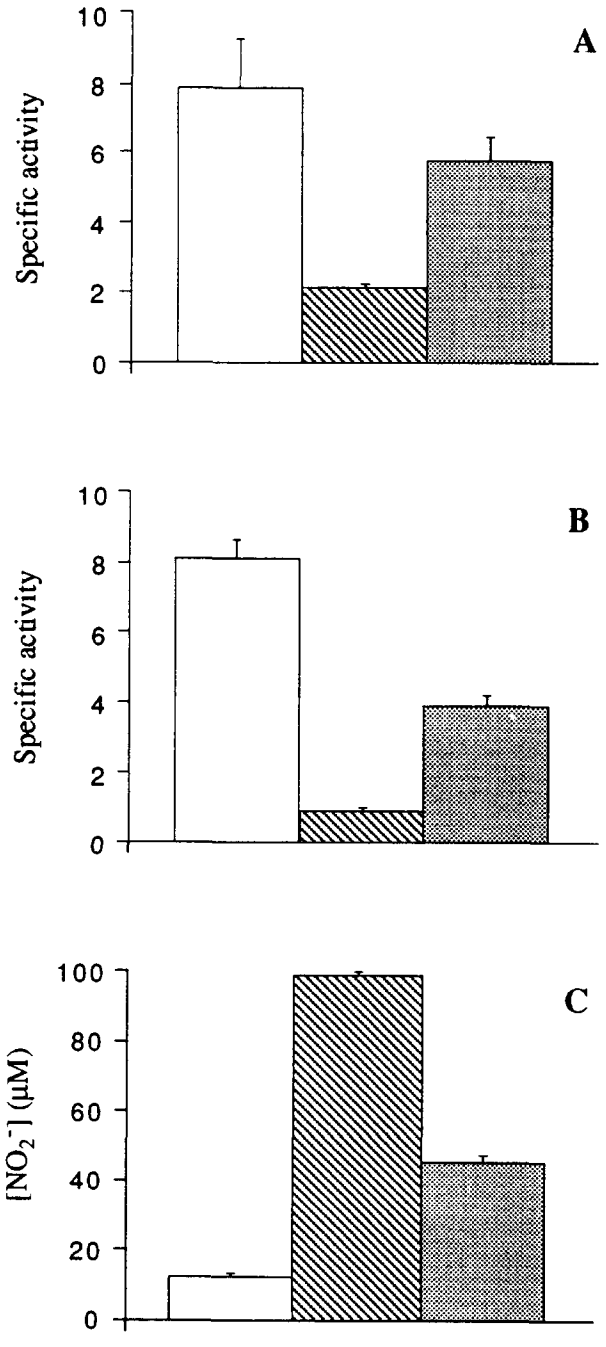

Fig. 2. Effect of cell stimulation in the presence of NO sinthase-specific inhibitor NMA. Cells were incubated for 24 h in DMEM alone (□) or in the same medium supplemented with 50 U/ml interferon-γ and 1 μg/ml bacterial lipopolysaccharide (▨) or with 50 U/ml interferon-γ, 1 μg/ml bacterial lipopolysaccharide and 1 mM NMA (■). NMA decreased the inactivation rate of both membrane-bound (A) and soluble PTPases (B) with respect to that observed in stimulated cells. In the NMA-treated cells we observed a reduced production of NO (C).

lar weight thiols, is first caused by the reaction of the very reactive Cys-12 with NO leading successively to the formation of the observed S–S bridge. Very reactive thiols were implicated by other authors in the reaction of enzymes [4–6] or membrane receptors [7] thiols to form *S*-nitroso-thiols. This paper demonstrates that macrophage PTPases, all containing a very reactive cysteine in the active site signature motif, are inactivated in vivo following cell stimulation by LPS and interferon-γ. It is known that cell activation causes NO production, as we also demonstrated in Fig. 1. The release of NO_2^- ions is related to NO production in the cell; NO_2^- concentration in the culture medium is not a measure of NO level in the cell, since NO has free radical structure, and it is a short-living specie. It reacts rapidly with water-dissolved oxygen to form NO_2, subsequently reacting with water producing both NO_2^- and NO_3^- ions. The intracellularly generated NO is often lower than nanomolar [16]; thus our findings indicate that the inactivation of PTPases occurs not only in vitro but also in vivo conditions. Since we demonstrate that the inhibition of low M_r PTPase can be reverted by low molecular weight thiols, we suggest that the inactivation of cellular PTPases is a transient phenomenon. These enzymes can regain their original activity through the action of intracellular low molecular weight thiols, like cysteine and reduced glutathione. In agreement with Girard and Potier [3], we think that the reversible covalent modification of reactive protein thiols, either to produce *S*-nitrosothiols or to produce S–S bridges, represents a new interesting type of regulation of biological functions. We underline that the continuously increasing NO_2^- concentration indicates that RAW-264.7 macrophages were still living during the time of our experiments; in fact, the death of these cells is accompanied by the decrease in the NO production rate as demonstrated by Stuehr and Marletta [17]. However, we performed viability tests 24 h after cell activation. We found that about 95% of the cells were alive.

Recently, Peranovich et al. [18] observed the in vivo inhibition of the EGF-receptor PTPase activity in the HER14 cell line incubated with sodium nitroprusside. These cells are murine fibroblasts expressing the human EGF-receptor. They found that increasing concentrations of NO are correlated with a gradual inhibition of PTPase activity in these cells, either in intact cells or in cell lysates. These findings suggest that the in vivo inhibition of PTPases by NO is not limited to the RAW 264.7 murine macrophages, but probably extended to all cells. NO is a small molecule involved in several physiological processes such as the inhibition of platelet aggregation, desensitisation of neurones to excess stimulation by glutamate, smooth muscle relaxation, and penile erection [1,2]. On the other hand, PTPases are known to be involved in the regulation of mitogenic signals acting on tyrosine kinase membrane receptors. Recently, we demonstrated that the low M_r PTPases are implicated in the down-regulation of the platelet derived growth factor receptor. In fact, the level of phosphotyrosine in BB-PDGF-stimulated NIH/3T3 fibroblasts overexpressing this enzyme is strongly reduced with respect to normal cells [19]. In addition, we found that a dominant negative gene (which produce a catalytically inactive enzyme that is still able to bind substrates) overexpressed in the above cell line forms a complex with the PDGF receptor [20].

Finally, we think that the action of NO on PTPases in vivo should be taken into great consideration, since more than forty different PTPases were discovered in the cells, most of them involved in the control of mitogenic signalling and others, such as the product of the cdc25 gene, essential in the control of the cell cycle and in other important biological functions.

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