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原　著

Reversible Inhibition of Protein Phosphatase1 by Peroxynitrite

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We investigated the effects of peroxynitrite on the activity of protein phosphatase 1 (PP 1), a metalloenzyme with putative important sulfhydryl groups for its activity. PP 1 activity was assayed colormetrically with a synthetic substrate of p-nitrophenyl phosphate. Peroxynitrite inactivated 16.7 nM PP 1 in a few seconds with an $IC_{50}=0.45\ \mu M$. *The peroxynitrite decomposition products, nitrite and nitrate, had no effect on activity. Inactivation was restored by* Mn^{2+} *or dithiothreitol but not by* Fe^{2+} *or sodium arsenite. These results show that peroxynitrite rapidly and reversibly inactivates PP 1 by affecting its sulfhydryl groups and* Mn^{2+}.

Key words: Peroxynitrite, protein phosphatase 1, acute organ failure

INTRODUCTION

The critical role of reversible phosphorylation in cellular signal transduction places protein phosphatases in a position of central importance because of their ability to reverse the action of protein kinases. Phosphatases that act on phosphorylated serine and threonine residues participate in the control of a wide range of cellular processes, including cell-cycle progression, cell proliferation, protein synthesis, transcriptional regulation, and neurotransmission[1,2]. Phosphatases are metalloenzymes that have two divalent metal ions at the center of the catalytic site[3]. Protein phosphatase 1 (PP1) has Mn^{2+} and Fe^{2+}, and in paticular, the recombinant type requires Mn^{2+} for its catalytic activity[4]. The catalytic site is coordinated by Asp 64, Asp 92, Asn 124 and His 66 of the mononucleotide-binding motif, His 173, and His 248, and is highly conserved[3]. No cysteine residue is found in the catalytic region, but this residue also seems to act in PP1 activation[5,6]. Mutagenesis of six conserved cysteine residues did not eliminate phosphatase activity completely, however, three of the six mutants significantly diminished activities correlated with lowered heat stabilities[7].

Peroxynitrite is a powerful biological oxidant that is produced by the diffusion-limited reaction of nitric oxide with superoxide[8]. Nitric oxide is the only known biological molecule that can be produced under pathological conditions in high enough concentrations to outcompete endogenous SODs for superoxide. At physiological pH, peroxynitrite decomposes through a proton-dependent mechanism to form an oxidant with the reactivity of hydroxyl radical plus nitrogen dioxide[9,10]. However, peroxynitrite much more rapidly oxidizes many biological targets such as sulfhydryls[11], and induces the oxidative inactivation of some sulfhydryl-containing enzymes including succinate dehydrogenase[12], caspase-3[13]and protein tyrosine phosphatases[14]. Furthermore, peroxynitrite reacts with metals to form a nitronium-like species (NO^{2+}) that results in the formation of nitrotyrosine[15]. Therefore, it is possible that peroxynitrite can potentially inactivate PP1 by the modification of cysteine or tyrosine residues.

In this study, we investigated the effects of peroxynitrite on PP1 activity, which was exceptionally inhibited by peroxynitrite, although this inhibition was recovered by dithiothreitol and Mn^{2+}.

MATERIALS AND METHODS

Protein phosphatase1, alpha-isoform (rabbit muscle, recombinant) and p-nitrophenyl phosphate were obtained from Calbiochem (San Diego, CA). All other reagents were from Sigma.

Peroxynitrite was prepared using quenched-flow reaction apparatus as described previously[16,17]. An aqueous solution of 0.6M sodium nitrite was rapidly mixed with an equal volume of 0.7M hydrogen peroxide containing 0.6M HCl and immediately quenched with the same volume of 1.5M NaOH. All reaction solutions were kept on ice. The con-

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centration of peroxynitrite was determined spectrally in 1.0 M NaOH (e302 nm=1700/M/cm). Stock solutions of peroxynitrite were stored at −80℃ for up to 6 months.

PP1 activity was measured using the synthetic substrate of p-nitrophenyl phosphate[18]. After 80μl buffer containing 50mM Tris/HCl, pH 7.0, 250mM NaCl and 0.05~0.1μg PP1 was treated with 1μl peroxynitrite or nitric oxide at 37℃, 0.2mM $MnCl_2$ was added. The reaction was started by adding 20μl of 10mM p-nitrophenyl phosphate at 37℃ for 30min. At the end of the incubation, the reaction was stopped by the addition of 0.9ml of 0.2 M NaOH. The extent of each reaction was estimated by measurement of the nitrophenol absorbance at 410nm. The PP1 was linear over a 0~0.2μg protein concentration over a span of 60min.

As alkaline conditions are required for peroxynitrite stability, it was diluted with 0.1M NaOH. To minimized pH changes in reaction solutions with PP1, 1μl dilutions of peroxynitrite were added to 80μl buffer at 37℃ to give the desired final concentration. The final pH of all reaction solutions was checked to ensure adequate buffering. The short half-life of peroxynitrite at pH7.0 necessitated the rapid mixing of reaction solutions. To avoid the decomposition of peroxynitrite before the solution was fully mixed, reaction solutions in 1.5ml microfuge tubes were rapidly mixed via a vortex mixer during bolus peroxynitrite additions. Either 0.1M NaOH or decomposed peroxynitrite was obtained by adding peroxynitrite to the pH7.0 buffer before the addition of protein (reverse order addition) as a control.

RESULTS AND DISCUSSION

Peroxynitrite inactivated PP1 in a concentration-dependent manner (Fig. 1), and the peroxynitrite concentration required for 50% inactivation (IC_{50}) was markedly dependent on phosphatase concentration. When the concentrations of phosphatase were 16.7 and 33.4 nM, the IC_{50} were 0.45 and 5.4μM, respectively. Ischiropoulos et al. showed that cysteine oxidation and tyrosine nitration are dependent on the concentration of protein reacted with peroxynitrite[19]. Therefore, our results may indicate that peroxynitrite-induced PP1 inactivation was due to cysteine oxidation and tyrosine nitration. The peroxynitrite solutions used contain some residual nitrite and hydrogen peroxide, depending on the synthetic reaction yield. In addition, nitrate was formed from the decomposition of peroxynitrite[20], however, as decomposed peroxynitrite obtained by adding peroxynitrite to the pH7.0 buffer before the addition of protein (reverse order addition) had no effect, peroxynitrite itself must be responsible for the inhibitory effects on PP1 activity.

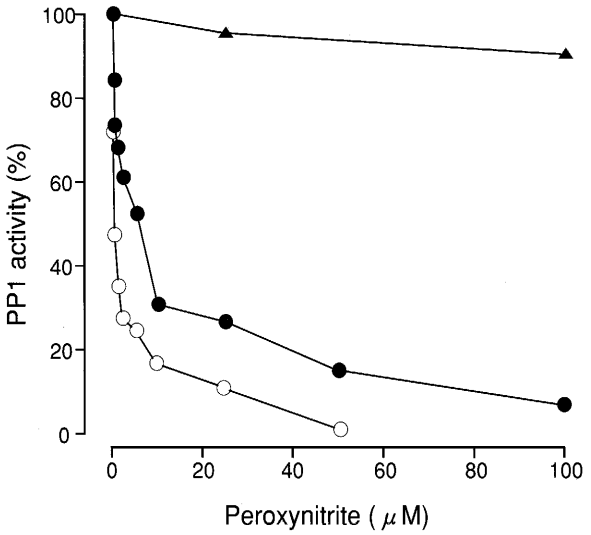

Fig. 1 : Protein phosphatase inactivation with peroxynitrite. Bolus addition of peroxynitrite (○ or ●) or decomposed peroxynitrite (▲; reverse order treatment) to protein phosphatases1 (PP1) was performed in pH7.0 Tris buffer at 37℃. Concentrations of phosphatase were 16.7 (○) and 33.4nM (●). The activity with 0.1 M NaOH alone, with which peroxynitrite was diluted, was taken as 100%. Data are the mean of at least two different experiments.

Pre-treatment of dithiothreitol defended phosphatase activity from 10μM peroxynitrite with 50% protection provided by 0.27mM dithiothreitol (Fig. 2). Even after the peroxynitrite treatment, dithiothreitol completely recovered PP1 activity (50% protection provided by 1.2mM dithiothreitol). Fig. 3 shows that PP1 activity inhibited by peroxynitrite was recovered by dithiothreitol but not by sodium arsenite. These results suggest that PP1 inactivation may occur via thiol oxidation by peroxynitrite resulting in disulfide formation, as dithiothreitol does not recover higher oxidation states such as sulfenic, sulfinic, or sulfonic acids (oxidation states of +2, +4 and +6, respectively), and sodium arsenite recovers sulfonic acids but not disulfide to thiol[10]. It was reported that the formation of a disulfide bond inhibited metal-binding to PP1 resulting in inactivation[5,6].

Recombinant-type PP1 requires Mn^{2+} for its catalytic activity[4]. Therefore, if peroxynitrite forms a disulfide bond and inhibits metal-binding to PP1 resulting in inactivation, it is expected that an excess addition of Mn^{2+} may recover the inactivation. As expected, the subsequent addition of manganese chloride recovered PP1 activity inactivated by

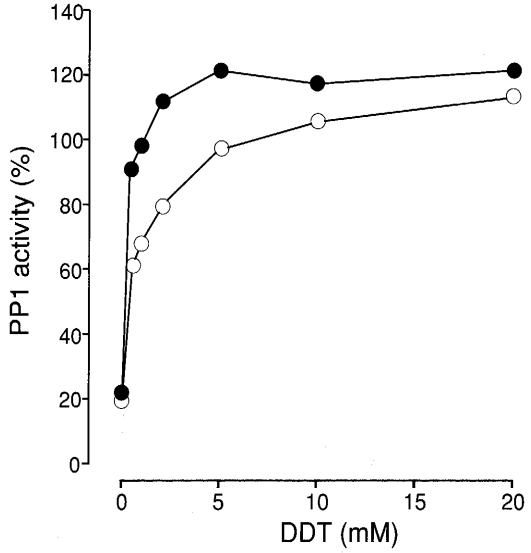

Fig. 2 : Effects of dithiothreitol to protein phosphatase1 inactivation with peroxynitrite.
Addition of dithiothreitol (DTT) before (●) or after (○) 10μM peroxynitrite treatment to protein phosphatase (PP1) in pH7.0 Tris buffer at 37℃. The activity with 0.1M NaOH alone, with which peroxynitrite was diluted, was taken as 100%. Data are the mean of at least two different experiments.

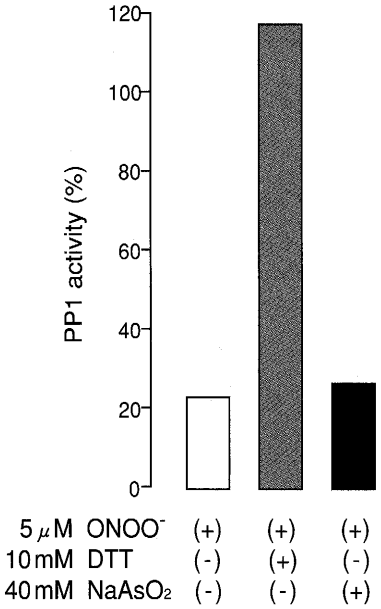

Fig. 3 : Effects of dithiothreitol or sodium arsenite to protein phosphatase1 inhibited by peroxynitrite.
Addition of dithiothreitol (DTT) or sodium arsenite ($NaAsO_2$) after 5μM peroxynitrite treatment to 16.7 nM protein phosphatase (PP1) in pH 7.0 Tris buffer at 37℃. The activity with 0.1M NaOH alone, with which peroxynitrite was diluted, was taken as 100%. Data are the mean of at least two different experiments.

peroxynitrite in a concentration-dependent manner (Fig. 4). Although it is well known that transition metals like manganese and iron also react directly with peroxynitrite and catalyze the nitration of tyrosines[15,21], ferrous ammonium sulfate did not further deteriorate PP1 activity inhibited by peroxynitrite (Fig. 4). Therefore, tyrosine nitration by peroxynitrite may not take part in PP1 inactivation. Also, in protein tyrosine phosphatases inactivated by peroxynitrite, no significant tyrosine nitration was detected on immunoblot analysis[14].

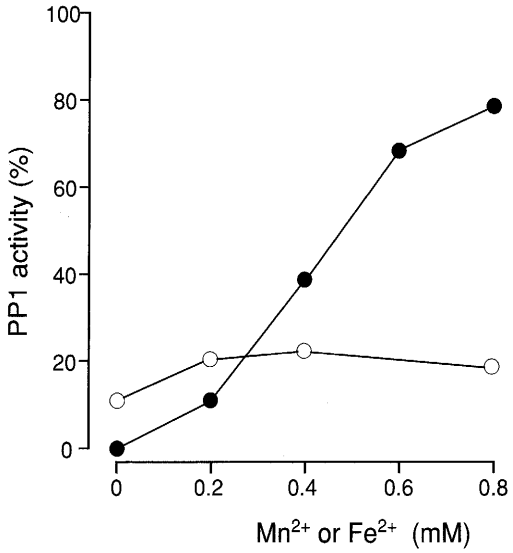

Fig. 4 : Effects of Mn^{2+} or Fe^{2+} on protein phosphatase1 inactivated by peroxynitrite.
Addition of manganese chloride (Mn^{2+}; ●) or ferrous ammonium sulfate (Fe^{2+}; ○) after 5μM peroxynitrite treatment to 16.7nM protein phosphatase (PP1) in pH 7.0 Tris buffer at 37℃. The activity with 0.1M NaOH alone, with which peroxynitrite was diluted, was taken as 100%. Data are the mean of at least two different experiments.

The presence of substrate p-nitrophenyl phosphate could partially protect PP1 activity from peroxynitrite (Fig. 5). The protective effects of p-nitrophenyl phosphate were concentration-dependent with 50% protection provided by less than 0.1mM p-nitrophenyl phosphate. However, even high concentrations up to 2mM p-nitrophenyl phosphate did not completely protect PP1 activity. These results may suggest that the target(s) of peroxynitrite is not in the catalytic region.

In conclusion, peroxynitrite inhibited protein phosphatase reversibly and we believe that its mechanism is the reversible inhibition of essential Mn^{2+} binding to PP1 by the formation of a disulfide bond at the cysteine residue outside the catalytic region. Peroxynitrite is produced by the reac-

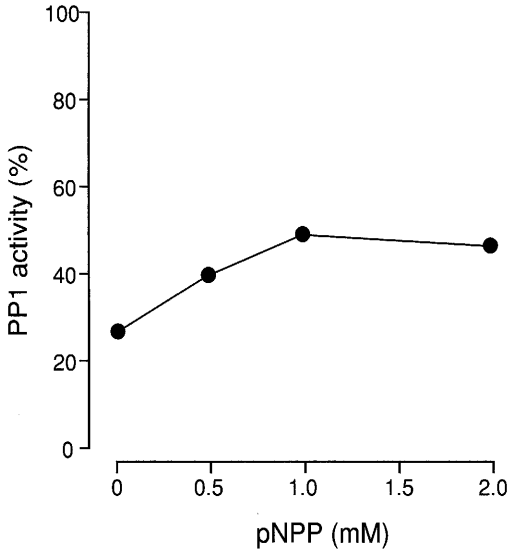

Fig. 5 : Effects of p-nitrophenyl phosphate on protein phosphatase inactivation with peroxynitrite.
p-Nitrophenyl phosphate (pNPP) was added before 5 μM peroxynitrite treatment to protein phosphatase (PP 1) in pH 7.0 Tris buffer at 37℃. The activity with 0.1M NaOH alone, with which peroxynitrite was diluted, was taken as 100%. Data are the mean of at least two different experiments.

tion of nitric oxide with superoxide under pathological conditions such as sepsis, pneumonia and adult respiratory distress syndrome[22,23]. Therefore, the inhibition of protein phosphatase by peroxynitrite may be one of the methods of pathogenesis, as protein phosphatase is important in the control of many cellular processes.

REFERENCES

1) Cohen P. The structure and regulation of protein phosphatases. *Annu Rev Biochem.* 1989 ; 58 : 453–508.

2) Shenolikar S. Protein serine/threonine phosphatases-new avenues for cell regulation. *Annu Rev Cell Biol.* 1994 ; 10 : 55–86.

3) Goldberg J, Huang H, Kwon Y, Greengard P, Nairn A and Kuriyan J. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature.* 1995 ; 376 : 745–753.

4) Zhang Z, Bai G, Deans-Zirattu S, Browner MF and Lee EY. Expression of the catalytic subunit of phosphorylase phosphatase (protein phosphatase-1) in Escherichia coli. *J Biol Chem.* 1992 ; 267 : 1484–1490.

5) Nemani R and Lee E. Reactivity of sulfhydryl groups of the catalytic subunits of rabbit skeletal muscle protein phosphatases1 and 2A. *Arch Biochem Biophys.* 1993 ; 300 : 24–29.

6) Chu Y, Lee E, Reimann E, Wilson S and Schlender K. Effect of activation of protein phosphatase1 on sulfhydryl reactivity. *Arch Biochem Biophys.* 1996 ; 334 : 83–88.

7) Zhang Z, Zhao S, Deans-Zirattu S, Bai G and Lee E. Mutagenesis of the catalytic subunit of rabbit muscle protein phosphatase-1. *Mol Cell Biochem.* 1993 ; 127–128 : 113–119.

8) Beckman J, Beckman T, Chen J, Marshall P and Freeman B. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci. U.S.A.* 1990 ; 87 : 1620–1624.

9) Koppenol WH, Moreno J, Pryor W, Ischiropoulos H and Beckman JS. Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem Res Toxicol.* 1992 ; 5 : 834–842.

10) Crow JP, Spruell C, Chen J, Gunn C, Ischiropoulos H, Tsai M, Smith CD, Radi R, Koppenol WH, Beckman JS. On the pH-dependent yield of hydroxyl radical products from peroxynitrite. *Free Radic Biol Med.* 1994 ; 16 : 331–338.

11) Radi R, Beckman JS, Bush K and Freeman B. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem.* 1991 ; 266 : 4244–4250.

12) Rubbo H, Denicola A and Radi R. Peroxynitrite inactivates thiol-containing enzymes of Trypanosoma cruzi energetic metabolism and inhibits cell respiration. *Arch Biochem Biophys.* 1994 ; 308 : 96–102.

13) Mohr S, Zech B, Lapetina E and Brune B. Inhibition of caspase-3 by S-nitrosation and oxidation caused by nitric oxide. *Biochem Biophys Res Commun.* 1997 ; 238 : 387–391.

14) Takakura K, Beckman JS, MacMillan-Crow L and Crow JP. Rapid and irreversible inactivation of protein tyrosine phosphatases PTP1B, CD45, and LAR by peroxynitrite. *Arch Biochem Biophys.* 1999 ; 369 : 197–207.

15) Beckman JS, Ischiropoulos H, Zhu L, van der Woerd D, Smith C and Chen J. Kinetics of superoxide dismutase-and iron-catalyzed nitration of phenolics by peroxynitrite. *Arch Biochem Biophys.* 1992 ; 298 : 438–445.

16) Reed J Ho H and Jolly W. Chemical syntheses with a quenched flow reactor. Hydroxytrihydroborate and peroxynitrite. *J Am Chem Soc.* 1974 ; 96 : 1248–1249.

17) Beckman JS, Wink DA and Crow JP. M. Feelisch and JS Stamler, ed. Nitric Oxide and Peroxynitrite, Methods in Nitric Oxide Research. New York: John Wiley & Sons; 1996 ; 61–70.

18) Barik S. Expression and biochemical properties of a protein serine/threonine phosphatase encoded by bacteriophage lambda. *Proc Natl Acad Sci. U.S.A.* 1993 ; 90 : 10633–10637.

19) Ischiropoulos H and al-Mehdi A. Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett.* 1995 ; 364 :

279–282.
20) Crow JP, Beckman JS and McCord J. Sensitivity of the essential zinc-thiolate moiety of yeast alcohol dehydrogenase to hypochlorite and peroxynitrite. *Biochemistry* 1995；34：3544–3552.
21) Ischiropoulos H, Zhu L, Chen J, Tsai M, Martin J, Smith C and Beckman JS. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch Biochem Biophys*. 1992；298：431–437.
22) Haddad I, Pataki G, Hu P, Galliani C, Beckman JS and Matalon S. Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *J Clin Invest*. 1994；94：2407–2413.
23) Kooy N, Royall J, Ye Y, Kelly D and Beckman JS. Evidence for in vivo peroxynitrite production in human acute lung injury. *Am J Respir Crit Care Med.* 1995；151：1250–1254.

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ペロキシナイトライトによる蛋白リン酸酵素1活性の抑制

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丹羽ひかる　広瀬雅之　大嶋和之
溝上真樹

細菌性毒素や炎症性サイトカイン等の刺激によりマクロファージなどで大量に産生される一酸化窒素（NO）は，感染や腫瘍に対し防御反応として作用する一方，宿主に対しても細胞・組織障害を引き起こすと考えられてきた．しかし，生体内ではNOそのものには従来言われているほどの高い化学反応性や毒性はなく，より反応性の高いペロキシナイトライト（$ONOO^-$）のような二次酸化物が障害因子の本体ではないかと考えられるようになった．そこで，蛋白合成や神経伝達に重要な役割を持つ蛋白リン酸酵素1（protein phosphatase1：PP1）活性に及ぼす$ONOO^-$の作用を検討した．

P–ニトロホスホフェノール（pNPP）がPP1によりニトロフェノールに変換される量を吸光度（410nm）により測定し，活性を評価した．

$ONOO^-$はPP1活性を容量依存性に抑制した（IC_{50}=0.45μM）．$ONOO^-$分解産物であるNO_2やNO_3にはPP1活性抑制作用はなかった．還元剤DTTにより抑制された活性は完全に回復したが，$NaAsO_2$によっては回復しなかった．また，PP1活性の必須金属であるMn^{2+}投与によって活性は回復した．酵素基質pNPP前処置は$ONOO^-$による抑制を一部のみ防御した．

$ONOO^-$は基質結合部位以外でジスルフィド結合させることによりMn^{2+}の結合を減弱し，PP1活性を抑制する．これは，急性臓器不全の病因の一つである可能性を示唆している．

キーワード：ペロキシナイトライト，蛋白リン酸酵素1，急性臓器不全