Nitric Oxide Regulates Prolidase Activity by Serine/Threonine Phosphorylation

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Abstract
Prolidase [E.C. 3.4.13.9], a member of the matrix metalloproteinase (MMP) family, is a manganese-dependent cytosolic exopeptidase that cleaves imidodipeptides containing C-terminal proline or hydroxyproline. It plays an important role in collagen metabolism, matrix remodeling and cell growth. Nitric oxide (NO), a versatile signaling molecule, regulates many processes including collagen synthesis and matrix remodeling and, thereby, may modulate angiogenesis, tumor invasiveness, and metastasis. Thus, we considered that prolidase may be an important target of NO regulation. In our study, SIN I and DETA/NO were used as NO donors. Both donors increased prolidase activity in a time-dependent and dose-dependent manner. Prolidase activity increased not only with NO donors but also with endogenous NO in cells transfected with iNOS. The effect of iNOS was abolished by treatment with S-methylisothiourea (SMT), a selective inhibitor of iNOS. However, with either exogenous or endogenous sources of NO, the increase in prolidase activity was not accompanied by increased prolidase expression. Therefore, we suspected phosphorylation of prolidase as a potential mechanism regulating enzyme activation. We observed increased serine/threonine phosphorylation on prolidase protein in cells treated with NO donors and in cells transfected with iNOS. To determine the pathways that may mediate prolidase induction by NO, we first used 8-Br-cGMP, a cGMP agonist, and found that 8-Br-cGMP strongly and rapidly stimulated prolidase activity accompanied by increased phosphorylation. Rp-8-Br-pCPT-cGMP, an inhibitor of cGMP, reduced NO donor-stimulated prolidase activity to control levels. To test whether the MAPK pathway is involved in this NO-dependent activation, we used an ERK1/2 inhibitor and found that it had no effect on prolidase activity increased by NO donors. These results demonstrate that NO stimulates prolidase activity by increasing serine/threonine phosphorylation through PKG-cGMP pathway, but independent of MAPK and suggest an interaction between inflammatory signaling pathways and regulation of the terminal step of matrix degradation. J. Cell. Biochem. 96: 1086–1094, 2005. © 2005 Wiley-Liss, Inc.

Key words: prolidase; Nitric oxide; cGMP; serine/threonine phosphorylation

Prolidase (E.C. 3.4.13.9) is a widely distributed cytosolic enzyme, which is required to hydrolyze imidodipeptides with C-terminal proline or hydroxyproline because the peptide bond nitrogen within the pyrrolidine ring is not susceptible to generic peptidases [Myara et al., 1984]. Catalyzing the terminal step in the digestion of proline-containing nutritional proteins and the degradation of proline-, hydroxyproline-rich matrix proteins, for example, collagen, by matrix metalloproteinases, prolidase supplies and recycles proline for protein synthesis and cell growth [Jackson et al., 1975; Myara et al., 1984]. Recent findings suggest that prolidase also supplies proline as substrate for generating reactive oxygen species by proline oxidase during proline-induced apoptosis [Donald et al., 2001; Maxwell and Rivera, 2003; Liu et al., 2005]. On the biochemical level, prolidase is a matrix metalloproteinase [Boright et al., 1989; Mock et al., 1990]. Unlike other enzymes in the family which bind tightly to Zn (II), prolidase binds tightly and is activated by Mn (II). Because of the activity requirements for metal ion, prolidase can be affected by various metal ions. Nickel, which can cause disease in humans with occupational exposure, is a potent inhibitor of prolidase activity [Miltyk et al., 2005].
The importance of prolidase in human physiology is emphasized by the clinical findings in humans with inherited prolidase deficiency. These individuals exhibit defective wound healing, extensive skin ulcerations and scarring, frequent infections due to immunodeficiency, and mental retardation. Although these associations are of considerable interest, the mechanisms underlying them are not well understood (Royce and Steinmann, 1993).

The participation of prolidase in the proliferative response is suggested by the regulation of prolidase by the activation of two receptors, β1 integrin (Palka and Phang, 1997) and IGF (Miltyk et al., 1998). Both of these receptors can initiate signaling cascades through MAP kinases—ERK1/2—to modulate prolidase expression. Although prolidase participates in the growth paradigm, and increased prolidase activity is associated with increased collagen synthesis, the mechanism for this association is not well understood. Whether these associations are due to a common stimulus or whether they are causally related remain to be elucidated.

Nevertheless, these findings do suggest that prolidase participates in the inflammatory response that is to injury as well as for immune competence. Therefore, we considered that nitric oxide (NO), a signaling molecule within the inflammation paradigm, might provide an important link. Certainly, NO is elevated in tissues undergoing repair (Shi et al., 2001; Witte and Barbul, 2002). Immune cells stimulated by cytokines secrete NO into the region (Marcinkiewicz and Chain, 1993; Marcinkiewicz et al., 1996; Marcinkiewicz, 1997; Giustizieri et al., 2002). Additionally, fibroblasts isolated from healing wounds synthesize NO autonomously and spontaneously (Schaffer et al., 1997).

Nitric oxide, a short-lived free radical, is a pleiotropic regulator, critical to numerous biological processes (Koshland, 1993) and acts at physiological and pathological levels (Bishop and Anderson, 2005; Ischiropoulos and Gow, 2005). NO has many implications as a potential regulatory effector for prolidase. Through its second messenger, cGMP, NO can regulate the activity of MMPs (Mei et al., 2002; Tsuruda et al., 2004; Liu et al., 2004). Although prolidase is a special type of MMP, it may be also regulated by NO because it catalyzes the terminal step in matrix breakdown. Finally, NO can modulate a MAPK pathway (Schroeter et al., 2002), which have been previously shown to affect prolidase functions.

The aforementioned considerations prompted us to investigate the effects of NO on prolidase activity. We found that both exogenous NO from NO donors as well as NO generated endogenously from transfected iNOS increased prolidase activity in a time- and dose-dependent fashion. The mechanism of this effect is due to serine/threonine phosphorylation rather than induction of prolidase expression. Furthermore, the effects on phosphorylation are mediated by cGMP signaling. Our findings provide evidence that NO regulates prolidase and that this regulation may be important as a component of NO signaling. Finally, the finding establishes a link between NO and prolidase for mediating cell-matrix interaction and angiogenesis, which is critical for inflammation and wound healing.

MATERIALS AND METHODS

Reagents

Dulbecco’s minimal essential medium (DMEM), fetal bovine serum (FBS), glutamine, penicillin, and streptomycin were obtained from Quality Biologicals, Inc., USA. NO donor SIN-1, iNOS inhibitor SMT (S-methylthiourea), 8-Br-cGMP (Guanosine 3′,5′-cyclic Monophosphate, 8-Bromo-, Sodium Salt), Rp-8-pCPT-cGMP (Guanosine 3′,5′-cyclic Monophosphorothioate, 8-(4-Chlorophenylthio)−, Rp-Isomer, Triethylammonium Salt), UO 126 were purchased from Calbiochem (San Diego, CA). DETA/NO (2,2′-(Hydroxynitrosohydrazono)bis-ethanimine) was purchased from Sigma (St. Louis, MO).

Cell Culture

All studies were performed using NIH 3T3 mouse fibroblasts, which were purchased from American Type Culture Collection, (Rockville, MD). The cells were maintained in DMEM supplemented with 10% FBS (HyClone Laboratories, Logan, Ut), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin at 37°C in a 5% CO2 incubator. Cells were counted in a hemocytometer and cultured at 2 × 10^5 cells per well in 2 ml of growth medium in 6-well plates (Costar, Corning, NY).

Determination of Prolidase Activity

The activity of prolidase was determined according to the method of Myara et al. (1982),
which is based on colorimetric determination of proline using Chinard's reagent. Cells were scraped off and centrifuged at 200g for 15 min and the supernatant was discarded. The cell pellet was suspended in 1 ml of 50 mM HEPES, pH 7.8, and sonicated for 3 × 10 s at 0°C.

Samples were then centrifuged (12,000g, 30 min) at 4°C and the supernatant was used for protein determination (Bradford method) and prolidase activity assays. Activation of prolidase requires incubation with Mn(II): 100 μl of cell extract supernatant was mixed with 100 μl of 50 mM HEPES, pH 7.8 containing MnCl₂ at a final concentration of 1 mM in the mixture. After incubation for 24 h at 37°C, the prolidase reaction was initiated by adding 100 μl of the activated mixture to 100 μl of 94 mM glycyl-proline (Gly-Pro) for a final concentration of 47 mM. After additional incubation for 1 h at 37°C, the reaction was terminated with the addition of 1 ml of 0.45 M trichloroacetic acid. To parallel blank tubes, trichloroacetic acid was added at time “zero.” Samples were centrifuged at 10,000g for 15 min. The released proline was determined by adding 0.5 ml of the trichloroacetic acid supernatant to 2 ml of a 1:1 mixture of glacial acetic acid: Chinard's reagent (25 g of ninhydrin dissolved at 70°C in 600 ml of glacial acetic acid and 400 ml of 6 M orthophosphoric acid) and incubated for 10 min at 90°C. The amount of proline released was determined colorimetrically by monitoring absorbance at 515 nm and calculated using proline standards. Enzyme activity was reported in nanomoles of proline released per minute per milligram of protein.

Preparation of Anti-Prolidase Antibody

Synthetic peptide consisting of an amino terminal cysteine followed by residues 195–211 of the human prolidase was conjugated with KLH. The coupled peptide was mixed with Freund’s adjuvant and two rabbits were immunized by multiple subcutaneous injections (Covance, Denver, PA). The serum was purified using protein G sepharose chromatography. Immunoreactivity of the prolidase antiserum was confirmed by Western immunoblots with purified prolidase as antigen. Titration of the antibody showed that 1:1,000 dilutions were optimal.

Western Blot Analysis and Immunoprecipitation

Equal amounts of total cellular protein extract were electrophoresed on SDS–PAGE gels and transferred by electrophoretic transfer onto nitrocellulose membrane (Bio-Rad, Hercules, CA). The primary antibodies used were anti-prolidase and anti-serine/threonine phosphorylation (Upstate, VA). Anti-mouse or anti-rabbit antibodies (Amersham Biosciences, Piscataway, NJ) were used as secondary antibodies. Blots were developed using the enhanced chemiluminescence procedure (Amersham Biosciences, Piscataway, NJ).

The methods for the immunoprecipitation assay were described in the kit from Sigma, (St. Louis, MO). Briefly, the cells were lysed in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM NaVO₃, 1.5 mM MgCl₂, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonfyl fluoride. Protein G-agarose was incubated prolidase antibody at 4°C for 1 h followed by incubating with 100 μg of protein extracts at 4°C overnight. The agarose mixture was pelleted and washed four times in lysis buffer. For immunoprecipitation-Western blotting, the agarose was resuspended in 50 μl of 1 x sample buffer (125 mM Tris, pH 6.8, 4% SDS, 0.005% bromphenol blue, 20% glycerol, 0.7 M β-mercaptoethanol), and 20 μl was loaded on 10% SDS–PAGE. Western blotting to phosphoserine/threonine was performed as described above.

Plasmid Constructs and Stable Transfection

The pcDNA3/iNOS was kindly provided by Dr. David Geller of the University of Pittsburgh (Taylor and Geller, 2000). Briefly, culture medium was removed from wells and the adherent cells washed twice with PBS. 0.5 ml of transfection complex in Opti-MEM I medium containing lipofectamine 2000 (2 μl) and plasmid (1 μg), was then added to each well. Cells were incubated for a further 24 h at 37°C, after which the medium was removed, replaced with normal culture medium containing 10% FBS, and incubated in a tissue culture incubator at 37°C for 24 h. In control experiments, cells were transfected with empty vector. Cells with the appropriate Geneticin vector stably integrated were selected in the presence of Geneticin (500 μg/ml for 1 week).

Statistical Analysis

In all experiments, the mean values for at least three assays ± SEM were calculated unless
otherwise indicated. The results were submitted to statistical analysis using the Student’s t-test, accepting *P < 0.05 and **P < 0.01, as significant.

**RESULTS**

Prolidase activity was measured in NIH 3T3 cells that had been treated with NO donor DETA/NO. As shown in Figure 1A, NO treatment increased prolidase activity in a dose dependent manner. At 250 µM DETA/NO increased prolidase activity about 80%. The increases in prolidase activity with DETA/NO also exhibited time dependence (Fig. 1B). The greatest increase in prolidase activity was observed after 24 h incubation with 250 µM DETA/NO. To confirm these findings we used another NO donor—SIN I, which also increased prolidase activity in a dose and time dependent manner (Fig. 2A,B).

Nitric Oxide, a potent second messenger and regulator, is generated from L-arginine by several nitric oxide synthases [Aktan, 2004]. To determine whether endogenous NO had similar effects, NIH 3T3 cells were stably transfected with recombinant iNOS. In those cells, prolidase activity was much higher (+75%) compared to cells stably transfected with empty vector (Fig. 3). This is consistent with aforementioned finding that NO from NO donors stimulated prolidase activity.

Additional evidence supporting the idea that the increase in prolidase activity depends on nitric oxide synthesis generated from iNOS expression comes from using the specific iNOS inhibitor: S-methylisothiourea (SMT). SMT had no effect on prolidase activity in control cells, but SMT treatment resulted in a concentration dependent decrease in prolidase activity in cells stably transfected with iNOS. SMT at 50 µM decreased the prolidase activity in iNOS transfected cells to the control level. (Fig. 4).
It is well established that cGMP-PKG pathway is the main mediator of NO affects. Increased NO from NO donors stimulated cGMP activity [McDonald and Murad, 1996]. To determine whether cGMP is involved in prolidase regulation, we stimulated cGMP activity using cGMP analog, 8-Br-cGMP. As shown in Figure 5A, 8-Br-cGMP increased prolidase activity in a dose-dependent manner. 8-Br-cGMP at 100 μM increased prolidase activity similar to that of NO donors. At a concentration of 100 μM, this cGMP analog increased prolidase activity in a time-dependent manner. Highest increase in prolidase activity was observed after 24 h incubation with 8-Br-cGMP (Fig. 5B).

Having demonstrated that NO from both endogenous and exogenous source stimulated prolidase activity, we then addressed the mechanism for NO stimulated prolidase activity. First we raised a polyclonal antibody against prolidase. Using this antibody, we monitored the levels of prolidase protein and found under aforementioned conditions the increase in prolidase activity was not accompanied by total prolidase protein levels (Fig. 6B–D). This suggested that perhaps the NO-stimulated prolidase activity was a post-translational event.

Phosphorylation of enzyme protein is a commonly occurring mechanism regulating enzyme activation. Prolidase has a number of serine/threonine sites, which can be potentially phosphorylated [Songyang et al., 1996; Yaffe and Smerdon, 2004]. To test this idea, prolidase was immunoprecipitated with anti-prolidase antibody and the immunoprecipitation analysis was carried out for phosphorylation using anti-phosphoserine/threonine antibody. After immunoprecipitation, we showed that a band in the
immunoprecipitant comigrating with prolidase was detected by antiphosphoserine/threonine antibodies (Fig. 6A).

In cells stably transfected with iNOS and further treated with or without SMT, we found correlation between increased prolidase activity and increased phosphorylation of prolidase protein (Fig. 6B). In contrast, immunodetectible prolidase protein was essentially unchanged under all the conditions tested. After these observations, we assayed extracts from cells treated with DETA/NO (Fig. 6C) or with 8-Br-cGMP (Fig. 6D). These time course experiments in Figure 6C,D correlate with those showing prolidase activities in Figures 1B and 5B. As shown in Figures 6C,D, with both treatments, prolidase phosphorylation increased whereas prolidase protein did not. Although the magnitude of phosphorylation generally reflected the increase in enzyme activity, the correlation was not complete. With DETA/NO, maximal increase in prolidase phosphorylation was observed after 8 h treatment, whereas with 8-Br-cGMP, the increased phosphorylation was detectible as early as 4 h and the effect plateaued by 8 h.

These findings convincingly showed that the increase in prolidase activity occurred by

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**Fig. 6.** Western immunoblot analysis of serine/threonine phosphorylated prolidase and total prolidase protein. To determine levels of serine/threonine phosphorylation, extracts from NIH 3T3 cells treated as described were immunoprecipitated with anti-prolidase antibody. The precipitates were analyzed by Western immunoblot using anti-phosphoserine/threonine antibody or with anti-prolidase antibody. A: Cells were treated with 100 μM 8-Br-cGMP for 0 h or 8 h. B: Cells stably transfected with empty vector (control) or iNOS cDNA were treated with or without 50 μM SMT. C: Cells were treated with 250 μM DETA/NO for various durations as in Figure 1B. Analysis for prolidase protein was performed on the whole extract. D: Cells were treated with 100 μM 8-Br-cGMP for various durations as in Figure 5B. Analysis for prolidase protein was performed on the whole extract.
serine/threonine phosphorylation and not by an increase in levels of prolidase protein.

Since nitric oxide can increase prolidase activity by two pathways that is by cGMP kinase mediated effects as well as via the MAP kinase pathway, we compared the respective effects of the two pathways by using selective inhibitors. As shown in Figure 7, Rp-8-pCPT-cGMP, which inhibits the cGMP pathway, had no effect on basal prolidase activity. However, it completely abolished prolidase activity stimulated by DETA/NO. By contrast, UO 126, a specific inhibitor of ERK1/2, had no effect on prolidase activity with or without DETA/NO treatment. Thus, the effect of nitric oxide appears to be mediated by the cGMP pathway but not by MAP kinases.

**DISCUSSION**

Our studies showed a definite link between NO levels and prolidase activity. In time course experiments with DETA/NO as the NO donor, a significant increase in activity was seen as early as after 4 h of exposure. However, the increase in prolidase activity was not accompanied by an increase in prolidase protein. Since previous work has shown that prolidase may be regulated by post-translational modification [Surazynski et al., 2001], we considered that regulation of prolidase by NO may also occur by a post-translational mechanism. In fact, prolidase contains at least three potential sites for serine/threonine phosphorylation as deduced by known consensus sequence required for serine/threonine phosphorylation and the primary structure of prolidase [Songyang et al., 1996; Yaffe and Smerdon, 2004]. Using an antiprolidase antibody developed in our laboratory, we immunoprecipitated prolidase protein and showed that a band in the immunoprecipitant comigrating with prolidase was detected by antiphosphoserine/threonine antibodies. Thus, we showed for the first time that prolidase is a phosphoserine/threonine protein and the NO mediated increase in phosphorylation suggests that this may be a mechanism for regulating prolidase activity in various physiologic conditions.

NO can activate proteins in several ways, but the known mechanisms resulting in increased phosphorylation are limited to the cGMP and MAPK pathways. We first used the longer acting analog of cGMP, 8-bromo-cGMP, and found that, indeed, treatment with the analog not only increased prolidase activity but also markedly increased phosphorylation of prolidase protein. An alternative mechanism for activation of phosphorylation by NO is the MAP kinase pathway. Published work showed that the activation of prolidase by integrin receptors might be mediated through MAP kinases [Surazynski et al., 2001]. However, when we used an inhibitor of the MAPK pathway, we found that the baseline activity as well as the NO-stimulated activity were unaffected. Thus, NO increased serine/threonine phosphorylation of prolidase through the cGMP pathway but not through the MAPK pathway.

Although NO is a potent downstream mediator of certain cytokines, it also has an immunomodulatory role by downregulating the expression of cytokines in circulating immune cells [Marcinkiewicz and Chain, 1993; Marcinkiewicz et al., 1996; Marcinkiewicz, 1997; Giustizieri et al., 2002]. A number of these cytokines have the X-Pro sequence at the N-terminus and they are activated or inactivated by dipeptidyl peptidase IV or its homologues, which cleave the cytokine at the penultimate proline [Flentke et al., 1991; Busek et al., 2003]. Interestingly, the derivatized imidodipeptide (Ala-boroPro) is a potent pharmacologic inhibitor of dipeptidyl peptidase IV [Flentke et al., 1991], leading to the hypothesis first proposed by Hechtman [2001] that these

![Fig. 7. Effect of inhibitors on DETA/NPO stimulation of prolidase activity in NIH 3T3 cells. The concentration of Rp-8-pCPT-cGMP, UO 126, and DETA/NO were 50 μM, 20 μM, and 250 μM, respectively. Duration of incubation was 24 h. Data represents the mean ± SEM of at least three experiments. Comparisons were made between values with and without DETA/NO treatment. **P < 0.01.](image-url)
Nitric Oxide Regulates Prolidase Activity

Enzymes can be inhibited by other X-Pro imidodipeptides. Our finding that NO stimulates prolidase, thereby decreasing the cellular content of imidodipeptides, provides a mechanism by which NO may modulate the availability of active cytokines to alter a variety of cellular responses.

Moreover, prolidase and the regulation of prolidase by NO may be important in the regulation of collagen turnover. Collagen, the most abundant protein in the body, constitutes more than a quarter of total body proteins. It is not only essential for maintaining musculoskeletal integrity, but also serves to maintain integrity of tissue architecture. Increased prolidase activity has been associated with increased rates of collagen synthesis, although the mechanism for this association is not fully understood. On the other hand, NO has been shown to play a role in regulating collagen metabolism. High NO concentrations are associated with increased collagen biosynthesis and modification, especially during wound repair. In fibroblasts derived from mice with the inducible NO synthase gene knocked-out, collagen synthesis, proliferation and contractility are markedly reduced as compared to WT fibroblasts [Shi et al., 2001; Witte and Barbul, 2002]. Since the mechanism linking NO to these phenomena are not understood, it is tempting to propose that the effect of NO on prolidase activity may provide a mechanistic link for these associations.

As a final point, the relevance of our finding to the clinical phenotype of prolidase deficiency is readily apparent. Wound healing is primarily an inflammatory response with markedly increased rates of collagen synthesis, although the mechanism for this association is not fully understood. On the other hand, NO has been shown to play a role in regulating collagen metabolism. High NO concentrations are associated with increased collagen biosynthesis and modification, especially during wound repair. In fibroblasts derived from mice with the inducible NO synthase gene knocked-out, collagen synthesis, proliferation and contractility are markedly reduced as compared to WT fibroblasts [Shi et al., 2001; Witte and Barbul, 2002]. Since the mechanism linking NO to these phenomena are not understood, it is tempting to propose that the effect of NO on prolidase activity may provide a mechanistic link for these associations.

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