Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite

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Abstract Peroxynitrite ($ONOO^-$) has recently been implicated in connective tissue destruction in vivo. We have studied the effect of $ONOO^-$ on the activity of tissue inhibitor of metalloproteinase-1 (TIMP-1) in vitro. The inactivation of TIMP-1 by $ONOO^-$ was dose dependent with 50 μM $ONOO^-$ reducing the inhibitory activity of TIMP-1 towards gelatinase-A by 50%. High concentrations of $ONOO^-$ (500 μM–5 mM) caused protein fragmentation whilst lower concentrations (< 250 μM) inactivated TIMP-1 without altering the molecular weight. Inactivation could be blocked by $ONOO^-$ scavengers but not by hydroxyl radical scavengers. Our results show that $ONOO^-$ is capable of inactivating TIMP-1, a process which could potentiate metalloproteinase-mediated tissue breakdown.

Key words: Tissue inhibitor of metalloproteinases; Peroxynitrite; Nitric oxide; Reactive nitrogen species; Inflammation; Reactive oxygen species; Connective tissue damage

1. Introduction

Oxidative stress has been implicated in many disease processes including inflammation, reperfusion injury, aging and cancer. In inflammation and ischaemic reperfusion significant amounts of superoxide ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$) are produced [1,2]. Peroxynitrite ($ONOO^-$) is formed rapidly by the interaction between $NO^{\bullet}$ and $O_2^{\bullet-}$ with a rate constant of 6.7×10^9 $M^{-1}\cdot s^{-1}$ in aqueous solution at pH 7.4 [3]. Many workers have demonstrated that $ONOO^-$ is an extremely reactive species which readily oxidises various biomolecules, such as low density lipoprotein [4], DNA [5] and α-1-proteinase inhibitor [6]. In the latter case, the oxidation of α-1-proteinase inhibitor causes inactivation of its proteinase inhibitory capacity. $ONOO^-$ can react with the tyrosine residues of proteins to form 3-nitrotyrosine [7] and this "marker" molecule has been detected at inflammatory sites as well as in body fluids [8,9]. Therefore, it has been suggested that $ONOO^-$ may contribute to tissue damage.

Tissue inhibitor of metalloproteinase-1 (TIMP-1) is a member of the naturally-occurring inhibitors of tissue-damaging matrix metalloproteinases [10]. It inhibits active metalloproteinases, such as stromelysins, collagenases and gelatinases, by forming a tight one-to-one stoichiometric complex [11]. Thus, the balance between the activities of TIMPs and active metalloproteinases is probably a critical factor in the control of connective tissue remodelling in both health and disease, and a change of this balance in favour of active metalloproteinases may be an important determinant in the disease process. Although the inactivation of TIMPs by either reactive oxygen species or proteinases has not been demonstrated in vivo, the potentiation of metalloproteinase activity through inactivation of TIMP-1 by neutrophil elastase has been shown in vitro [12]. In this study, we have demonstrated a mechanism by which oxidative stress might potentiate metalloproteinase activity by TIMPs inactivation. We report that $ONOO^-$ is capable of inactivating TIMP-1 effectively.

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Abbreviations: TIMP, tissue inhibitor of metalloproteinases; APMA, (4-aminophenyl) mercuric acid; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

2. Materials and methods

2.1. Human TIMP-1, gelatinase-A and stromelysin-1

Human recombinant TIMP-1 was expressed and purified from C127 cells [13,14]. Human recombinant progelatinase-A [15] and recombinant prostromelysin-1 [16] were generously donated by Dr. G. Murphy, Strangeways Research Laboratories (Cambridge, UK). Progelatinase-A was activated by incubation with 2 mM (4-aminophenyl)mercuric acetate (APMA) for 1 h at 25°C. Prostromelysin-1 was activated by incubating with 2 mM APMA for 4 h at 37°C. The conversion of proenzymes to active enzymes was confirmed by SDS-PAGE and activity assays.

2.2. Determination of enzyme activity

Gelatinase-A was assayed using the synthetic peptide DNP-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH2 as described by Stack and Gray [17]. The synthetic peptide (26 μM) was cleaved by gelatinase-A (25 nM) in 50 mM Tris/100 mM $NaCl_2$/10 mM $CaCl_2$/0.05% Brij35 (pH 7.4). The rate of change in fluorescence of tryptophan (excitation at 280 nm with emission at 346 nm) was monitored for a period of 7 min at 37°C.

The activity of stromelysin-1 was measured using [^{14}C]acetylated casein as substrate in 40 mM Tris-HCl (pH 7.6) with 12 mM $CaCl_2$ as described by Cawston et al. [11]. Reaction mixtures were incubated for 20 h at 37°C and incorporated 0.93 μg/μl stromelysin. One unit of stromelysin activity produced 1 μg of casein fragments (soluble in 3% (w/v) trichloroacetic acid) in 1 min at 37°C. The specific activity of stromelysin after activation by APMA was 739 U/mg.

2.3. Determination of TIMP-1 activity

TIMP-1 activity was determined by measuring residual enzyme activity after incubating enzymes with TIMP-1 for 30 min (gelatinase-A) and 2 h (stromelysin-1) at 25°C.

2.4. Synthesis of $ONOO^-$

$ONOO^-$ was synthesised from 0.2 M $NaNO_2$ and 1 M H_2O_2 in 0.5 M HCl [18] and stored in 1.5 M KOH at −70°C. Excess H_2O_2 in the $ONOO^-$ solution was removed by MnO_2, and the H_2O_2 concentration was determined by the horseradish peroxidase assay [19]. High concentrations of $ONOO^-$ (>200 mM) were obtained by freeze fractionation [6]. The concentration of $ONOO^-$ was determined by its absorbance at 302 nm ($\varepsilon_{302nm} = 1670$ $M^{-1}\cdot cm^{-1}$) in 1.5 M NaOH.

2.5. TIMP-1 inactivation by $ONOO^-$

TIMP-1 (final concentration of 5.5 μM) was incubated with

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$ONOO^-$ (1 μM, 5 μM, 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, 1 mM and 5 mM in 375 mM KOH) in 0.1 M phosphate buffer pH 7.0 (final volume of 100 μl) for 20 h at 20°C. The final pH of the reaction mixture was 8.0. The effect of scavengers on the inactivation of TIMP-1 by $ONOO^-$ was also examined. For this purpose, mannitol, thiourea, benzoate, desferrioxamine, methionine, histidine, lysine, proline, tyrosine and tryptophan were included in the reaction mixture. The molar ratio of scavengers to TIMP-1 was fixed at 182:1.

2.6. Examination of TIMP-1 molecular form

SDS-PAGE was carried out in a Tris/glycine buffer system using 10–20% gradient gels. Samples were treated in SDS-containing sample buffer with or without 2-mercaptoethanol, and bands were revealed by silver staining. When a gel was used for Western blotting, proteins were detected by Ponceau S (0.03% w/v) prior to incubating with anti-TIMP-1 monoclonal antibody (1 μg/ml, Oncogen Science, MA) or anti-nitrotyrosine monoclonal antibody (1 μg/ml, Upstate Biotechnology Inc. NY).

3. Results

When human TIMP-1 (5.5 μM) was treated with $ONOO^-$ (1 μM to 1 mM) for 20 h at 20°C, the inhibitory activities of TIMP-1 towards both gelatinase-A and stromelysin-1 were significantly decreased in a dose-dependent manner (Fig. 1). The concentrations of $ONOO^-$ causing 50% inactivation of TIMP-1 were approximately 50 μM and 250 μM for gelatinase-A and stromelysin-1, respectively. The difference between the dose–response curves generated by the two metalloproteinases was thought to be a result of differing assay methodologies.

$ONOO^-$ solutions may contain both nitrate and nitrite contamination. In order to examine the effect of these contaminants on TIMP-1 activity, 0.8 mM decomposed $ONOO^-$ was made by adding $ONOO^-$ to 100 mM potassium phosphate (pH 7.4) at 37°C. There was no remaining $ONOO^-$ in solution as determined at 302 nm. When decomposed $ONOO^-$ was incubated with TIMP-1 under conditions identical to the above dose–response experiments (Fig. 1), it had no effect on TIMP-1 as detected using both gelatinase-A and stromelysin-1 assays.

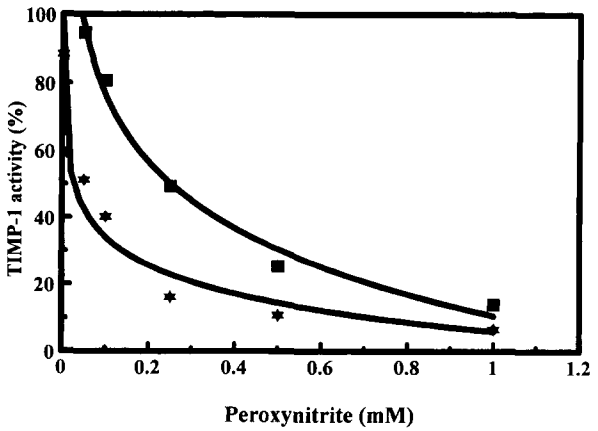

Fig. 1. Effect of peroxynitrite ($ONOO^-$) on the ability TIMP-1 to inhibit gelatinase-A and stromelysin-1 activities. TIMP-1 (5.5 μM) was exposed to $ONOO^-$ (1 μM, 5 μM, 10 μM, 50 μM, 100 μM, 250 μM, 500 μM and 1 mM in 375 mM KOH) in 100 mM phosphate buffer (final pH of solution was 8.0) for 20 h at 20°C. For the measurement of gelatinase-A inhibitory activity, 2.5 μM $ONOO^-$-treated TIMP-1 was reacted with 2.5 μM gelatinase-A for 30 min at 37°C (★). For the measurement of stromelysin-1 inhibitory activity, 2.5 μM $ONOO^-$-treated TIMP-1 was incubated with 0.15 μM stromelysin-1 for 2 h at 37°C (■). After incubating with enzymes, the residual enzymic activities were assayed (see section 2). The proteinase inhibitory activity of TIMP-1 samples incubated in the absence of $ONOO^-$ was taken as 100% activity.

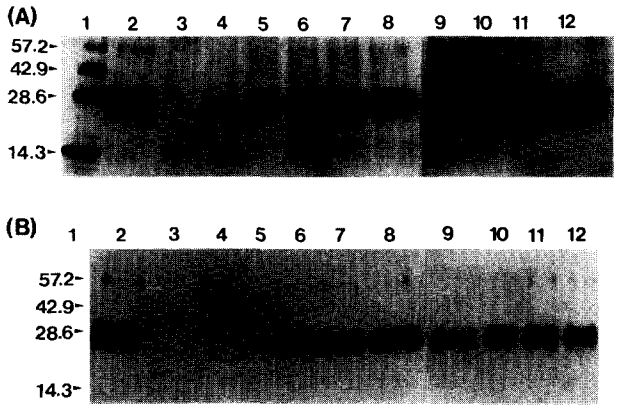

Fig. 2. Modification of the molecular form of TIMP-1 after treatment with peroxynitrite ($ONOO^-$). Samples from Fig. 1 experiments were examined in parallel by 10–20% gradient SDS PAGE with (A) silver staining, and (B) Western blotting with anti-TIMP-1 monoclonal antibody (1 μg/ml). Lane: 1, molecular weight markers and molecular weight values (kDa) given to the left of gels. Lane 2, TIMP-1 (5.5 μM) incubated in the absence of $ONOO^-$. Lane 3 to 11, TIMP-1 (5.5 μM) incubated in the presence of decreasing concentration of $ONOO^-$: lane 3, 5 mM; lane 4, 1 mM; lane 5, 500 μM; lane 6, 250 μM; lane 7, 100 μM; lane 8, 50 μM; lane 9, 10 μM; lane 10, 5 μM; and lane 11, 1 μM. Lane 12, 5.5 μM TIMP-1 incubated with 0.8 mM decomposed $ONOO^-$.

The molecular forms of TIMP-1 after $ONOO^-$ treatment were also examined by SDS-PAGE with silver staining and Western blotting with an anti-TIMP-1 monoclonal antibody [20]. We used parallel TIMP-1 samples from the activity experiments (for details see Fig. 1). The human recombinant TIMP-1 used in this study is a 28 kDa protein with a trace amount of dimer contamination (56 kDa) seen by both silver staining and Western-blotting (Fig. 2, Lane 2). Both direct protein detection (Fig. 2A) and antibody detection (Fig. 2B) showed similar results. High concentrations of $ONOO^-$ (5 mM and 1 mM) completely degraded TIMP-1 (both the 28 kDa protein and the 56 kDa dimer) to low molecular weight fragments which were not detected by either silver staining or anti-TIMP-1 antibody detection. However, using 500 μM $ONOO^-$, TIMP-1 protein bands were seen by both protein and antibody staining, but these bands were very weak. The intensity of these TIMP-1 bands remained the same as the concentration of added $ONOO^-$ was progressively lowered (250 μM down to 1 μM). Moreover, decomposed $ONOO^-$ did not affect TIMP-1 (Fig. 2, Lane 12). No evidence of further polymerisation of TIMP-1 was found by either protein or antibody detection.

Comparing the results of the activity assays (Fig. 1) with the SDS-PAGE results (Fig. 2), it appears that protein fragmentation is not directly responsible for TIMP-1 inactivation. $ONOO^-$ at a concentration of 250 μM reduced the inhibitory activity of TIMP-1 by 80%. However most of this TIMP-1 was still detected by both protein and antibody detection (Lane 6 in Fig. 2).

TIMP-1 (5.5 μM) inactivated by various doses of $ONOO^-$ was also Western blotted and examined by an anti-nitrotyrosine monoclonal antibody [21] to detect nitrosylation of TIMP-1 tyrosine residues by $ONOO^-$. Only TIMP-1 samples

exposed to 500 μM $ONOO^-$ showed a single band at the 28 kDa position (data not shown). The doses tested which were higher and lower than 500 μM did not produce nitrotyrosine formation that was detectable by this method. A possible explanation for these results is that high concentrations of $ONOO^-$ (1 and 5 mM) do cause the formation of nitrotyrosine within TIMP-1, but also degrade the protein to fragments which are not detected on 10–20% SDS-PAGE. Lower doses of $ONOO^-$ (250 μM or lower) may generate small amounts of nitrotyrosine which are not detectable by the current staining method.

We studied the ability of various scavengers to block the inactivation of TIMP-1 by $ONOO^-$. Based on the results of the dose–response study (Figs. 1 and 2), 500 μM $ONOO^-$ was chosen to use throughout the course of the scavenger study. The legend of Fig. 3 summarises the details of this study. Fig. 3 demonstrates that thiourea, desferrioxamine, tyrosine and tryptophan inhibited TIMP-1 inactivation induced by 500 μM $ONOO^-$ to the extents of 94.2%, 91.1%, 91.8% and 75.3%, respectively. Methionine offered 50.9% protection against $ONOO^-$-mediated inactivation. However, mannitol, benzoate, lysine, proline and histidine did not provide protection against TIMP-1 oxidation by $ONOO^-$.

4. Discussion

The balance between the activity of proteinase inhibitors and proteinases is thought to be a critical factor in extracellular matrix turnover. TIMP-1 is often co-expressed with metalloproteinases from connective tissue cells [22,23]. Okada et al demonstrated the proteolytic inactivation of TIMP-1 by neutrophil elastase [12] and suggested that neutrophils, which infiltrate inflammatory sites, may contribute to TIMP-1 inactivation. The lack of inhibitory capacity of the natural inhibitor of neutrophil elastase, α-1-proteinase inhibitor, at inflammatory sites has been described [24].

In the present study, we have demonstrated that $ONOO^-$ is able to inactivate TIMP-1. The oxidation of TIMP-1 was evidenced by blocking with scavengers of reactive oxygen species, such as desferrioxamine and thiourea, and direct detection of 3-nitrotyrosine formation in the TIMP-1 protein. Concentrations of $ONOO^-$ as low as 50 μM inactivated 50% of the TIMP-1 as detected by gelatinase-A (Fig. 1) and concentrations over 500 μM readily degraded TIMP-1 protein to fragments (Fig. 2). In agreement with our observation of TIMP-1 fragmentation, recently Ischiropoulos and Al-Mehda have shown that $ONOO^-$ can cause partial fragmentation of fatty acid-free bovine serum albumin [25].

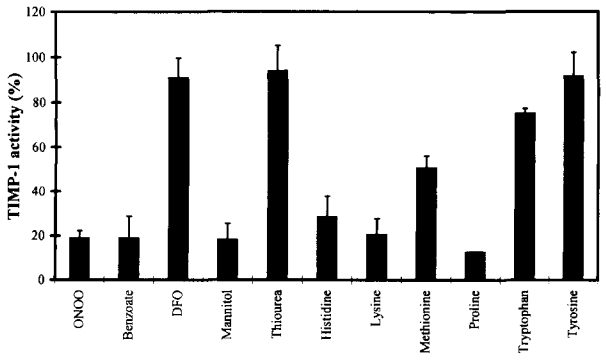

Fig. 3. The protective effect of a range of scavengers and amino acids on TIMP-1 inactivation by peroxynitrite ($ONOO^-$). Each reaction mixture containing TIMP-1 (5.5 μM), $ONOO^-$ (500 μM), and 1 mM scavenger/amino acid was incubated as detailed in Fig. 1. The TIMP-1 activity was determined by measuring residual gelatinase-A activity (except tryptophan experiments, in which stromelysin-1 was used). Values are presented as mean ± S.D. calculated from duplicate runs of three independent experiments (except tryptophan where two independent experiments were performed). DFO represents desferrioxamine.

Evidence for the generation of $ONOO^-$ in vivo has recently been obtained by the detection of nitrotyrosine in inflammatory sites [9,21] and body fluids [8]. It has been estimated that a phagosome in an activated macrophage may generate $ONOO^-$ at a rate of approximately 500 μM/min [26]. On the other hand, in response to inflammatory cytokines $NO^\bullet$ levels can be elevated by the induction of inducible nitric oxide synthase [27]. Recently, it has been shown that $NO^\bullet$ can also be generated in ischaemic conditions by direct reduction of nitrite to $NO^\bullet$ [2]. Thus, production of $NO^\bullet$ together with $O_2^{\bullet-}$ during inflammation and ischaemic reperfusion may elevate the formation of $ONOO^-$, and subsequent damage to biomolecules such as TIMP-1 could occur under these pathological conditions.

TIMP-1 inactivation by $ONOO^-$ could be blocked by the addition of thiourea, tyrosine, desferrioxamine, tryptophan and methionine. It is known that thiourea, desferrioxamine, and methionine are able to inhibit $ONOO^-$-mediated oxidation [1,6,28,29]. Proline, lysine and histidine were ineffective at preventing TIMP-1 inactivation by $ONOO^-$ despite the susceptibility of histidine to free radical attack [30]. Tyrosine, tryptophan and methionine are known to react directly with $ONOO^-$ [7,25] and these amino acids blocked TIMP-1 inactivation very effectively. We found that $ONOO^-$-mediated TIMP-1 oxidation was not blocked by the classic $^\bullet OH$ scavengers, mannitol and benzoate. Similar results with mannitol and benzoate have been reported before [6,28]. Inactivation of TIMP-1 could only be inhibited by agents which rapidly react with $ONOO^-$ ie tyrosine, tryptophan, methionine, thiourea and desferrioxamine.

In conclusion, our results suggest that $ONOO^-$ is capable of inactivating TIMP-1, a possible mechanism which may contribute to the regulation of connective tissue remodelling in health and disease.

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